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Development of a Mouse Model of NY99 West Nile Virus-Induced Neurological Changes

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Development of a Mouse Model of NY99 West Nile Virus-Induced Neurological Changes

by

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Dedication

I would first like to dedicate this work to my wife, Catherine May Fulton, who has been an absolute joy to know and be married to. She has helped push me into being a better scientist and person, while also being the best support I could have ever asked for. She has been my best friend throughout all of this and never doubted me, and has been my staunchest supporter, while also knowing exactly when I needed to be pushed and motivated. She's an amazing scientist and person.

I would also like to dedicate this to my family. I have been incredibly lucky to be related to brilliant and supportive people. My parents, Greg and Helen Fulton, have been supportive of my pursuits since I was young, and have always been ready with a supportive ear and encouragement (even if sometimes I can't talk about all my work while having dinner with them). My brothers, Lowell and Kevin Fulton, have been my best friends for my whole life and always are ready to support me with insight or the occasional joke. My extended family, including my grandparents, cousins, aunts, and uncles have all supported me more than they had any reason to. They have provided encouragement, advice, moments of relaxation, and more scientific jokes than I can remember. It has been a wonder to be a member of this family, and to get to learn from all of them, including Ingrid, Carson, Samuel, and Rhiannon.

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Development of a Mouse Model of NY99 West Nile Virus-Induced Neurological Changes

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West Nile virus is a mosquito-borne flavivirus with worldwide distribution that can cause severe neurological disease in those infected. After recovery, as many as 60% of patients report neurological deficits that can persist for the rest of their lives. Some of the most common sequelae are depression, memory loss, motor incoordination, and trouble processing information. Although recent research has studied possible mechanisms behind these sequelae in mouse models, these relied on an attenuated virus inoculated directly into the brain. The studies presented here aimed to reproduce these neurological changes in a mouse model using peripheral inoculation of wild-type virus to mimic human infection more closely. The aims were to determine if this form of infection caused neurological deficits, if there were inflammatory changes after infection, and if the viral RNA persisted in the brain. Mice infected with WNV that were euthanized over one month post-infection tended to perform worse than uninfected mice on memory testing, and had persistent inflammatory lesions and viral RNA in multiple brain regions, though both were most commonly found in the hindbrain. WNV-infected mice also showed significant changes in the levels of pro-inflammatory cytokines in the cortex and hindbrain, indicating persistent

changes in these regions. Microgliosis and viral RNA of certain brain regions correlated with changes in behavioral tests investigating depressive-like behavior, sensorimotor gating, and motor learning, though there was no correlation with memory function. These studies show that this mouse model of WNV infection causes long-term changes in the brain that can manifest as behavioral changes, which are in part related to inflammation and persistent viral RNA in the brain.

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List of Abbreviations

UTMB	University of Texas Medical Branch
GSBS	Graduate School of Biomedical Science
TDC	Thesis and Dissertation Coordinator
2WAA	Two-way active avoidance
Aβ	Amyloid-beta
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
ASR	Acoustic startle response
BBB	Blood-brain barrier
BDV	Borna disease virus
BSA	Bovine serum albumin
BSC	Biosafety cabinet
BSL	Biosafety level
CD3	Cluster of differentiation-3
CDV	Canine distemper virus
CNS	Central nervous system
CVO	Circumventricular organs
DENV	Dengue virus
DI	Defective interfering particle
DMEM	Dulbecco's modified Eagle's medium
DPI	Days post-infection
GFAP	Glial fibrillary acidic protein
HD	Huntington's disease
HIV	Human immunodeficiency virus
HSV-1	Herpes simplex virus-1
Iba1	Ionized calcium-binding adapter molecule 1
IC	Intracranial
IDO	Indoleamine 2,3-dioxygenase
IL-1β	Interleukin-1β
IHC	Immunohistochemistry
LTP	Long-term potentiation
MDD	Major depressive disorder
MS	Multiple sclerosis
MV	Measles virus
NCS	Neonatal calf serum
NY99	New York 99 strain of West Nile virus
PD	Parkinson's disease

PFU	Plaque-forming units
PNS	Peripheral nervous system
PPI	Prepulse inhibition test
PRR	Pattern recognition receptor
SHIRPA	SmithKline Beecham Pharmaceuticals; Harwell, MRC Mouse Genome Centre and Mammalian Genetics Unit; Imperial College School of Medicine at St Mary's; Royal London Hospital, St Bartholomew's and the Royal London School of Medicine; Phenotype Assessment
TBI	Traumatic brain injury
TBS	Tris-buffered saline
TLR	Toll-like receptor
TMEV	Theiler's murine encephalomyelitis virus
TGF-β	Transforming growth factor- β
TNF-α	Tumor necrosis factor alpha
TST	Tail suspension test
WNE	West Nile encephalitis
WNF	West Nile fever
WNM	West Nile meningitis
WNND	West Nile neuroinvasive disease
WNP	West Nile paralysis
WNV	West Nile virus

Chapter 1: Introduction[1]

NEUROINVASIVE VIRUSES AND LONG-TERM SEQUELAE

Acute encephalitis has an incidence of up to 15 cases per 100,000 people per year in the developed world[1]. Viruses are a major cause of acute encephalitis. which causes severe damage that can limit quality of life in surviving patients. Neuroinvasive viruses are found in many viral families, including *Herpesviridae, Rhabdoviridae, Paramyxoviridae, Picornaviridae*, and *Togaviridae*. Neuroinvasive viruses often have a high mortality rate due to the sensitive nature of the central nervous system (CNS). Another common aspect of these viruses is the development of neurological sequelae following infection. The CNS is largely well-differentiated and the cells there do not replicate as much as most other organ systems. This means that immune responses are often blunted to prevent collateral damage and that damage is often permanent. These factors make the nervous system particularly susceptible to viral infections and chronic, irreparable damage. This damage can manifest sudden neurological changes and death. The damage can be more progressive and chronic than peripheral infections, which can manifest as neurological changes that affect the patient's life, termed "sequelae." The mechanisms behind the acute and chronic pathology vary from virus to virus but generally they are either induced through direct viral infection of neuronal circuits or through overt, uncontrolled inflammatory responses in the brain[2].

The incidence of viral encephalitis is estimated to be 3.5-7.5 per 100,000 people[3], though this varies based on region, industrialization level, average age, and other factors. Some of the most common neuroinvasive viruses, such as measles virus (MV) and mumps virus, have decreased in incidence with vaccination, while others, such as Epstein-Barr virus, have increased in incidence. One family of viruses, *Flaviviridae,* has many members

[1] A portion of this introduction has been previously published.

that cause high rates of encephalitis and have been showing increasing incidence worldwide. Flaviviruses are positive-sense, single-stranded RNA viruses that have often been associated with neuroinvasion. Most flaviviruses associated with neurological disease are arthropod-borne viruses (arboviruses). This relationship with arthropods makes them more likely to cause seasonal outbreaks. When these viruses become endemic, they can have high rates of incidence[3]. The family includes viruses such as Zika virus (ZIKV), Japanese encephalitis virus (JEV), and Powassan virus. West Nile virus (WNV), a flavivirus, is one of the most widespread arbovirus in the world, and is considered one of the most important causes of encephalitis in the world[4]. WNV can cause a severe neurological infection called West Nile neuroinvasive disease (WNND) in infected patients when it enters the CNS causing severe disease and possibly long-term neurological sequelae.

WEST NILE VIRUS

Virus information

WNV is a member of the family *Flaviviridae*, of the genus *Flaviviridae*. It is a member of the JEV serocomplex. WNV has a positive-sense RNA genome of roughly 11 kb. The genome encodes a single polyprotein that is cleaved into ten separate proteins using host proteases. The viral particle is enveloped in a phospholipid bilayer and is made of three structural proteins: the capsid (C) protein, the pre-membrane (prM) protein, and the envelope (E) protein. The C protein binds the RNA in the viral particle and directs viral assembly[5]. The prM and E proteins embed in the lipid bilayer and form the outer layer of the virion. The E protein provides the putative binding site for the cell surface receptor and facilitates fusion with the host membrane[6]. The genome encodes seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4Bm and NS5[4]. The nonstructural proteins serve multiple functions, including immune modulation and viral replication and assembly[7,8].

WNV is maintained in an enzootic cycle between birds and mosquitoes. Over 60 species of mosquito can carry the virus although the predominant transmitting species varies by region[9]. Over 300 species of birds can be infected with WNV, serving as reservoirs and amplifying hosts. Humans are considered dead-end hosts as their viremia does not reach high enough levels to infect mosquitoes[10]. When an infected mosquito bites a human, they can transmit the virus into the host's skin, leading to infection.

History and spread

West Nile virus (WNV) is one of the most widespread arboviruses in the world. WNV was first discovered in 1937 in Uganda. Initially, WNV was considered a mild illness and no fatalities were reported after infection[11]. A serosurvey performed in Egypt in 1950 indicated that over 70% of those older than four years old at testing had antibodies against WNV, indicating widespread infection, though only mild disease was reported[12]. In 1951, WNV was isolated in Israel[13], with more outbreaks occurring in 1952 and 1953[4,14]. Seropositivity for WNV was first found in Asia during testing in India in 1954[15]. Neurological involvement of the virus was first reported in 1957 in an outbreak in Israel[16]. An outbreak of WNV occurred in Albania in 1958 as evidenced by serosurvey, and further outbreaks occurred in France[17]. Further evidence of spread in Europe was detected in other Mediterranean countries in the 1960s and 1970s. The virus was detected spreading further in Asia when WNV was detected in Myanmar in 1966[18]. Antibodies against WNV were detected in Iran in 1970[19], and in Turkey[20] and Israel[21] in 1977, indicating active transmission of the virus in those regions. More severe outbreaks associated with neuroinvasive disease occurred in Ukraine in 1985, followed by epidemics in Romania in 1996[22] and in Russia in 1999[23], all of which showed evidence of neurologic disease. The outbreak in Russia led to hundreds of patients diagnosed with WNND. Another outbreak occurred in Turkey in 2010 with 40 reported cases of WNND[24]. From 2010-2012, there

were outbreaks in northern Africa, including Morocco[25], Tunisia[26], and Egypt[27], although only mild symptoms were reported following infection in these cases.

WNV spread to the U.S. in 1999. An outbreak that began in New York state led to 62 reported cases and seven deaths, though an estimated 2.6% of the population near New York City were infected[28]. The starting point of this outbreak has not been determined, but the initial strain is closely related to one found in Israel[29], and is believed to have been brought in via an infected bird or mosquito[30]. The strain isolated from this outbreak (NY99) was associated with a large number of cases of viral encephalitis. While the initial outbreak was limited to the state of New York, the virus was found in multiple surrounding states within a year (**Fig. 1**), including human cases[31]. By 2001, WNV was found across the Eastern seaboard and into the Midwest. By 2002, WNV had spread to almost all the continental U.S. with most of those states reporting human cases. Since then, WNV has become endemic across the continental U.S. It is difficult to determine the number of infections, but there have been consistently

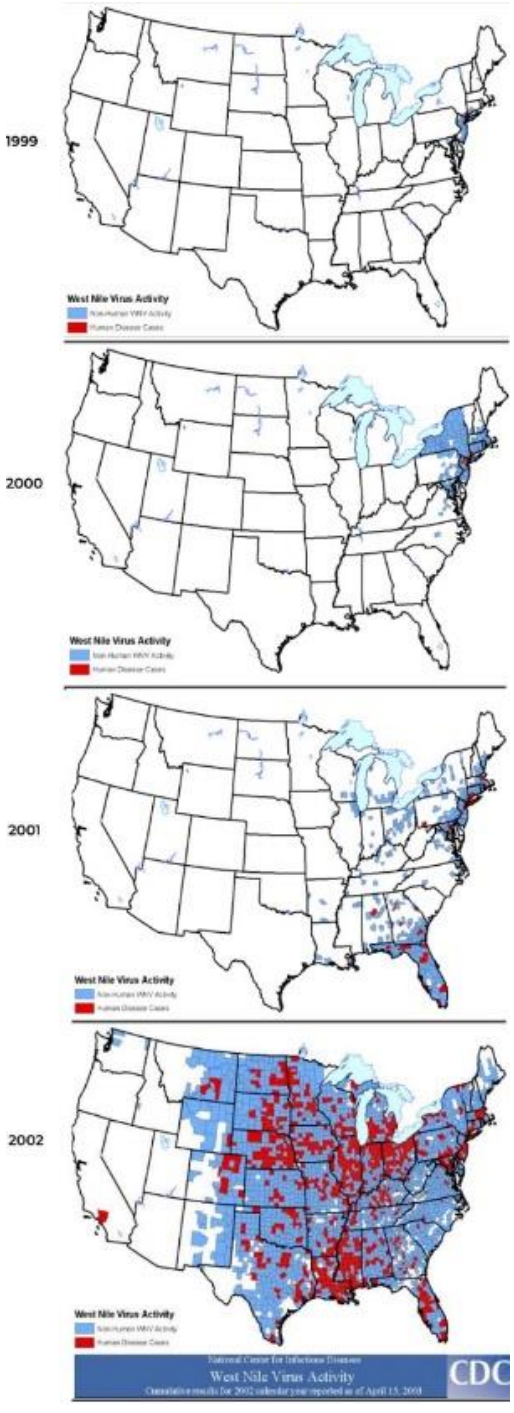


Figure 1: Spread of WNV through the U.S. from 1999 to 2002. Blue areas indicate detection in non-human species Red (darker) areas indicate human disease cases. Adapted from Roehrig, Viruses, 2013.

reported cases of WNND since WNV's introduction to the U.S. Since 2012, there have been at least 1000 confirmed cases of WNND per year in the U.S. The distribution of viral infections in each region varies from year to year, though certain states such as Texas, Colorado, and California regularly have higher numbers of cases. WNV has also been found in Canada and parts of South America indicating a wide dispersal of the virus. WNV is now endemic in the U.S., with large numbers of infections every year. Some regions of the U.S. show higher incidence of WNND, though this varies from year to year (as seen in **Fig. 2**). The factors that lead to these outbreaks are still being determined.

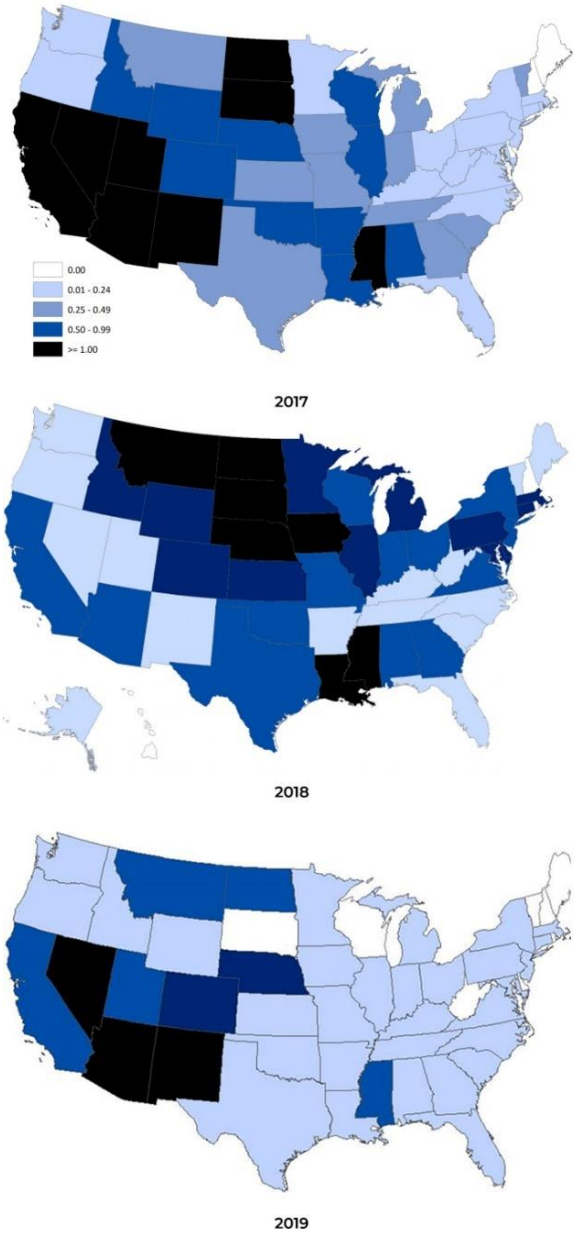


Figure 2: Incidence of WNND by state, 2017-2019.
Colors indicate number of cases of WNND per 100,000 population each year. Image modified from ArboNET, Arboviral Diseases Branch, Centers for Disease Control and Prevention.

Outside of the Americas, WNV causes outbreaks in Africa, Asia, Australia, and Europe, leading to thousands of cases of WNND each year. There have been numerous outbreaks in Europe, including in Ukraine, Romania, Russia, Italy, and France. Since the 1990s, outbreaks with neurological involvement have become more prevalent in European countries, and the cases have become reported in more regions, including parts of Siberia[4].

Viral neuropathogenesis

Based on mouse models, WNV initially replicates in the skin after transmission from the infected mosquito, and then uses resident phagocytic cells to spread to the lymph nodes. The virus enters the bloodstream via the thoracic duct and enters the CNS around five days post-infection (dpi). The virus is cleared from the peripheral organs between six and eight dpi, though it is replicating in the CNS during this time[32].

WNV has been shown to enter the CNS through multiple pathways, including transsynaptic spread, breakdown of the blood-brain barrier (BBB), transendothelial spread, and infection of immune cells that cross into the brain parenchyma[33]. The roles that each of these forms of entry play in overall pathogenesis are still being studied and may not be shared across all strains of WNV. Endothelial cell infection has been shown *in vitro*[34] but has not been recapitulated *in vivo*. WNV infection has been shown to decrease surface expression BBB molecules important to maintaining cell-cell adhesion including claudin-1, occludin, JAM-A, and β-catenin, with an increase in matrix metalloproteases[35]. This leads to increased permeability through the BBB and increased extravasation of immune cells. This has been attributed as a potential cause of direct viral crossover into the CNS and as a mechanism for infected peripheral cells to enter the CNS. Axonal transport of WNV has also been shown in hamsters, and WNV appears to be able to move both retrograde and anterograde[36], indicating another possible mechanism for spread to the CNS.

The envelope protein of WNV mediates entry into cells through clathrin-mediated endocytosis[37], with the WNV envelope protein as the receptor-binding protein. The receptors for the virus have not yet been determined, though there is evidence that DC-SIGNR[38], $\alpha_v\beta_3$ integrin[39], and laminin-binding protein[37] have been shown to facilitate entry into cells. Within the CNS, the virus primarily replicates in neurons[40], but there is evidence that it can infect glial cells including astrocytes[41].

The immune response to WNV in the CNS starts with recognition of the virus through pathogen recognition receptors including Toll-like receptors (TLRs). Signaling through the TLRs is necessary for the survival of the host through the acute phase of WNV infection[42]. TLR3 has been shown to be protective in infection[43] but also crucial for allowing viral entry into the CNS[44]. TLR7 is important for both innate and adaptive immune responses[45]. These initially signal through microglia, which leads to the recruitment of peripheral immune cells including monocytes and T cells. The type-I interferon (IFN) response is crucial to the survival of the host during the acute phase of infection. Necessary elements include upregulation of Ifitm3[46,47] and interferon-response factor-3[48] and -7[49], all of which reduce viral load in the CNS.

WNV has been shown to induce the accumulation of misfolded, ubiquitinated proteins[50], which leads to neuronal malfunction and death. This has been linked specifically to the activity of the capsid protein, which inhibits the autophagy pathway by inducing degradation of AMP-activated protein kinase[51].

There are multiple lineages of WNV, which is may play a role in the development of long-term neurological sequelae resulting from WNV infection. The most widespread is lineage 1, which is distributed globally. Lineage 2 is the next most common and is primarily found in Africa and Europe[52]. The neuropathogenicity varies from strain to strain within a lineage. The outbreak in New York in 1999 was of lineage 1 and led to 63% of those affected showing ongoing clinical signs one year post-infection[53]. In addition, the strains of WNV present in the U.S. have been shown to be mutating with potential changes to pathogenicity[54]. Some variants show different virulence and pathogenicity in hamsters[55], mice[56,57], and birds[57,58], but whether these lead to changes in long-term outcomes remains to be seen. The Kunjin subtype from Australia is also grouped in lineage 1, and shows little to no neurovirulence[59–61]. The Kunjin strain shows significant differences from the more pathogenic IS98 strain[61]. While the IS98 strain of WNV is more lethal in mice, the Kunjin virus induced neuronal apoptosis in the brain in many regions. The hippocampus showed

different areas of susceptibility as well: Kunjin virus localized more to the CA1 region, while IS98 localized more to the dentate gyrus. The brains of mice infected with Kunjin showed greater levels of T cell infiltration and inflammatory lesions in the cortex striatum. This indicates that apoptosis and inflammatory cell infiltration are crucial for early control of the virus and that evasion of these responses can dictate viral pathogenicity.

Some WNV strains from lineage 2 have been shown to be neuroinvasive and neurovirulent based on data from outbreaks in Russia, Greece, Italy, and Hungary[62–65]. One follow-up study in Greece showed neurological sequelae of similar frequency and severity to the U.S. outbreak one year after infection with a lineage 2 strain of WNV[66]. In experimental models, the lineage 2 strains of WNV are generally less neuroinvasive in hamsters and mice, though this varies based on strain[67]. Whether infections with different strains of WNV lead to different risks of the development of sequelae is still being determined.

Clinical information

Blood donations have been used to screen for the rate of symptomatic cases of WNV. The majority (70-75%) of human WNV infections are asymptomatic[68,69]. About one in four infections manifests as a non-specific, flu-like illness called West Nile fever (WNF). In roughly one in every 150 infections, the virus invades the CNS, causing a severe neurological disease called West Nile neuroinvasive disease (WNND)[70,71]. Cases of WNND manifest as one or more of the following, based on the spread of the virus: West Nile encephalitis (WNE), infection of the brain parenchyma; West Nile meningitis (WNM), infection of the meninges; and West Nile paralysis (WNP), infection of the spinal cord leading to poliomyelitis[72]. These are diagnosed through a combination of clinical signs and laboratory confirmation of viral infection of the CNS, including analysis of cerebrospinal fluid for virus or antibodies against the virus[70,73]. Based on U.S. data, WNND has a 10% case fatality rate[74,75]. Treatment is generally limited to supportive care, though

there have been successful, small-scale trials using intravenous gamma globulin from survivors of WNV infection[76,77]. Since 1999, there have been over 20,000 diagnosed cases of WNND in the U.S., with over 1,000 cases per year each year since 2012[75].

WNV can infect many different regions of the CNS. Post-mortem, viral proteins have been detected via immunohistochemistry (IHC) in the hippocampus, cerebellum, basal ganglia, thalamus, midbrain, and pons[78,79]. The cranial nerves and spinal nerves may be involved, as well[80]. WNV infection causes neuronal cell death, gliosis, reactive astrocytosis, perivascular cuffing, and infiltration of monocytes and lymphocytes from the peripheral blood into the CNS[78]. Outside of the brain, WNV has been localized to the spinal cord, dorsal root ganglia, and peripheral motor neurons[81].

WNV-Induced Neurological Sequelae in Patients

WNV infection can induce severe, life-altering neurological sequelae in patients that survive infection. The prevalence of sequelae among survivors of WNV infection varies between studies, but it is generally between 30% and 60%[82–85]. The most commonly reported sequelae include memory loss, muscle weakness, depression, and fatigue[86] (**Fig. 3**).

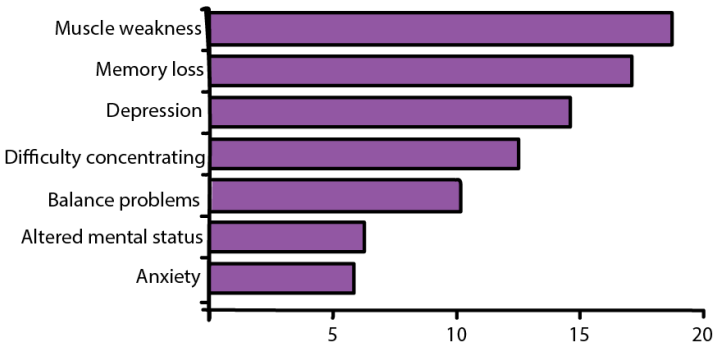


Figure 3: Commonly reported neurological sequelae following WNV infection in patients relevant to our behavioral studies.
The y-axis represents percentage of patients reporting the listed sequelae post-infection, from 19 studies.
Source: Patel et al, 2015. Lancet

These may last for months or for the rest of the patient's life. The mechanisms that underlie these sequelae are poorly understood.

Some factors have been conclusively linked to an increased risk of developing WNND during WNV infection, including old age[87], immune dysfunction, concurrent chronic diseases such as diabetes or hypertension[88], and mutations in the CCR5 gene[89]. Old

age appears to increase the risk of developing sequelae[82,86,90], while younger age correlates with a greater chance of full recovery[91]. Gender appears to play a role as well: men are less likely to develop depression[92], and men tend to recover cognitive function faster than women[83]. This may be due to the difference in immune response to WNV between men and women. Men maintain a more prolonged elevation of peripheral cytokines and reported fewer symptoms during the acute phase of illness[93], but this has not yet been correlated to incidence of sequelae.

Whether the initial clinical presentation reflects the risk of WNV-related neurological sequelae is controversial. It has been reported that sequelae occur more commonly in patients diagnosed with WNND[94]; however, it has also been reported that the severity of initial disease, including hospitalization, does not increase risk[95]. Specific to WNND, those with WNM tend to show complete recovery, while those with WNE and WNP have greater risks of long-term sequelae[96]. WNE tends to be associated with the highest risk of developing long-term neurological sequelae, with up to 86% of patients presenting with abnormal neurological exams up to three years post-infection[97].

Initial CNS invasion does not appear to be necessary for development of sequelae, as patients with WNF have reported development of neurological sequelae. One study showed that 27% of patients diagnosed with WNF without diagnosed WNND had neurological abnormalities up to three years post-infection[97]. When re-interviewed between eight and eleven years post-infection, 57% of subjects reported new abnormalities. Neurocognitive sequelae appear to occur equally in patients diagnosed with WNND compared with patients diagnosed with WNF[86,98], or at an increased rate[94,99,100], depending on the study. Other studies have shown that patients diagnosed only with WNF have measurable deficits in one or more neuropsychological functions, including memory, executive function, depression, and motor coordination over one year post-infection[95,100]. One notable difference is that motor sequelae persist more in patients initially diagnosed with WNND[84,95]. Most of the studies agree that neurological sequelae do occur in some

patients after WNF. This could indicate either that the virus can enter the CNS without causing overt clinical signs of neurological disease, or that peripheral inflammatory responses lead to neurological malfunction. Further research needs to be done to determine the extent of CNS inflammation in patients with sequelae following WNF.

The most commonly reported sequelae are associated with motor functions, including fatigue, myalgia, and generalized or limb weakness[86]. Motor issues are generally attributed to damage to peripheral motor neurons or the dorsal horn of the spinal cord, from which sensory neurons emanate. In cases where the patient succumbed to infection, the virus has been found in the cerebellum and substantia nigra[81]. The cerebellum is responsible for fine motor coordination[101], while the substantia nigra regulates the initiation of voluntary movements[102]. Damage to either region could lead to motor incoordination or deficits. In cases of WNP, about one-third of those affected attain complete or near-complete recovery, one-third show partial recovery, and one-third show little to no improvement[84]. Weakness tends to persist even when other symptoms resolve[72]. In those without complete recovery, the weakness is generally not associated with sensory loss. One study[103] indicated that, despite reporting weakness and numbness of the extremities, about one-fifth of the patients showed normal electromyelography (EMG) results, indicating that the peripheral weakness and numbness was likely of central origin. In the remaining 80% of patients, the abnormal EMG results were attributed to WNP, neuromuscular junction disorder, or sensory/sensorimotor polyneuropathy. Two of the patients showed signs of chronic and active denervation, indicating an ongoing inflammatory process. Patients with long-term paralysis or weakness often show decreased motor amplitudes on electrodiagnostics. This indicates damage to motor neurons or cells of the anterior horn of the spinal cord[104] which may be due to excitotoxic mechanisms or persistent viral infection. As with other sequelae, the severity and persistence of motor deficits vary from patient to patient. These findings indicate that WNV can cause motor weakness through both central and peripheral mechanisms, though the exact mechanisms need further study.

Fatigue is a commonly reported sequela in WNF/WNND patients[86]. One reported mechanism is the persistently elevated levels of pro-inflammatory cytokines up to five years post-infection[105]. Some of the cytokines correlated with fatigue included IFN-γ, IL-2, and IL-6. Besides the roles these cytokines play in controlling acute WNV infection[64,106,107] and in ND, they have been implicated in chronic fatigue syndrome (CFS). CFS has previously been associated with viral infections[108], including Epstein-Barr virus and herpesvirus-6. Elevated levels of IFN-γ have been associated with CFS[109], and higher levels of the cytokine in serum correlates with increased severity of disease[110]. Plasma IL-6 is elevated in patients with CFS[111], and has also been associated with major depressive disorder (MDD)[112] and fibromyalgia[113]. These studies indicate a common pathway leading to similar symptoms in patients suffering from CFS and those reporting fatigue after WNV infection.

Memory loss is a reported in almost of 20% of patients surviving WNV infection[86]. One study[96] correlated neurological function (via neuropsychological testing) with the MRIs of patients who survived either WNF or WNND. Patients ranged from three to eight years post-infection. Almost half of the participants had abnormal neurological findings, including weakness, abnormal reflexes, tremors, and immediate or delayed memory loss. One-fifth showed neuropsychological impairments including short-term and long-term memory deficits. A subset of these patients received an MRI, which showed cortical thinning in multiple brain regions, including the posterior cingulate cortex, superior frontal cortex, and the para-hippocampal region. Memory loss correlated with the thinning of the caudal middle frontal gyrus, rostral middle frontal gyrus, and supramarginal gyrus of the left hemisphere only. The middle frontal gyrus plays a role in maintaining attention[114] and the supramarginal gyrus is involved with multiple processes including cognitive functions[115], both of which could account for changes in memory function. Although the functional changes only correlated with thinning of the cortices in the left side, it seems unlikely that laterality would always play a role. This study was based on 30 patients, so

whether these regions are specifically affected or play a significant role in neurocognitive sequelae needs to be studied further.

Patients have reported developing depression after WNV infection to the level of MDD. The incidence of MDD among WNV survivors varies between different studies, but ranges between 21% and 56% of patients[83,85,92,116]. In one study, 75% of patients with reported depression scored positively for mild to severe depression using the Center for Epidemiologic Studies Depression scale[116]. However, one study reported that MDD occurred at higher rates in patients diagnosed with WNF compared to those diagnosed with WNND[72], though this has not been consistent across all studies. There have not been any studies investigating potential mechanisms for depression in patients surviving WNV infection. Many other viruses have been associated with depression following infection, including influenza, varicella-zoster, human immunodeficiency virus[117], herpes simplex virus 2, and cytomegalovirus[118], and the mechanisms by which these viruses cause depression should serve as a starting point for WNV research..

Whether the virus persists in the human CNS after the acute neuroinvasive infection has not been sufficiently demonstrated. WNV has been shown to persist in the CNS of an immunocompromised patient for up to four months post-infection[119], and there is indirect evidence for persistent infection in that some patients maintain high levels of anti-WNV IgM in the cerebrospinal fluid and blood[120]. It appears that WNV can also persist in the kidneys of some patients with other underlying chronic conditions for years after infection, and people with viral persistence in their kidneys tend to have higher rates of neurological sequelae[121]. This may be due to consistent viral shedding or a more disseminated infection in these patients. Further research needs to be done to determine how often the virus persists in patients and how this impacts the development of sequelae. However, technical limitations to CNS virus detection are an impediment. Detection of live, replicating virus or viral nucleic acids indicating persistent infection requires invasive procedures such as biopsies that allow for histopathologic or molecular (RT-PCR) analyses targeting the virus.

Biopsies of the brain or spinal cord require full anesthesia and present significant risk to patients. Pre-mortem, non-invasive methods to detect WNV are needed to determine the persistence of the virus and what role it plays in the development of sequelae.

Animal models of WNV infection

Many vertebrate species can be infected with WNV. These species show active replication of the virus in different organs leading to varying levels of disease severity, viral replication, and organ tropism for the virus. Different species show different susceptibility to the virus, meaning some serve as better models than others.

Passerine bird species serve as a reservoir and amplifying host for WNV, as the virus can replicate to high titers in several species[122]. More than 300 avian species can be infected with WNV[123]. Different species show different susceptibilities to infection. Corvids, such as crows and ravens, are susceptible to infection, showing high rates of mortality and viremia[122,124], and likely serve as an amplifying host. Death in corvids is attributed to multiorgan failure, as there are largely not lesions in the CNS[125,126]. Other species such as sparrows and house finches develop similar levels of viremia, but do not show the same levels of mortality[122,127]. These species are some of the most important hosts of the virus, as they breed in large numbers, reach high levels of viremia, and do not die of the virus.

WNV commonly infects horses in regions where outbreaks occur. Though they are dead-end hosts for the virus, they can manifest severe disease including WNND. They have been found to be infected in outbreaks since the 1960s[17]. More than 28,000 cases of WNV in horses have been reported in the U.S. since the initial outbreak in 1999. The percentage of symptomatic infections is around 10%, similar to that in humans. When they do show signs, horses develop severe fevers and neurological signs such as paresis, tremors, and skin and muscle fasciculations. The case fatality rate during the initial outbreak was 38%, but is now estimated to be between 38% and 57%[128]. Horses are the only species with

licensed vaccines against WNV at this time, including a recombinant canarypox-vectored vaccine and an inactivated whole-virus vaccine[129]. Although horses recapitulate many of the signs of human disease, the expense of the animals and necessity for specialized housing make it an unlikely model for research.

Dogs and cats can be infected with WNV, but rarely show clinical signs. Both species can become viremic in response to infection, but do not seem capable of transmitting it to mosquitoes[130].

Sheep and alpacas are susceptible to WNV-associated neurological disease. Alpacas appear more susceptible, showing clinical disease roughly on par with horses[128,131,132]. Sheep show lower clinical attack rates but viral infection has been associated with abortion, stillbirth, and teratogenesis[133].

Non-human primates (NHPs) are generally considered the gold standard for studying viral diseases of humans due to their genetic similarities to humans. NHPs have been used for studying WNV but their use is limited as they largely do not show signs of neuroinvasion[134–136]. The virus does replicate and is able to persist in the CNS of NHPs, but does not show the severe signs associated with human disease. WNV antibodies have been detected in NHPs in laboratory and natural settings. This included a primate research center in Louisiana that found that 36% of the NHPs screened showed antibodies to WNV during an outbreak, though all animals were asymptomatic[137]. One case of an aged macaque developing encephalitis after WNV infection has been reported[138]. In the wild, gorillas and mandrills in the Congo Basin have shown neutralizing antibodies to WNV[139]. Given the lack of clinical signs, it is unlikely that NHPs would serve as a good model of WNV-induced neurological sequelae.

Rodent models provide an attractive option for testing putative mechanisms of WNV-induced CNS damage. WNV commonly invades the CNS of rodents, including mice and hamsters, resulting in disease outcomes that align well with human WNV pathology. In mice[61] and hamsters[140], it has been shown that WNV infects similar regions of the brain,

spinal cord, and peripheral nerves, as reported in humans. WNV infection also causes similar pathologic changes in rodents as those found in human cases: inflammatory peripheral immune cell infiltration, reactive astrocytosis, neuronal cell death, and gliosis[141]. Specific types of hamsters and mice show survival post-infection, allowing for the study of neurological sequelae. Established neurobehavioral tests can be used to study WNV-induced neurological sequelae. The numerous genetically modified mouse strains allow for investigating the roles of specific genes in the occurrence of these sequelae.

The hamster model of WNV infection was initially used to study the acute phase of disease, but hamsters have since been used to study other aspects of WNV infection. Hamsters can have persistent WNV infection in the CNS and in the kidneys, like humans. The virus has been found in the CNS for up to three months post-infection and in the urine up to eight months post-infection[142]. Behavioral testing on hamsters after the acute phase has shown long-term neurological deficits in memory[143] and motor function[144]. Memory function was found to improve with treatment using a WNV-neutralizing monoclonal antibody administered at four dpi. Four dpi is after the virus invades the CNS, indicating that initial viral invasion is not the only cause of memory loss. Motor weakness correlated with motor neuron death between 10 and 26 dpi. Viral RNA and envelope protein were detected in the cortex, hippocampus, midbrain, cerebellum, and spinal cord of hamsters up to 90 dpi, indicating an active infection in the CNS. The WNV envelope protein was primarily detected in regions of inflammation, indicating an association between ongoing infection and the potentially damaging inflammatory response[144].

The mouse is a commonly used animal model of WNV infection, and the C57BL/6J strain is commonly employed. Outcomes from multiple studies suggest that WNV can persist in the CNS of infected mice and that the host maintains an immune response against it. Live, replicating WNV has been recovered from the brains and spinal cords of mice up to four and six months, respectively, after peripheral inoculation with WNV[145]. Similarly infected mice had increases in B cells and T cells in the brain up to 12 weeks post-infection.

Antibody-secreting cells specific for WNV and virus-specific T cells were found in the brain up to 16 weeks post-infection. These cells were found in mice with and without clinical signs during the acute phase of infection[146]. These studies indicate that WNV can persist in the CNS and that the host maintains an immune response against it.

WNV appears to have multiple mechanisms to enter the CNS. There is evidence that it can travel transsynaptically, from peripheral neurons to the brain[147,148]. It also induces matrix metalloprotease production in inflammatory cells that break down the BBB[35,149]. There is also evidence that the virus can infect inflammatory cells such as monocytes, neutrophils[150,151], and T cells[152] that cross into the CNS, which allows for spread through what is called the "Trojan Horse" mechanism. WNV has been shown to infect endothelial cells like those lining the BBB *in vitro*, and can cross the endothelium to allow for neuroinvasion[153]. This finding has not been recapitulated *in vivo* in mice or horses[33]. Administration of IFN-λ was shown to reduce BBB permeability and WNV neuroinvasion in a mouse model, which was attributed to tightening of the BBB via increased tight junction protein expression[154].

The exact cellular and molecular mechanisms by which WNV induces cell death and inflammation are still being determined. In the CNS, WNV generally only infects neurons[155], leading to varying degrees of apoptosis and necrosis. This is likely due to variations in neuronal phenotype. Different neuronal subtypes have shown different basal levels of cytokines, which can affect their susceptibility to WNV[156]. Some evidence suggests that it infects astrocytes in humans[157]. This has been recapitulated *in vitro* using primary mouse astrocytes[158], though cultured astrocytes show different activity than those *in vivo*. WNV has been shown to induce the accumulation of misfolded, ubiquitinated proteins leading to neuronal malfunction and death[50]. The related tick-borne encephalitis virus shows a similar mechanism by causing protein aggregation in the presynaptic regions of neurons[159].

One mouse model that has provided unique insight into the pathogenesis of WNV-induced memory loss used a recombinant, engineered virus with an introduced mutation in non-structural protein 5 (NS5). The NS5 protein protects the virus from the IFN response by generating a 5' cap on viral RNA using a 2'-O methyltransferase[160]. This mutation attenuated the viral antagonism of the IFN response by removing the activity of the 2'-O methyltransferase[161]. Following direct inoculation into the cerebral ventricles of C57Bl/6J mice (i.e. direct delivery of the virus to the brain), the NS5 mutant caused reduced mortality compared to wild-type WNV. When infected mice were tested for hippocampus-dependent memory function 46 dpi with the Barnes maze, they performed worse than uninfected controls. Post-mortem analysis of these mice demonstrated that WNV-induced memory loss was caused by microglial phagocytosis of presynaptic termini in the CA3 region of the hippocampus, which was confirmed when mice lacking microglia did not demonstrate memory loss. Mechanistically, it was demonstrated that the complement protein C1q bound the termini to promote phagocytosis[162]. The microglia were stimulated to perform phagocytosis through IFN-γ that originated from CD8+ T cells that had infiltrated the CNS[163]. This indicates that peripheral immune cells crossed the BBB, though whether this is a continuous process has not been determined. This infection model also exhibited astrocyte release of interleukin-1 beta (IL-1β, leading to diminished neurogenesis and increased astrocytosis in the hippocampus[164].

WNV and neurodegenerative diseases

One intriguing aspect of WNV infection is its overlap with neurodegenerative diseases (NDs) such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and multiple sclerosis (MS). Animal models show that survival of the acute phase infection of the CNS with WNV requires an inflammatory response in the brain, including the production of cytokines[106,165] and the infiltration of circulating monocytes and T cells[166]. Investigating the neuropathology of WNV infections as a form of ND and

potentially labeling the sequelae as a ND may be a good first step toward helping patients recover from this debilitating disease.

NDs and WNV infection appear similar pathologically. Microglial nodules have been found in the brainstem, midbrain, medulla, and pons of WNV patients[81] and in the hippocampus of mice post-infection[162]. Microglial nodules[167] and perivascular cuffing[168,169] are commonly found in cases of MS. WNV has been associated with PD-like signs, including damage to the substantia nigra[170], similar to PD. JEV, which is a member of the same serogroup as WNV, shows tropism for the substantia nigra in patients and a rat model[171]. Inflammatory T cells infiltrate the brain in both WNV and in NDs[172]. In NDs, these T cells can induce neuronal cell dysfunction[173] and death[174], activation of inflammatory cells[175], and pro-inflammatory cytokine release[176]. Pathogenic T cells have been shown to induce pathologic synaptic pruning in a mouse model of WNV[163], and may contribute to long-term damage in other ways.

Permeability of the BBB is a hallmark of NDs because BBB dysfunction allows for increased movement of immune cells and pro-inflammatory molecules into the CNS, which exacerbates neurological damage[177]. However, none of the studies about long-term neurological sequelae following WNV infection have investigated the role the BBB plays. Given that WNV has been shown to affect the BBB so dramatically, it seems likely that this plays a role in ongoing neurological damage.

WNV infection has been shown to disrupt normal protein turnover through inhibition of the autophagy pathway[50,51]. This mechanism has been shown to cause neurodegeneration in a model of human immunodeficiency virus (HIV) as well[178]. NDs are often associated with dysregulated protein turnover[179]. WNV has been shown to induce the production of proteins associated with NDs. Alpha-synuclein (α-syn) is a presynaptic protein that is associated with PD. It forms into protofibrils that are toxic to neurons and its secretion can cause damage to neighboring cells[180]. Accumulation of α-syn causes neuronal apoptosis and can decrease neurogenesis.[181] In a mouse model, α-syn becomes

elevated in the brain after infection, and is protective against WNV infection[182]. This may indicate that it has a role as an antiviral peptide, and that continued secretion of α-syn leads to pathogenic responses.

AD is characterized by the formation of plaques made of amyloid beta peptide (Aβ) and neurofibrillary tangles of tau protein[183]. These proteins, when aggregated, can disrupt neuronal homeostasis and induce cell death. *In vitro*, WNV has been shown to induce Aβ production in human neurons[184] indicating a possible connection. A tau neurofibrillary tangle was found in two patients who succumbed to WNV infection[170,185], though whether this was linked to the infection has not yet been determined.

The TLRs involved in the initial recognition and response to WNV in the brain have been linked to exacerbation of NDs. Artificial activation of TLR3 increases Aβ levels in the hippocampus and causes cognitive deficits in mice[186], and is elevated in microglia near Aβ rich plaques in AD patients[187]. TLR7 expression has been associated with regions of neurodegeneration in mouse models of AD[188] and amyotrophic lateral sclerosis (ALS)[189]. Activation of TLR7 can induce neuronal apoptosis and microglial activation in the brain[190]. Mice infected with WNV show increased levels lipocalin-2[191] and α-synuclein[182] in the brain. Lipocalin-2 is normally a bacteriostatic protein, but in the brain it is released from astrocytes and acts as a neurotoxic molecule[192]. α-synuclein is protective against neuroinvasive WNV infection, but is also associated with PD, where it forms neurofibrillary tangles responsible for neuronal degeneration and death[180].

Many of the cytokines that are important for surviving acute WNV infection can have detrimental effects on normal CNS function. IL-1β[165], IFN-γ[106], CCL2, and tumor necrosis factor-alpha (TNF-α)[166] have been shown to be essential in early antiviral activities against WNV infection. These same cytokines have been associated with NDs, causing peripheral immune cell infiltration[193], glutamate toxicity[194], and neuronal dysfunction and death in NDs[195] including AD, PD, HD, and MS. IL-1 signaling has been shown to exacerbate dopaminergic neurodegeneration in mice[196], and IL-1β induces

excitotoxic neurodegeneration in MS patients[197]. IL-1 signaling recruits peripheral leukocytes to the CNS[198]. Despite IL-1β being protective during the acute phase of WNV infection, it reduces hippocampal neurogenesis and increases astrocytosis in the long-term, leading to memory loss[164]. To be activated, pro-IL-1β needs to be cleaved by an activated inflammasome. Given that the inflammasome is activated in both NDs and in acute cases of WNV infection, it is worth investigating if there is chronic inflammasome activation in any model of WNV-induced neurological sequelae. The formation of the inflammasome through NLRP3 activation is necessary for viral clearance[165] but activation of the NLRP3 inflammasome has also been shown to exacerbate NDs such as AD[199], PD[200], and MS[201]. TNF-α and IL-1β have also been shown to be released from pro-inflammatory, neurotoxic astrocytes in NDs[202] with genes that mark these specific astrocytes upregulated in a model of WNV infection, and that these astrocytes are the primary source of IL-1β post-WNV infection[164]. IL-2 appears to play a more beneficial role during AD, through stimulation of immunomodulatory T cells and the reduction of amyloid plaques[203]. IFN-γ is associated with increased neurodegeneration in a stroke model[204] and increases microglial activation and peripheral monocyte infiltration[205], which can be protective or detrimental, depending on the model[206]. IFN-γ can also induce neuronal apoptosis via astrocyte production of neurotoxic molecules[207], and direct apoptosis in the neurons in response to Aβ[208]. This all indicates that neurons may serve as bystanders in the immune response to WNV infection, and that neurodegeneration occurs in response to the pro-inflammatory cytokine signals necessary for control of WNV. It may also indicate that persistent immune stimulation due to persistent viral infection or stimuli leads to a persistent inflammatory state in the CNS.

The elimination of synapses in the hippocampus via complement-mediated microglial phagocytosis has been found to occur in mouse models of AD[209] and hippocampal synapse loss correlates with early AD and cognitive impairment[210]. Reduced neurogenesis in the hippocampus has been found in AD[211] and in the substantia nigra in PD[212]. Decreased neurogenesis in the hippocampus has been linked with major depressive

disorder[213] and reduced neurogenesis is an early event in AD and PD in mouse models[214,215]. This seems to be a common finding in NDs and indicates a common thread among viral infection, inflammation, and neurological dysfunction.

A summary of the similarities between WNV infection and NDs in rodent models is provided in Figure 4. The animal models indicate that multiple mechanisms underlie WNV-induced neurological deficits, and that these overlap in many cases with findings in NDs. The rodent models show many of the same pathological hallmarks of WNV infection and some of the neurological deficits as are seen in human WNF/WNND patients. Memory loss seems to mimic other NDs, by inducing synaptic loss, astrocytosis, and a decrease in neurogenesis. Motor deficits seem to correlate with inflammation and persistent viral infection. However, many of the reported neurological sequelae in humans have not been reported in rodent models yet. More animal studies using a variety of behavioral, neurological, and pathological techniques are needed to understand the mechanisms underlying the long-term changes WNV can cause.

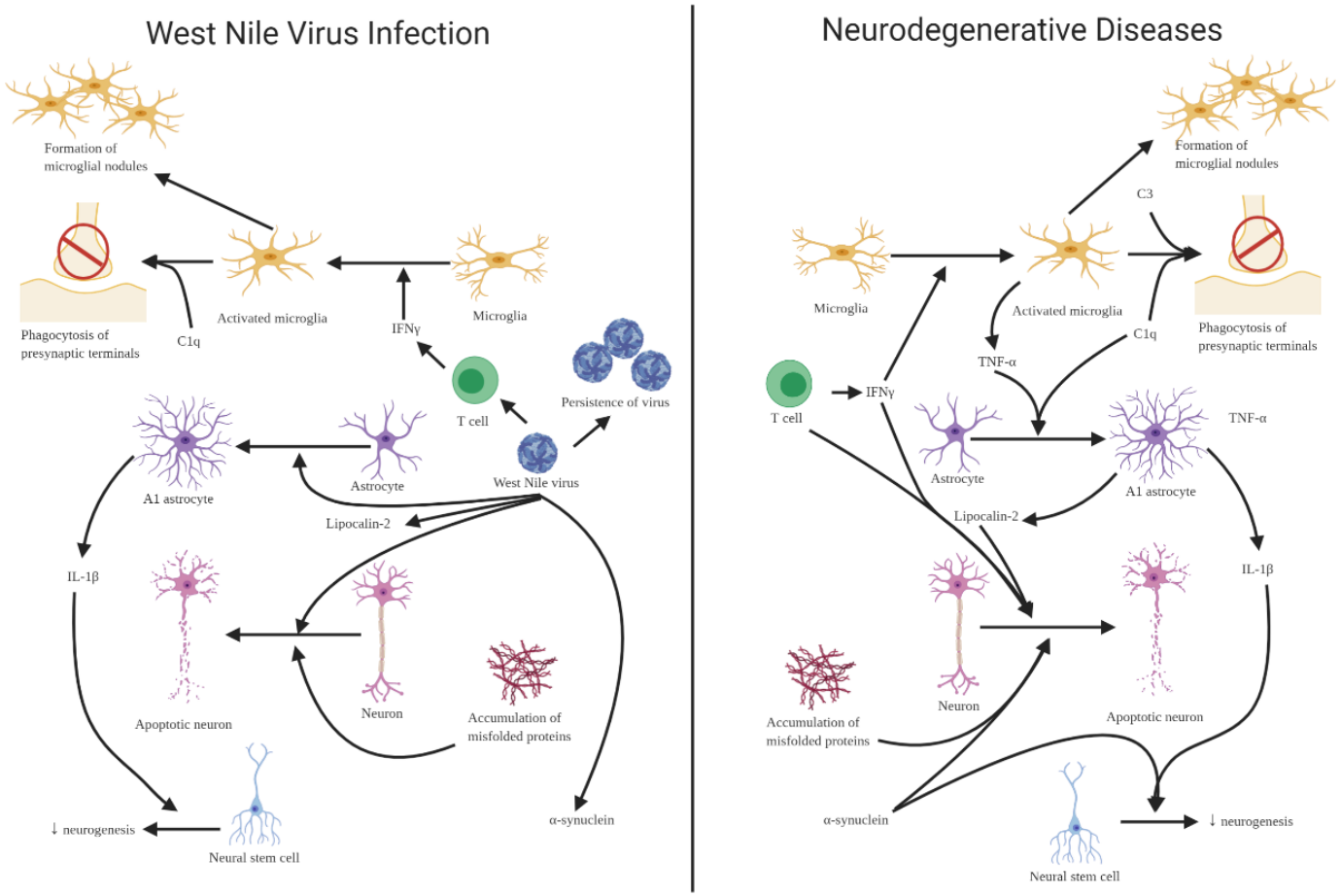


Figure 4: Comparison between WNV infection and NDs: long-term effects and pathways involved.
WNV infection (left) and NDs (right) are associated with long-term inflammation leading to neuronal damage and dysfunction. Common mechanisms between multiple NDs, including AD, PD, MS, and ALS, are included in the figure on the right. Infiltrating T cells activate microglia through IFN-γ signaling. In NDs, T cells and IFN-γ have been shown to be directly neurotoxic. In both cases, activated microglia proliferate and form nodules. The activated microglia phagocytose presynaptic terminals that are marked with C1q, and C3 in the case of NDs. This causes neurological deficits including memory loss. In NDs, activated microglia produce pro-inflammatory cytokines including TNF-α, which activates astrocytes. In both WNV and NDs, astrocytes are signaled to become A1 astrocytes, which produce active IL-1β, stimulating astrocytosis and decreased neurogenesis in the hippocampus. Lipocalin-2 is upregulated in the brain in both cases, and this has been shown to come from A1 astrocytes in NDs. Astrocyte-derived lipocalin-2 is neurotoxic in ND. Both WNV and NDs induce the accumulation of misfolded proteins in neurons, inducing neuronal dysfunction and apoptosis. α-synuclein is upregulated in both NDs and WNV infection. In ND this has been shown to cause neuronal apoptosis and decreased neurogenesis from neural stem cells.

CONCLUSIONS AND FURTHER WORK NEEDED

There has been an increase recently in the amount of research being done on long-term neurological sequelae following viral infections, including WNV. Most of this work has been done using an attenuated model that only looked at one region of the brain (the hippocampus) and one neurological function (memory). This research only reflects a small portion of the possible outcomes in patients following WNV infection and does not delve deeply into other regions that may be affected following infection.

This project was designed to investigate how wild-type, neuroinvasive WNV disperses, persists, and causes damage to the CNS in the long-term, based on an established rodent model. The aim of these studies was to perform neurobehavioral testing to determine how the virus affects function in mice and to compare this to human cases. The studies would also characterize inflammation in the brain past the acute phase of infection to study if WNV causes persistent, long-lasting inflammation similar to NDs that cause similar symptoms. Finally, these studies aimed to localize viral persistence to regions of the brain to determine if there is tropism for specific brain regions, and to see if viral persistence correlated with either inflammation or behavioral changes. These aims would investigate potential mechanisms of long-term neurological sequelae in patients and establish a reproducible mouse model that could be used for researching mechanisms and interventions.

Chapter 2: General Methods

Viruses

Two viruses were used during these experiments, both based on the consensus sequence of NY99 WNV. For all experiments using the wild-type NY99 WNV, mice were infected with stocks of first-passage WNV that had been transfected using two plasmids on Vero cells. For experiments using the G331A strain of WNV, a seed (from Maria Alcorn, PhD) was used to grow new stocks of virus using Vero cells.

Cell culture

African green monkey kidney cells (CCL-81, referred to as Vero cells) were used for all experiments, including stock generation and plaque assays. Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM; ThermoFisher, Waltham, MA, D5796) supplemented with 5% heat-inactivated fetal bovine serum (FBS; ThermoFisher, 26140), 1% penicillin-streptomycin (P/S, 100 U/ml and 100 µg/ml respectively; ThermoFisher, 15140122) at 37°C with 5% CO_2.

Generation of stocks

All viruses used in these studies originated from stocks that were generated by DNA transfection into Vero cells. These stocks were kept at -80°C until used for infection, RNA extraction, or growth of new stocks of virus.

To derive new stocks, 500 µl of a previous stock was thawed at 37°C and added to a T-150 flask containing 60-80% confluent Vero cells. These were left rocking at room temperature for one hour. After this, 25 ml of DMEM + 5% FBS + 1% P/S was added to the flask, and the flasks were kept at 37°C and 5% CO_2. Flasks were monitored daily for cytopathic effect. After three days, supernatant was removed from the cells and aliquoted into 500 µl samples. All samples were stored at -80°C then thawed at 37°C for use.

PLAQUE ASSAYS

All stocks and viral inocula were quantified using plaque assays. Samples were centrifuged at 10,000 rotations per minute (rpm) for five minutes at room temperature to clarify sample. 100 µl of clarified supernatant was added to almost confluent (70-90%) monolayers of Vero cells on 12-well tissue culture plates. Plates were left rocking at room temperature for one hour. Two mL of a solution containing one-half 1% agar and one-half 2X minimum essential medium Eagle (EMEM; ThermoFisher, 670086) + 8% heat-inactivated neonatal calf serum (NCS; ThermoFisher 16010167) + 2% P/S was applied to each well. The plates were then transferred to an incubator set to 37°C with 5% CO_2. Two days later, an additional 1 ml of one-half 1% agar and one-half 2X EMEM + 8% NCS + 2% P/S with an added 1% neutral red (ThermoFisher, N3246) was added to each well, and the plates were returned to the incubator. Plaques were visualized 24-48 hours later and counted to quantify number of plaque-forming units (pfu).

Animals

NY99 STUDIES

Eight- or sixteen-week-old female C57BL/6NTac mice from Taconic Biosciences were used for all experiments. Mice were housed five per cage. They were kept in a 12h/12h day/night cycle and provided ab libitum food and water along with nesting material. No other enrichment was provided to prevent confounding behavioral testing. All mice were kept in animal biosafety level-3 (ABSL-3) facilities. All mice were transferred to the ABSL-3 and allowed to acclimate for three days prior to any behavioral testing or other handling. All animals were kept between 20-23°C. All animal experiments were conducted in accordance with University of Texas Medical Branch Institutional Animal Care and Use Committee (UTMB-IACUC) approved protocols. For dosage studies, two groups (n=10 per group) of eight-week-old mice and two groups (n=10) of sixteen-week-

old mice were randomly assigned into different dosages: 10 or 1000 pfu (for the eight-week-old mice) or 100 or 1000 pfu (for the 16-week-old mice). For behavioral studies, a total of 68 mice were randomly assigned to either uninfected control (n=20) or 100 pfu WNV-infected (n=48) groups. All mice were tested across a total of three experiments. The first was done in conjunction with the 1000 pfu study using 16-week-old mice, and the data from ten control mice were used. The remaining two studies each had five uninfected controls and 23 or 25 WNV infected mice. One infected mouse was euthanized during the study for humane reasons unrelated to WNV infection and was not used in data analysis.

G331A STUDIES

Three-week-old female Swiss-Webster mice from Envigo Biosciences were used for all experiments. Mice were housed in the same facilities as those in the NY99 studies and were housed similarly. For these studies, two groups of mice were randomly assigned to either uninoculated control (n=5), intracranial (IC) saline inoculation (n=10), or IC G331A WNV inoculation (n=20).

Infection

For the studies using the NY99 WNNV, eight- or sixteen-week-old C57BL/6NTac mice were used. The mice were deeply anesthetized using inhaled isoflurane using an airtight, plastic chamber. Five mice were placed in the chamber at a time. Oxygen flow was set to 0.5 L/min and isoflurane dose was set to 3.5%. When mice were non-responsive to gentle prodding, they were moved one at a time and placed on a nose cone for dosing. Anesthesia depth was confirmed by pinching the hind paw of the mouse on the nose cone and looking for spinal reflexes. When spinal reflexes were abolished, the anesthesia depth was considered adequate. Virus was delivered via footpad inoculation of 10, 100, or 1000 plaque-forming units (pfu) of NY99 WNV in 20 µl of saline solution, based on previous work[145]. Uninfected controls were inoculated with an equal volume of saline solution.

For studies using the G331A WNV strain, three-week-old Swiss-Webster mice were used. The mice were deeply anesthetized using inhalant isoflurane anesthesia. The incomplete sutures of the skull were recognized visually, and 100 pfu was inoculated via IC inoculation.

Disease monitoring

All mice were weighed on the day of infection to determine baseline weight. Mice were weighed once daily until 21 dpi to monitor for weight loss. Mice that lost >25% of their body weight were euthanized. Mice were checked once daily and clinical score was recorded based in **Table 1**.

Score (1-5)	**Description of Animal**
1	Healthy
2	Ruffled fur, lethargic (triggers second observation, 6-8 hours after first)
3	A score of 2 plus 1 additional clinical sign such as hunched posture, orbital tightening, or > 10% weight loss
4	A score of 3 plus 1 additional clinical sign such as reluctance to move when stimulated, or severe neurologic signs (single limb paralysis, etc.), or >15% weight loss
5	>25% weight loss, seizures, bilateral paralysis, inability to reach food/water normally, or moribund – euthanasia

Table 1: Clinical scoring guidelines for WNV-infected mice.

If the clinical score of at least one mouse was recorded as 2 or above, a second observation of all mice six to eight hours after the first observation was performed.

Behavioral testing

All behavioral testing was performed during the light phase. All mice were handled daily prior to behavioral testing. All behavioral testing was performed in the ABSL-3 room in which the mice were housed, using a biosafety cabinet. Behavioral testing began at 21 dpi and was completed at 34 dpi.

COLD PLATE TEST

Testing for allodynia and hyperalgesia was performed in one set of mice at 7, 21, 27,40, 49, and 77 dpi. The test was performed using an Ugo-Basile hot/cold plate (Ugo-Basile, 35150, Comerio, Italy). The plate was set up inside the biosafety cabinet (BSC), and the temperature was preset to 0°C. Cages of mice were taken into the BSC and one mouse at a time was placed inside of the chamber of the hot/cold plate via the top of the chamber. A timer was started for two minutes, and the time to the first jump (defined as all four paws leaving the cold surface of the plate) and first cold-based behavior (defined as shivering or forepaw rubbing), as well as the number of jumps, were recorded. After two minutes, the mouse was removed from the apparatus via the top and replaced into its cage.

TAIL SUSPENSION TEST

Depression testing using the tail suspension test (TST) was performed 21 dpi. The tail suspension test was performed using two perpendicular bars set with one parallel to the working surface, approximately 55-65 cm above the surface. An autoclavable container was placed below the mice during testing to catch feces or the mice if the tape failed. Mice were placed inside of the BSC and suspended by their tails 55-65 cm above the work surface using cloth tape to adhere the mice to a metal pole. The tape was placed

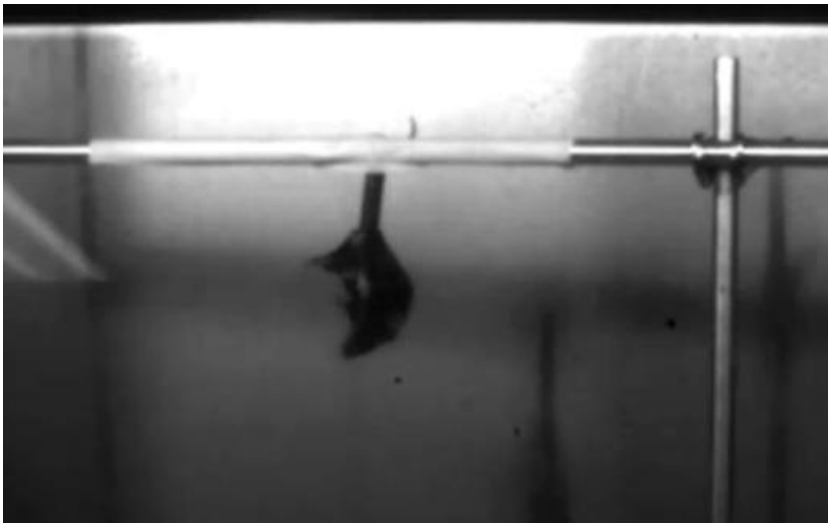


Figure 5: Example of tail suspension test layout.
Note the perpendicular bars being used as scaffolding. Cloth tape is used to hold the mouse's tail, and there is a 1.5-2.5 cm piece of plastic straw used to cover the base of the tail.

approximately halfway between the tip and base of the tail. A piece of plastic straw about 1.5 cm long was placed over the base of the tail to prevent tail climbing. Mice were recorded using a digital camera (The Imaging Source, DMK22AUC03) connected to a laptop computer in the room to record the mice for six minutes. AnyMaze software (Stoelting Co., Wood Dale, IL) was used to record video. Videos were edited to be exactly six minutes long and then renamed and randomized to prevent analysis bias. Time immobile was recorded by observer blinded to the infection status of the mice.

ROTAROD

Rotarod testing was performed using the single mouse rotarod (MK-630B, Muromachi Kikai, Co., Ltd., Tokyo, Japan) that was small enough to fit into a BSC and could easily be broken down and decontaminated when finished. For each day of testing, the rotarod was brought into the BSC and assembled, and broken down and decontaminated at the end of testing. Once assembled, the machine was placed into an autoclavable container to contain mice when they fell from the rod. Testing used the 9 cm diameter rod designed for rats. The machine was 26 cm wide x 40 cm deep x 42 cm tall with a falling distance of approximately 25 cm. The width between the flanges was 9 cm, which allowed mice to turn during testing.

Mice were screened for motivation to remain on the rotarod at 22 dpi, prior to rotarod testing, by being put on the rod, with a set rotation of 4 rpm. They were given three attempts to stay on for one minute. Each mouse received at least five minutes between each attempt, with screening being performed on each mouse before second or third attempts were made. If the mouse did not remain on for the whole minute during any of the three attempts, they were not tested for the next nine trials.

Mice that passed the screening test were tested three times daily starting at 23 dpi, for three days in a row. Each mouse was placed on the rod and given 30 seconds to acclimate. When testing was about to start, they were gently encouraged to face away from

the direction of rotation. If mice fell during the acclimation period, they were placed back on the rod and given another 30 seconds to acclimate. The rotarod began rotating at 4 rpm and accelerated to 40 rpm over five minutes. Mice that fell within the first fifteen seconds were immediately placed back on the rod and the timer was restarted. The time it took until the mouse fell was recorded. Each mouse was given at least one hour between trials with testing being performed on each mouse before the next trial was performed. Mice were allowed to turn freely during testing, and if they fell while facing toward the direction of rotation, this was recorded but considered a normal fall.

Grip Strength Test

Grip strength was measured using a grip strength meter (Ugo Basile, 47200, Comerio, Italy) with the attached t-bar for gripping. The machine was set up inside of the BSC. One mouse at a time was removed from their cage and placed into a plastic container. The grip strength meter was set to start measuring using the provided monitor. The mouse was gripped by the tail and removed from the container and dangled near the t-bar. When the mouse gripped the t-bar with both forepaws, it was slowly pulled away in a direction parallel to the work surface of the BSC. When the mouse released its grip, the grip strength meter would automatically record the maximum amount of force exerted on the bar. The mouse was given 20-30 seconds and then retested a total of three times. If the mouse only grasped with only one leg or grasped the bar with a hind leg, the mouse was given the 20-30 second rest period and then tested again until three total tests had been performed.

Two-Way Active Avoidance

The two-way active avoidance (2WAA) testing was performed using the Ugo Basile Active Avoidance shuttle-box (Ugo-Basile, Comerio, Italy, 40532) with included controller (Ugo-Basile, 40500-001). The external measurements of the shuttle box were 57 cm x 27cm x 30 cm. Each chamber of the shuttle box was 24 cm x 20 cm x 22 cm. The

shuttle box was placed inside of the BSC, with the control computer kept outside on a cart. A digital camera (The Imaging Source, Charlotte, NC, DMK22AUC03) was connected to two perpendicular poles over the enclosure to allow for visualization of testing using AnyMaze software (Stoelting Co, Wood Dale, IL). The wires connecting the shuttle box to the control computer were run over this same perpendicular rod to prevent them from pressing on the pivoting floor of the testing apparatus.

Each mouse received five sessions (one session/day, 30 trials/session). The protocol had random intertrial intervals (20-40 s) followed by a four-second tone (~75 dB) and light (conditioned stimulus, 4 s) followed by a 0.2 mA foot shock through the metal floor (unconditioned stimulus, 4 s). The foot shock turned off if the mouse transitioned from one side of the avoidance box to the other during the shock. The shock was avoided entirely if the mouse transitioned during the conditioned stimulus. These were considered "avoidances," and were recorded as a measure of memory function. One infected mouse did not move from one side for five trials in a row, and so was removed from testing, and this was recorded. An example of a test is presented in Fig. 5.

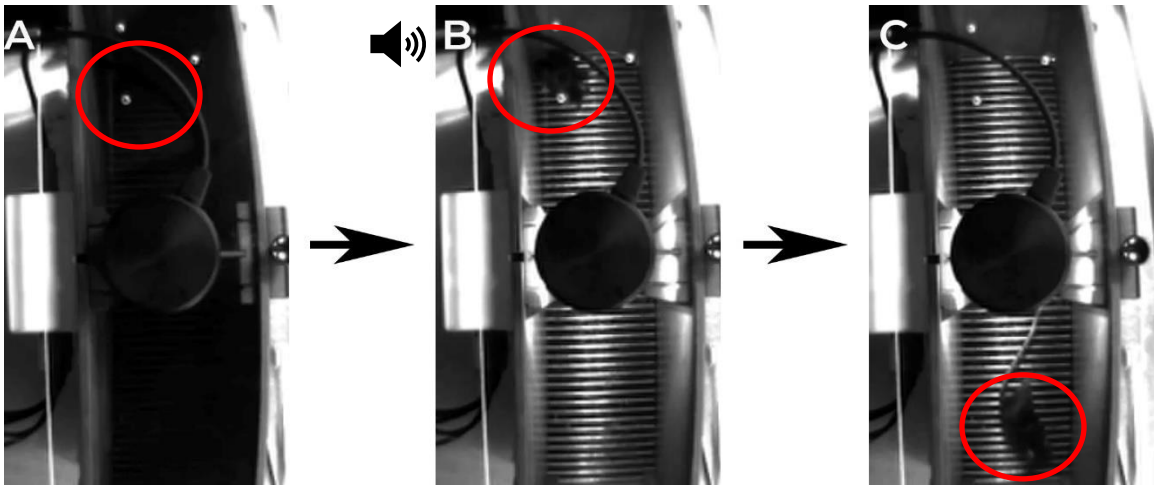


Figure 6: Example of two-way active avoidance testing.
The mouse (in the red circle) starts in the upper chamber with no stimulus (A). A light and sound go off, signaling the conditioned stimulus (B). The mouse then moves to the other chamber (C) due to either receiving a foot shock or due to learning to avoid the foot shock (considered an avoidance). This test was repeated 30 times per day per mouse.

ACOUSTIC STARTLE AND PREPULSE INHIBITION

Acoustic startle response (ASR) and prepulse inhibition (PPI) testing were performed using the SR-Lab Cabinet from San Diego Instruments (San Diego, CA, USA) and accompanying SR-LAB software. The testing equipment consisted of an ABS isolation cabinet, a small animal enclosure (internal dimensions: 12.5 cm x 4 cm) and a laptop computer that coordinated testing and collected the data. Prior to each testing session, the sound stimulus level inside of the ABS isolation cabinet enclosure was standardized using the provided software and decibelometer to ensure equal sound stimulus levels. Testing was performed outside of the BSC because mice were entirely contained, and the SR-Lab Cabinet did not fit into the BSC. Mice were loaded into the animal enclosure inside of the BSC. This was then surface decontaminated and transferred into the ABS isolation cabinet.

Each mouse was transferred to the testing chamber, which was taken out of the BSC and placed in the AS cabinet. The mouse was allowed to acclimatize to the chamber and background noise (set to 65 dB) for four minutes. The mouse then underwent 50 trials in a random order with five trial types, each performed ten times: background noise, acoustic startle (AS), 71 dB + AS, 77dB + AS, and 83 dB + AS. Prepulse and AS were set to 20 ms in duration with an interstimulus interval of 100 ms. Acoustic startle intensity was set at 120 dB, and the prepulse was 6, 12, and 18 dB above the background levels. Mice were tested pre-infection and at 14 and 28 dpi.

Euthanasia and necropsy

After infection, mice were monitored at least once daily for 21 days and weighed once daily. When mice began showing clinical signs including ruffled fur, weight loss, and hunched posture, monitoring was increased to twice daily, both during the light cycle. If mice were found to have any neurological signs, >25% body weight loss compared to pre-infection weight, or were showing hesitancy to move, a third observation of all mice was performed during the dark cycle. For the initial five studies, mice were euthanized if they

showed severe neurological signs (defined as seizures, two or more limbs paralyzed, or being moribund), an inability to reach food or water, or if >20% initial weight loss was recorded. For the final three behavioral studies, up to 25% weight loss was allowed before euthanasia. For the initial dosage studies, all mice were euthanized at 21 dpi. For the behavioral testing studies, one group was euthanized at 120 dpi, another group was euthanized at 80 dpi, and all remaining mice were euthanized at 35 dpi.

For euthanasia, all mice were deeply anesthetized using isoflurane anesthesia. Intracardiac exsanguination was performed while under anesthesia, and euthanasia was confirmed by cervical dislocation. The brain was removed and cut along the midline. One half was put into formalin for IHC. The second half was placed onto a dampened KimWipe on a cold plate set to 0°C for dissection. Using anatomical landmarks, the brain was separated into the cerebellum, hindbrain, midbrain, forebrain, hippocampus, and cortex (**Fig. 6**). As each section was removed, it was placed into a homogenization tube, which was kept on dry ice until transferred to the -80°C freezer.

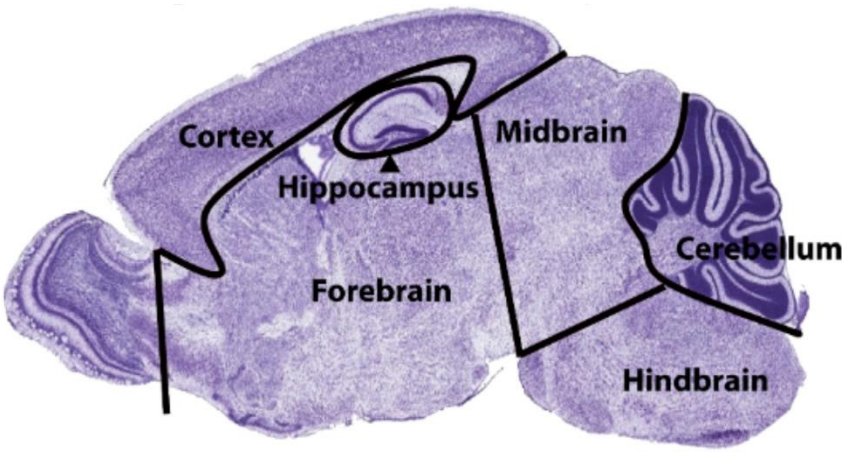


Figure 7: Anatomic landmarks for dissection of brain into separate functional regions.
Modified from Allen Brain Atlas.

Homogenization media was made in-house, and consisted of 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L PMSF, 0.05% Tween-20, and a cocktail of protease inhibitors (Roche), as described in previous work[216]. A sterilized metal bead (15 mm diameter) was added to each sample. Each cortex had 600 µl of solution added to it, and all other samples had 300 µl added. These were homogenized at room temperature for 30

seconds at 24 Hz using a TissueLyser (85300, Qiagen, Hilden, Germany). Samples were either used immediately or frozen at -80°C.

RNA Extraction and real-Time qPCR

If not performed before freezing, all homogenates were thawed at 37°C, then re-spun at 10,000 rpm for five minutes. A 100 µl sample from the supernatant of each regional homogenate was added to 900 µl of Trizol and left at room temperature for ten minutes inside the BSC. This was then transferred into the BSL-2 lab space. RNA was extracted from each regional sample using the Qiagen RNeasy extraction kit (Qiagen, 74136).

RNA from each brain region was tested for viral RNA using Roche Virus Master ReadyMix. Each sample was tested using primers targeting the envelope gene of WNV, as previously described[217]. The primers for detection are listed in **Table 2**.

Primer Target	**Sequence**
WNV Env Fwd	TCAGCGATCTCTCCACCAAAG
WNV Env Rev	GGGTCAGCACGTTTGTCATTG
WNV Env Probe	TGCCCGACCATGGGAGAAGCTC

Table 2: Primers for detecting WNV E protein.

To quantify total copies in the samples tested, a standard curve was created using a DNA plasmid containing the WNV envelope gene. Standard dilutions were made, and qPCR was performed to determine the sensitivity and correlation of Cq to concentration. RealTime Ready RNA Virus Master Mix (Roche) was used to make master mix. All samples were kept on ice until aliquoted into a 96-well plate. Reagents were added in the concentrations listed in **Table 3.**

Reagent	**Amount for one reaction (µl)**
Water (molecular grade)	2.6
Enzyme blend	0.4

Reaction buffer	4
Forward primer (10 µM in molecular grade water)	1
Reverse primer (10 µM in molecular grade water)	1
Probe (10 µM in molecular grade water)	1
Total	10

Table 3: Reagents concentrations for qRT-PCR.

An aliquot of 10 µl of extracted RNA from each region was added to each appropriate well. A negative control of water and a positive control of extracted viral RNA from viral culture was used for each run.

After applying clear film, the plate was spun at 500 rpm for two minutes.

The plate was loaded into a LightCycler 96.

RT-PCR was run using the steps listed in **Table 4**.

Step	**Temp (°C)**	**Time (s)**	**Cycles**	**Ramp rate (°C/s)**
Reverse transcription	52	480	1	4.4
Heat activation	95	30	1	4.4
Denaturation	95	1	50	4.4
Annealing	60	20	50	2.2
Elongation	72	1	50	4.4
Cooling	40	30	1	

Table 4: RT-PCR steps including time, temperature, and cycle number.

LightCycler measurement was set to be FAM (quant factor 10, melt factor 1.2).

Bioplex analysis

For multiplex analysis, the homogenate samples from the cortex and hindbrain were thawed and clarified. An aliquot 100 µl of supernatant from each sample was removed and separated into two separate wells of a 96-well plate. Samples were then processed according to manufacturer instructions using the Th17 cytokine kit from Bio-Rad (Bio-Rad, St Louis, MO, M60-00007NY). Briefly, the antibody-coupled beads were diluted as described in manufacturer instructions and 50 µl was added to each well. The plate was washed twice using a magnetic plate wash and manufacturer supplied wash solution. For

samples, 20 µl of sample were added in duplicate to wells of a 96-well plate. Then 80 µl of homogenization media (with 0.5% bovine serum albumin) was added to each well. Each well was mixed and then 50 µl of each diluted sample was transferred to the plate containing the beads. The standards were made according to manufacturer instructions and 50 µl was added to the appropriate wells. The plate was then washed three times with the magnetic plate washer. Each well had 25 µl of detection antibody added and the plate was left to shake at RT at 850 rpm for 30 minutes. The plate was then washed three more times with the magnetic plate wash. Each well had 50 µl of 1x streptavidin-PE added and the plate was left shaking at room temperature at 850 rpm for ten minutes. The plate was then washed three times, each well had 125 µl of assay buffer added, and then the plate was left to shake at RT at 850 rpm for 30 seconds. The plate was then transferred to a Bioplex-200 machine and read using the standard settings.

Histology

Samples were kept in formalin at room temperature in the BSL-3 for at least 24 hours, followed by a formalin change and an additional 48 hours at room temperature. All samples were taken to the UTMB Histopathology Core where they were processed and embedded in paraffin. Serial sections of 5 µm thickness were taken from each brain and collected on charged slides. Sections were selected to be approximately 0.48-1.48 mm lateral from Bregma to get consistent samples showing major anatomical structures, including the hippocampus, cerebellum, brainstem, striatum, and a majority of the cortex.

Paraffin was removed by baking the slides at 54-57°C in a hybridization oven for at least one hour, and then being dipped in xylene three times for five minutes each. Rehydration was performed by dipping the slides in 100% ethanol three times for five minutes each, followed by five minutes in 95% ethanol. Antigen retrieval was performed by putting the slides in a citrate bath (pH 6.0) for twenty minutes at 95-100 C. Primary antibody, diluted in Tris-buffered saline (TBS) + 1% bovine serum albumin (BSA) to the

listed concentration, was applied, and slides were left for one hour at room temperature in a humidified chamber. Slides were washed three times using TBS with 0.01% Triton X-100. Endogenous peroxidase activity was blocked using 0.1% hydrogen peroxide for five minutes. Slides were then washed in tap water three times. Secondary antibody, diluted in TBS + 1% BSA to the listed concentration, was applied and left for one hour at room temperature in a humidified chamber. Slides were then washed three times using TBS with 0.01% Triton X-100. Chromogen was applied for seven minutes (diaminobenzidene) or 25 minutes (Vector Red), then washed in water three times. Counterstaining was performed by dipping slides in Harris hematoxylin for one minute, then washing in tap water three times. Dehydration was performed by dipping slides in 95% ethanol, followed by three dips in 100% ethanol, and then three dips in xylene.

The antibodies were used at the concentrations listed in **Table 5**.

Target	**Species**	**Concentration**	**Secondary Antibody**	**Expected Target**
GFAP	Rabbit	1:1000 (Abcam, ab7260)	Goat anti-rabbit (1:500, Abcam, ab6721)	Astrocytes
Iba1	Rabbit	1:500 (Thermo-Fisher, PA5-27436)	Goat anti-rabbit (1:500, Abcam, ab6721)	Microglia
Phospho-tau S396	Rabbit	1:1000-1:8000(Abcam, ab109390)	Goat anti-rabbit (1:500, Abcam, ab6721)	Neuronal damage
Complement protein C3	Chicken	1:50-1:400 (Abcam, ab48581)	Goat anti-chicken (1:500, Abcam, ab6878)	A1 astrocytes

Table 5: Antibodies used for IHC, including manufacturer, concentration, and expected targets.

Positive and negative controls were included in each batch of staining. Samples from mice euthanized during the acute phase of infection were used for positive controls and samples from uninfected mice were used as negative controls.

RNA *in situ* hybridization was performed using RNAScope 2.5 (Advanced Cell Diagnostics, Newark, CA, #475091) using the manufacturer's instructions. Tissue sections

were deparaffinized by being put through two steps of xylene for five minutes each, followed by two steps of 100% ethanol for one minute each. Slides were boiled for 30 minutes in RNAScope Target Retrieval reagents using a vegetable steamer, and then incubated with RNAScope Protease plus for 30 minutes. Endogenous peroxidase activity was then quenched using hydrogen peroxide for 10 minutes. The probe targeting WNV was created by Advanced Cell Diagnostics. A positive control (mouse brain acutely infected with WNV) and negative control (human spinal cord from confirmed non-infected patients) were run simultaneously. Tissues were counterstained with Gill's hematoxylin[218].

Statistical analysis

For behavioral analysis, different statistical analyses were used to assess different tests. The rotarod, 2WAA, and body weights were compared using a repeated-measures two-way analysis of variance (ANOVA) and were analyzed using GraphPad 8.1. For the TST and PPI, a regression estimate analysis was performed to determine if infection status, viral RNA positivity in a region, or microglial lesion positivity in a region correlated with differences in behavioral outcomes. For the rotarod, a regression estimate analysis was performed using trials four through nine (24-25 dpi) using the same criteria, as well as having a repeated-measures two-way ANOVA to compare the groups. For the 2WAA, a regression estimate analysis was performed using data from 32-34 dpi using the same criteria, as well as a repeated-measures two-way ANOVA to compare the groups.

For the analysis, all four of the behavioral outcomes (rotarod, TST, 2WAA, and PPI) were assessed as continuous variables, with independent variables being infection status, lesions, and RNA. For lesions, each region of the brain was considered separately, but the hippocampus region was excluded because all subjects had the same response. Similarly, for RNA each region was dichotomized by those having any RNA and those with zero. These analyses were done by generalized estimating equations accounting for

correlation between time points and all three independent variables were included in each model to adjust for each other. These analyses were completed using SAS 9.

For inflammatory cytokine analysis, normalcy of data was first determined using the Shapiro-Wilks test for each group of samples. Student's t-test was performed to compare infected and uninfected respective regions and cytokines. A one-way ANOVA was performed to compare cytokine levels in the hindbrain and cortex of brains based on microglial lesions or persistent viral RNA. All of these statistical analyses were performed on GraphPad Prism 8.0.

For all tests, results were considered significant if the p-value was below 0.05, and they were considered to have a trend if the p-value was between 0.05 and 0.12.

Chapter 3: Behavioral Analysis

INTRODUCTION

Behavioral testing is one of the best ways to reliably study the neurological effects of damage to the nervous system, including damage stemming from viral infection. Some viruses show tropism for certain brain regions, leading to changes in specific neurological functions. For example, respiratory syncytial virus has a tropism for olfactory sensory neurons[219], which allows the virus to spread into the brain, where the virus infects other regions such as the hippocampus[220]. ZIKV has a tropism for neural stem cells in the brain, especially of the neocortex[221]. This causes reduced brain volume in patients with specific thinning of the cortex along with visual and motor abnormalities[222]. Even when viruses spread to the brain in the same way, they can show tropism for different regions and neurons[223]. Studies often rely on pathological changes as hallmarks of neurological damage. However, infection and pathological damage do not always correlate with neurological changes. In some cases, there are redundant neurological circuits, or the brain can adapt to damage, meaning that pathology does not necessarily lead to dysfunction. Conversely, damage and neuronal dysfunction are not always apparent with gross pathology, and neurological dysfunction can occur without obvious damage. There has been some work using rodents as models of WNV-induced neurological damage. Other viruses have been tested in different models and these can help direct studies when that aim to investigate the neuropathogenesis of WNV.

Previous models of virus-induced behavioral changes

Numerous other viruses induce long-term neurological changes, and some have been studied using behavioral testing in animal models to elucidate the mechanisms behind the neurological changes. These studies served as a starting point for our studies and the

common mechanisms found among the different viruses could indicate possible mechanisms for WNV.

One of the most studied viral infections of the CNS is HIV. HIV can induce a disease called neuroAIDS, which has increased in incidence with the increase in combined antiretroviral therapy. NeuroAIDS manifests as severe inflammation of the CNS with concurrent neurological deficits, including memory loss, emotional instability, and motor coordination loss. The mechanisms behind these sequelae are being studied, and animal models using behavioral tests have played a large role in this. Different models of neuroAIDS have been used and have indicated that there are multiple mechanisms behind the sequelae. One model using cats inoculated intracranially with feline immunodeficiency virus as a model showed that infection could induce memory loss and motor deficits which correlated with increases in pro-inflammatory cytokine levels[224].

Both the gp120 and Tat proteins of HIV have been specifically associated with neurological changes and behavioral deficits following inoculation in rodent models. Memory loss in mice after inoculation with the Tat protein has been associated with increased production of pro-inflammatory cytokines; neuronal death (specifically in regions of the hippocampus)[225]; loss of synapses and dendritic spines[226,227]; and activation of astrocytes including increased phagocytosis[227,228]. Some studies in mice showed expression of HIV proteins in the brain decreased memory function and decreased long-term potentiation (LTP)[227,229], a measure of synaptic activity changes in response to strong signals. LTP is a major mechanism behind the formations of memories. Mice inoculated with the HIV Tat protein showed an increase in depression, which was associated with increased transcription of IL-1β, markers of astrocytes and microglia, and indoleamine-2,3-dioxygenase (IDO)[230]. IDO is an enzyme involved with the breakdown of tryptophan, and it has been previously associated with neurological diseases in human patients, including schizophrenia, bipolar disorder, and neuroAIDS[231].

Borna disease virus (BDV) is the only member of the family *Bornaviridae,* and it has been studied extensively as a neuroinvasive virus in animals. There is evidence that it causes neurological disease in humans as there is some association between infection and schizophrenia[232–234], though this has not been fully established. BDV infection of rodents is commonly used as a model of neuroinflammation in rodents and has been used to model autism and schizophrenia. One study found that inoculation with the virus into neonatal rats caused significant motor deficits[235]. Infection caused increases in transcription of pro-inflammatory cytokines and pro-apoptotic genes and decreases in neurotropic and anti-apoptotic genes[235]. Multiple studies have shown that BDV can induce memory deficits in rats and mice. This was associated with deterioration of the dentate gyrus including neuronal apoptosis, increased levels of the pro-inflammatory chemokines IP-10 and RANTES, and direct viral infection of neurons[236]. BDV infection inhibits activity-dependent synaptic vesicle recycling, which is important for the formation of memories through LTP[237–239]. The P protein of BDV has been associated specifically with memory loss. One study found a decrease in the transcription of neurotropic genes in the hippocampus and cerebellum when only the P protein was inoculated, which correlated with memory loss and aggression[240]. When the P protein was specifically expressed in the dentate gyrus of the hippocampus of mice, the mice showed decreased contextual memory[241]. Similar to HIV, IDO expression has been found to be increased in neonatally infected rats up to four weeks post-infection in the hippocampus, striatum, and cerebellum[242], though behavioral changes were not investigated.

Theiler's murine encephalomyelitis virus (TMEV) is a picornavirus of mice that is normally an enteric pathogen. When inoculated into the brains of mice, TMEV can induce either a persistent, intermittent seizures or an acute demyelinating disease, depending on the strain of mouse used[243]. TMEV induces memory loss in some mouse models, as measured through the Morris water maze and novel object recognition test. These neurocognitive changes have been correlated with loss of hippocampal neurons,

inflammatory cell infiltrates, and loss of dendrites of hippocampal neurons, especially in the CA1 region[244,245]. The neurons undergoing apoptosis in these animals often are not infected, indicating a pro-inflammatory state that induces apoptosis in uninfected neurons[246]. Loss of memory function has also been associated with infiltration of inflammatory monocytes, but not infiltrating neutrophils[247,248].

Flaviviruses other than WNV can induce neurological deficits in patients and animal models. Given how much more closely related these viruses are to WNV than the viruses previously mentioned, the pathology they cause may be more similar to that found in WNV cases, and the mechanisms are more likely to overlap.

ZIKV is a flavivirus most commonly associated with microcephaly in newborns whose mothers were infected during pregnancy. ZIKV infection in adults has also been associated with Guillain-Barré syndrome, a severe autoimmune neurological disease. Multiple models have been used to study its neurological effects. Mice are commonly used as models of ZIKV infection. Mice infected *in utero* have shown severe motor deficits, which correlate with decreased brain volume and loss of neurons in the hippocampus[249] and cerebellum[250]. The motor deficits have also been associated with necrosis and dystrophic calcification of multiple regions including the hippocampus, striatum, and thalamus up to three months post-infection[251]. Similar to other viruses, there was an increase in pro-inflammatory cytokines, including IL-6, TNF-α, and IL-1β. Also like HIV, IDO was found to be upregulated in infected neonatal rats up to one month post infection[252], though this was not specifically associated with behavioral deficits.

Two studies have used non-human primates as models of infection. One study found that neonatal macaques infected with ZIKV subsequently showed decreased anxiety in response to unrecognized intruders at six and twelve months of age[253]. This behavioral change correlated with generalized inflammation, astrocytosis, and the formation of glial nodules in the cortex and basal ganglia. The second study found that congenitally infected rhesus macaques showed loss of balance, decreased visual recognition memory, and

decreased social behavior[254]. This correlated with enlarged lateral ventricles and changes in the functional connectivity between the hippocampus and amygdala, and the hippocampus and multiple areas of the cortex.

JEV is one of the viruses most closely related to WNV as they are within the same serogroup of the flaviviruses[255]. JEV is endemic to southeastern Asia, where it causes severe encephalitis with a 25% case fatality rate. There are an estimated 68,000 cases of JEV infection each year, and JEV is the main cause of viral encephalitis in many countries. Up to 50% of survivors of the encephalitis show permanent neurologic sequelae. When inoculated into rats, JEV induces spatial memory loss at up to five weeks post-infection. This is associated with decreased expression of cholinergic neurons in the frontal cortex, hippocampus, and cerebellum[256]. Infected rats also show decreased motor function as measured through spontaneous locomotor activity, grip strength, the rotarod, and the pole test[257]. This is associated with damage to the substantia nigra up to 25 weeks post-infection, leading to neuronal loss and gliosis. Tyrosine-hydroxylase positive neurons are particularly susceptible to JEV infection, with a significant reduction in numbers detectable up to 25 weeks post-infection[258,259]. Neurotransmitter production is also disrupted in multiple brain regions. Dopamine is reduced in the corpus striatum, frontal cortex, and midbrain up to 20 dpi, and remains decreased in the striatum up to 25 weeks post-infection. Norepinephrine production is reduced in the same regions up to 25 dpi, and in the hippocampus and medulla oblongata up to 25 weeks post-infection. Further work showed that infected rats had decreased norepinephrine and dopamine-related neurotransmitters in the corpus striatum, frontal cortex, striatum, and midbrain, which was associated with inflammation, necrosis, and neuronal shrinking in these regions at 10 and 20 dpi[260]. Dopamine remained decreased in the striatum and norepinephrine remained decreased in the hippocampus and medulla oblongata up to 25 weeks post-infection, which correlated with loss of motor coordination[261]. The rats in this study also showed decreased tyrosine hydroxylase-positive

neurons in the substantia nigra and neuronal loss with gliosis in the thalamus and basal ganglia.

The different models of viral infection leading to neurobehavioral changes in animal models indicate common pathological changes. Loss of neurons via apoptosis and necrosis, maintenance of an inflammatory state in the brain, and loss of normal neuronal activity (including synapse loss) appear to be common mechanisms leading to long-term neurological changes.

These studies indicated potential mechanisms worth investigating for this model, but also highlighted the difficulty in creating a reproducible animal model. Many of the mentioned models relied on IC inoculations or expression of specific viral proteins in particular brain regions. The number of possibly affected regions, and the associated pathways involved, complicated matters further. The approach taken in these studies was to begin with wide screening for possible behavioral changes using multiple methodologies. These could be used to narrow the subsequent investigation of pathological and neurological changes in the brain.

DEVELOPMENT OF MOUSE WNV DISEASE SURVIVAL MODEL

Mice are a commonly used animal model for studying WNV infection[262]. Initial studies focused on determining what roles cytokines, chemokines, and inflammatory cells played in neuroinvasion[263]. Later studies have shown that lower doses of virus can lead to a mouse model that survives infection[145,146]. Model development at this point aimed to replicate these studies to see if WNV infection led to behavioral or inflammatory changes in the brain, and how persistent virus was distributed.

The C57Bl6 strain of mouse was selected as it is the most commonly used inbred strain for both neurological and virological studies. It was important that an inbred strain be selected to reduce the role that genetic variation would play on virus distribution and behavioral changes as much as possible.

The previously mentioned NS5 mutant model of WNV infection[162,163] presented a challenge to the development of our project as that research had the same aim as this dissertation was aiming to study: namely, determining the mechanisms behind behavioral changes caused by WNV infection and the overlap with NDs. While these studies did provide important information, there were certain shortcomings that these studies were aimed at addressing.

The use of an attenuated mutant virus with a reduced ability to antagonize the interferon response gave a reduced lethality. The interferon response plays a major role in restricting the virus' pathology and tropism[47,264] meaning that changes to the response may change the way the virus spreads in the brain. The first study also reported that persistent virus was not detected and therefore did not play a role in pathogenesis[162]. Antagonism is crucial for the maintenance of persistent viral infection in other viruses such as lymphocytic choriomeningitis virus[265], so the attenuated virus may allow for easier clearance of WNV. This model has only been used to study memory function with a focus on the hippocampus. WNV causes many other sequelae in humans and is known to affect many other regions in both mice and humans. The use of IC inoculation ensured that virus was present in the CNS but did not account for how the virus would naturally spread after primary or secondary viremia, which could affect what regions of the CNS the virus can invade. Considering that viral persistence has been shown to occur and that different regions show different susceptibilities to viral infection, it is reasonable to assume that certain regions are more likely to show persistent virus.

It was important for these studies to study mimic natural infection as closely as possible to determine how the virus naturally distributed and persisted in the mouse brain, and to determine how translatable this would be to human patients. Some considerations to reduce mortality, such as treatment with antibodies or use of an attenuated strain of virus, were considered but ruled out as making the model less translatable.

Dosage and aging studies

The first step to developing a mouse model of WNV-induced behavioral changes was to determine the optimal dose. The infectious dose needed to be high enough to induce disease and ensure some amount of neuroinvasion, but low enough to allow for the survival of sufficient numbers of mice for behavioral testing.

Two initial studies were performed using two groups (*n*=10) of 8-week-old female C57BL/6NTac mice from Taconic. These mice were infected with either 10 or 100 pfu of NY99 WNV in the footpad to mimic natural infection and spread of the virus from a peripheral, subcutaneous infection site. Mice were observed for 21 days, over which time clinical signs were recorded. Both groups had high rates of mortality (**Fig 8**), with 60% and 66% of mice needing to be euthanized at the 10 and 100 pfu doses, respectively. This mortality rate was considered too high to use for studying sequelae. Due to these findings, the age of the mice was increased to 16-weeks-old, and another study was performed.

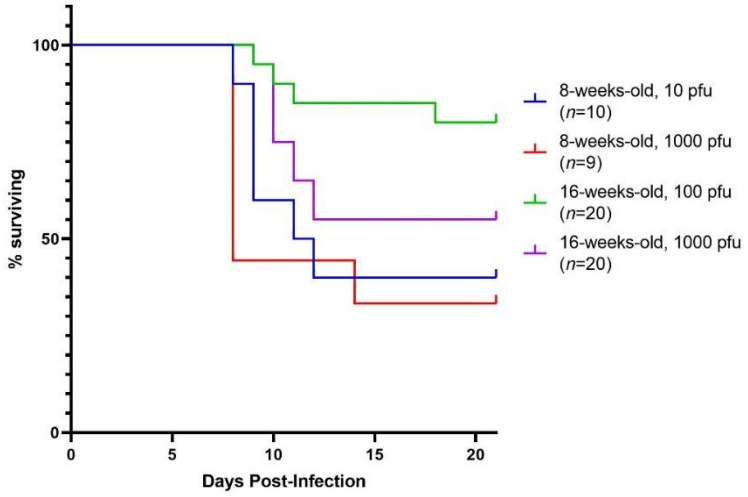


Figure 8: Survival curves of mouse dosing studies using different doses of NY99 WNV virus inoculated into footpad and two different ages of mouse.
Groups of eight- or sixteen-week-old mice were inoculated with different doses of NY99 WNV to determine optimal dosing for development of a survival model. Older mice showed greater survival, and mice infected with 100 pfu of WNV had mortality of about 20%.

Two groups of 16-week-old female mice (*n*=10) were inoculated with 100 or 1000 pfu of NY99 WNV in the footpad. These mice had 20% and 60% mortality, respectively.

The lower dose of virus showed a much higher survival rate, which would allow for behavioral studies. This same group of mice was used for the first set of behavioral tests. While the mortalities occurred mostly within the equivalent timespan as the other studies, one mouse was euthanized later than in other studies, at 20 dpi. The surviving mice from these initial studies were kept for either 80 or 120 dpi to determine if additional mortality, neurological changes, or recovery occurred. These mice did not show clinical changes past 21 dpi. Because of the lack of changes in clinical severity and the changes made to the behavioral testing schedule (see next section), the schedule was adjusted to perform behavioral testing from 21-34 dpi and euthanize the mice on 35 dpi. This would allow for description of pathology immediately after behavioral testing to correlate neurological changes with pathological, virological, and immune analyses. A summary of all of the studies is listed in **Table 6**, and a summary of the final mouse model used is seen in **Fig. 9.**

Year of Study	**Age of Mice Used**	**Infectious Dose**	**Date of Euthanasia**	**Behavioral Tests Performed**
2017	8-weeks-old	10 pfu	21 dpi	None
2017	8-weeks-old	1000 pfu	21 dpi	None
2017	16-weeks-old	100 pfu	120 dpi	Rotarod, ASR/PPI, 2WAA, cold plate, SHIRPA, grip strength
2017	16-weeks-old	1000 pfu	80 dpi	Rotarod, ASR/PPI, 2WAA, cold plate, grip strength, TST
2018	16-weeks-old	100 pfu	35 dpi	Rotarod, ASR/PPI, 2WAA, TST
2019	16-weeks-old	100 pfu	35 dpi	Rotarod, ASR/PPI, 2WAA, TST

Table 6: Summary of all mouse studies using NY99 WNV and C57Bl/6 mice. Multiple studies were performed to optimize viral dose, age of mouse, time to euthanasia, and behavioral testing. All mice used were female. The age of mice was changed to allow for greater survival rates. Behavioral testing began in a group that showed enough survival to warrant behavioral assessment. Testing was performed at different times for different studies, and some tests were removed based on feasibility of testing.

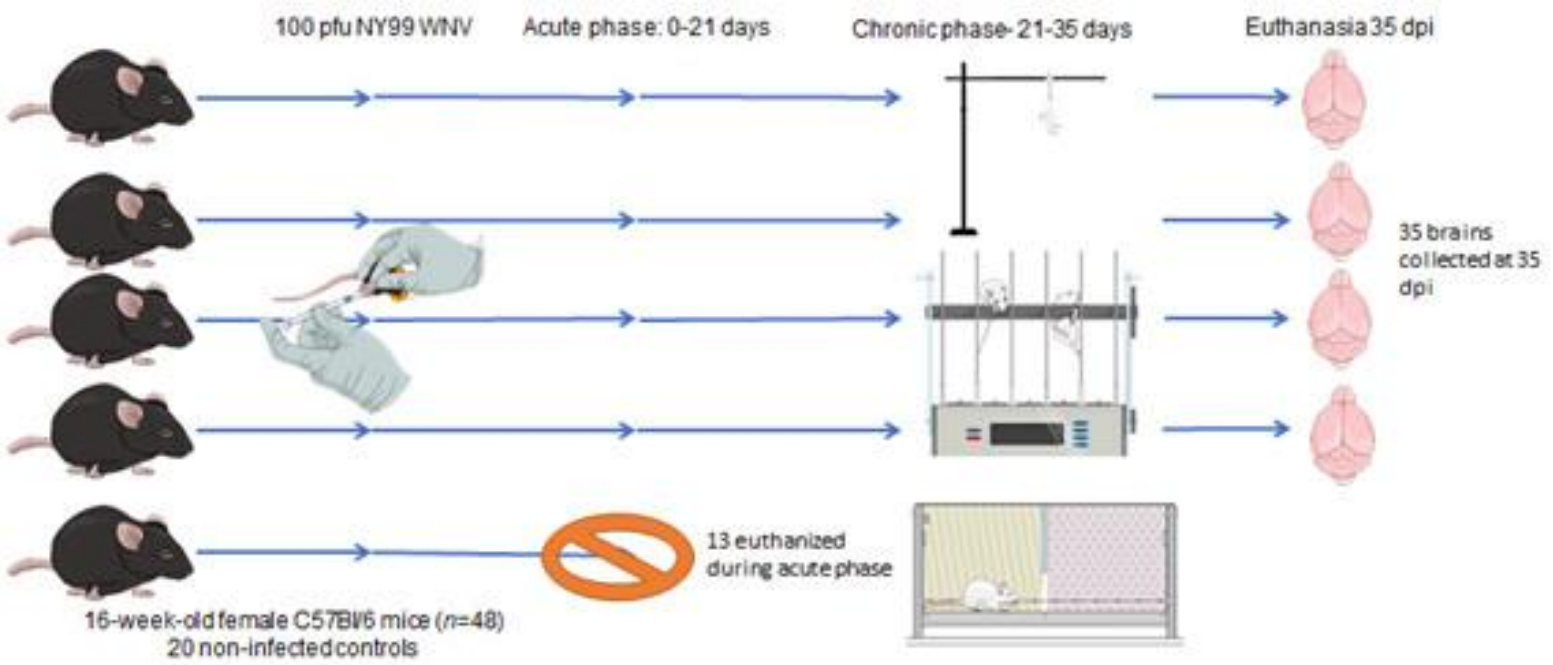


Figure 9: Diagram of final study design for viral dosing and behavioral testing.
A total of 48 female 16-week-old C57Bl/6 mice were inoculated with NY99 WNV in the footpad, with 20 uninfected mice as controls. Mice were observed daily for 21 days to determine mortality, and thirteen were euthanized. The remaining infected and uninfected underwent behavioral testing until 35 dpi, when all were euthanized and the brains were removed for analysis.

Clinical scoring and weight loss

All mice were observed at least once daily for clinical signs and weight loss. Following the preliminary dosing studies, clinical scoring criteria were changed to allow mice to lose up to 25% of total body weight and to show paresis or paralysis in one limb before being euthanized (see section 2 for clinical scoring chart).

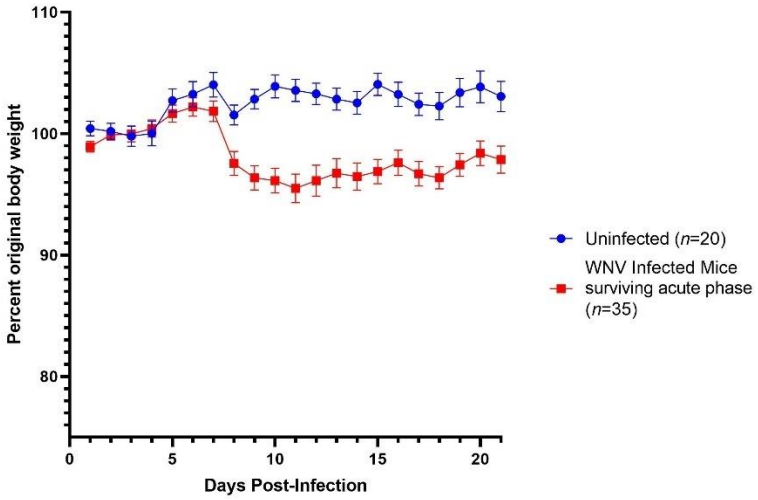


Figure 10: Body weight loss in uninfected vs NY99 WNV-infected mice that survived past 21 dpi.
Error bars represent mean +/- standard error of the mean.

Most of the infected mice lost weight, though not all. Peak weight loss in occurred at 11 dpi, with an average weight loss of 4.5% in WNV-infected mice on that day (**Fig. 10**).

None of the mice that showed clinical signs more severe than ruffled fur survived past 20 dpi. Four mice showed single limb paralysis during the acute phase. All of these mice, and any mice that developed a hunched posture or other signs indicating more severe disease, developed more severe

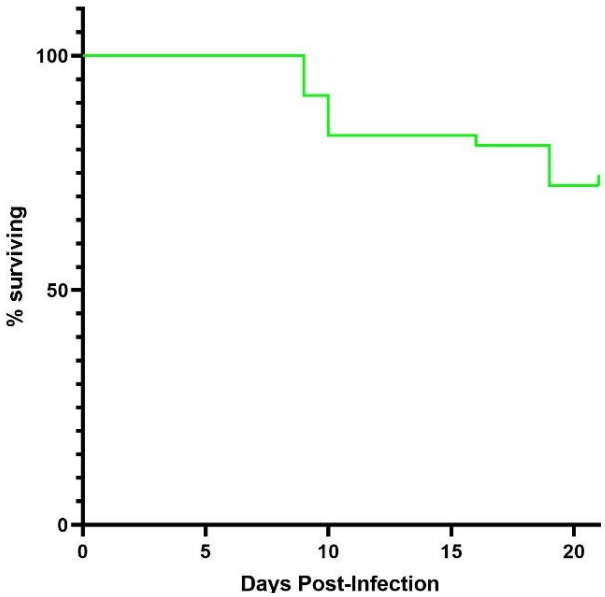


Figure 11: Survival curve of mice (n=48) used for behavioral analyses.
Of the 48 mice inoculated with NY99 WNV, 73% survived through the first 21 dpi. Peak mortality was seen at 9-10 dpi.

disease through the study. Four of these mice survived past peak mortality and began to recover, but required euthanasia at 19 dpi due to sudden recurrence of clinical signs and becoming moribund. A total of thirteen mice were euthanized during the acute phase, leading to a total of 73% (35/48) of the NY99 WNV-infected mice surviving long enough to be tested for behavioral changes (**Fig. 11**).

Behavioral tests

BEHAVIORAL TEST SELECTION

One of the earliest decisions made was what behavioral tests to use. Using reports on human patients to determine what the most commonly reported sequelae were[86], the behaviors chosen were memory loss, depression, motor coordination, motor learning, sensorimotor gating, hearing loss, and hyperalgesia.

After selecting these behavioral paradigms to study, the next decision was which tests to use for these paradigms so that they would fit within the confines of the laboratory environment. Given that the work would be with a BSL-3 agent, all tests had to be able to be done within BSC or in a fully contained enclosure. The tests could also not use large amounts of water, as this would require prohibitive amounts of bleach for decontamination.

Originally, the SHIRPA (SmithKline Beecham Pharmaceuticals; Harwell, MRC Mouse Genome Centre and Mammalian Genetics Unit; Imperial College School of Medicine at St Mary's; Royal London Hospital, St Bartholomew's and the Royal London School of Medicine; Phenotype Assessment) battery of tests[266] was to be used for semi-quantitative analysis of multiple behavioral categories. This battery of tests has been used in other mouse models, including infectious disease models, to determine neurological changes. This involved handling and observing the mice, and quantifying behaviors based on certain parameters. After the first experiment using this, it was determined that the SHIRPA protocol was not sensitive enough to study the subtle changes WNV could induce, and too time-consuming for the large numbers of mice to be tested on a regular basis.

The ASR and PPI use the same apparatus and similar paradigms for testing. They both use an acoustic startle chamber- a soundproofed box with a container for the mouse inside. Mice are placed inside, given time to acclimate, and then exposed to a series of sounds. The ASR uses one loud sound to test an animal's response to a sudden noise by measuring the amount of motion after a loud sound. This tests an animal's ability to hear and respond to a sound. Changes can indicate loss of hearing, loss of signaling from auditory centers to motor centers, or loss of signaling from the motor centers to the musculature. The PPI test is based on sensorimotor gating, where extraneous sensory information is filtered out in the brain. The paradigm uses a similar mechanism to the ASR test: the mouse is exposed to a loud noise, and the amount of motion is measured. In the PPI test, a quieter sound of varying degrees of intensity above baseline is produced just prior to the loud sound. In normal animals, this reduces the amount of motion when compared to the ASR, as the sensory information from the loud sound is shunted through a different pathway and filtered. The reduction in movement when compared to the ASR is calculated as a percentage and used as a measure of sensorimotor gating.

The rotarod is a test commonly used to measure motor coordination that has recently been used to study motor learning[267]. The mouse is placed onto a rod high enough above the work surface that the mouse is motivated to remain on the rod. The rod begins to rotate and continues rotating until the mouse falls off. In some studies, including our own, the rod accelerates over time. Mice with motor deficits remain on the rod for shorter periods of time, and mice with motor learning deficits do not show as steady of an increase in the latency to falling[267]. Motor skill learning relies on the basal ganglia[268], motor cortex[269], hippocampus, and striatum[270,271].

The parallel rod floor test was selected to complement the rotarod as a measure of motor coordination without a motor learning component. In the parallel rod floor test, a mouse is allowed to explore an open area that has a floor composed of several small metal rods with spaces between them. When the mouse places its paw in between the rods, it is

counted as a “footslip.” The number of footslips is divided by the total distance traveled to indicate general motor coordination, and total distance traveled can be an indication of anxiety.

The grip strength test was selected as a measure of motor weakness to determine if there might be spinal cord or motor nerve involvement. The mouse is lifted by its tail and allowed to grasp a bar. This grasping motion is a natural response of the mouse when it is being held aloft. The mouse is then gently pulled away from the bar until it releases its grip. The amount of force required to make the mouse let go is recorded and this can be compared. The test is used as a measure of muscle strength, motor neuron coordination, and sensory input as the mouse needs to maintain a grip. Deficits in grip strength have been attributed to the cerebellum[272], the cortex[273], and white matter injury[274] in the brain. There are options for testing, including whether to test the forelimbs, hindlimbs, or all four limbs. The initial studies used a grid that would allow for testing of all four limbs. This allowed for too much variability as some mice would not use all four feet to grip the grid, and so was switched to using a t-bar to only test the forelimbs.

The 2WAA was selected to test for memory function. Other paradigms including the Morris water maze, Barnes maze, and fear conditioning tests were ruled out due to their inability to be used inside of a BSC. The 2WAA test uses a Pavlovian conditioning paradigm to train mice to respond to a light and sound. In Pavlovian conditioning, there is a conditioned stimulus, unconditioned stimulus, and a response. For the 2WAA, a mouse is put into a two-chamber system that it can freely move between. After a period of acclimation, the mouse is exposed to a light and sound (the conditioned stimulus). If the mouse does not move to the opposite chamber (considered the response), it receives a foot shock until it does move or for ten seconds (the unconditioned response). This is repeated multiple times per day for multiple days. Mice with functional memory learn to avoid the shock by moving in response to the light and sound, before the foot shock. This test uses multiple pathways and forms of memory, and many of them are still being elucidated. The

basal ganglia[275], hippocampus[276], and cortex[277] have all been shown to play a role 2WAA performance.

Most tests for depression in mice require either water (such as the forced swim test) or specialized apparatuses that would not fit inside of a biosafety cabinet. The TST uses a similar paradigm as the forced swim test: it tests a despair mechanism in the mice. In both cases, mice are put in an uncomfortable situation from which they cannot escape- either being in water or being held by their tail. The mouse struggles to escape and will give up multiple times throughout the session. The amount of time the mouse spends immobile is a correlate of depression[278,279]. The TST was selected because it could be done without specialized equipment, it could be performed inside of the BSC, and it did not require the large amount of water that the forced swim test would.

The cold plate is designed to measure hyperalgesia and allodynia, which are signs of neurogenic or chronic pain. The mouse is placed onto a chamber with a floor set chilled to 0°C for two minutes. The number of times the mouse jumps, the latency to its first jump, and the number of cold-based behaviors, such as paw rubbing or shivering, are quantified and compared[280]. The apparatus for this was selected because it could fit into the BSC and chronic pain is a commonly reported sequela of WNV infection.

BEHAVIORAL TEST OPTIMIZATION

An important part of these studies was optimizing the behavioral testing paradigms and the schedule in which they were performed. Initially, the studies were planned to perform repeated tests on infected mice to track how the behavior change over time and map this as neurological dysfunction. This decision was changed as more data were acquired and indicated that the mice would learn from the tests and have various responses to it that could lead to confounding memory function with any of the tests. The amount of time each test took needed to be considered, as most of the tests required at least 20 minutes per mouse. This meant that only one test could be performed per day. The stress that each

test induced in the mice needed to be considered as well. Highly stressful tests such as the 2WAA and TST needed to be separated from each other to prevent changing the behavioral phenotypes in the mice.

The rotarod was a test that required multiple optimizations. Initially, testing was performed once weekly using the smaller, mouse-sized rod as a measure of motor coordination. The mice would cling to the smaller rod rather than falling off, leading to skewed results. This behavior was eliminated when the 10 cm rod designed for rats was used, as has been done in previous work[267]. The willingness of mice to stay on the rod was another factor that had to be accounted for. Because the rotarod system needed to be able to fit into a BSC, it was smaller and the distance the mice fell was less than in other rotarods. Some mice appeared not to be motivated to stay on the rod, even with repeated testing. To screen these mice out from the experimental groups, all mice underwent pre-testing screening after the acute phase. This removed mice that were not motivated by the height or fall. The role that memory and learning played in the rotarod test also needed to be addressed. Mice learn and generally improve their ability to stay on the rod with repeated testing, and were found to learn even when the trials were separated by a week. The mice reached a peak of performance, after which their performance either plateaued or decreased. To address the role memory and learning played in testing, a previous protocol was adapted using the rotarod as a measure of motor learning[267] rather than strictly as a measure of motor coordination.

The tail suspension test was initially performed multiple times throughout the study, each two weeks apart. Past research had shown the repeated testing does affect the behavior, but this had been performed within 24 hours of each other[281]. Control mice spent more time immobile after only one trial, indicating that the mice learned from the first test. These changes in behavior would introduce a confounding variable of memory to the phenotype of depression.

Initially, memory testing using the 2WAA test was performed at 50 dpi in the mice kept until either 80 or 120 dpi. Because most of the other tests showed attenuation after the first test, testing was performed on a subset of mice until 35 dpi to determine if there were significant differences between early and late testing, similar to what was performed in Vasek *et al*[162]. The mice were found to show little difference in memory performance when tested on 30 or 50 dpi, so all testing was abbreviated to finish by 35 dpi (**Fig. 12**).

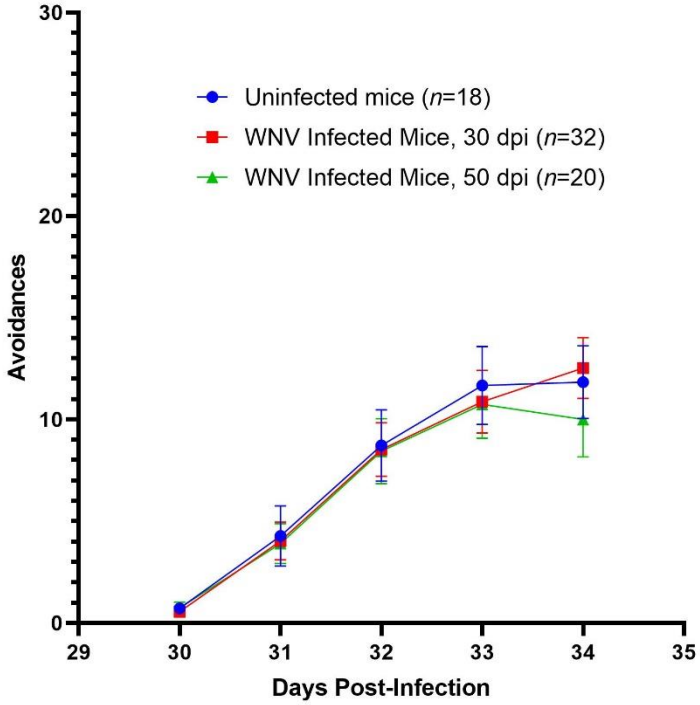


Figure 12: Comparison of 2WAA performance between uninfected mice, mice 30 dpi with NY99 WNV, and mice 50 dpi with NY99 WNV. One group each of WNV-infected mice were tested either 30 or 50 dpi to determine if time post-infection affected the memory phenotype. When compared together, there are no significant differences on any single day in 2WAA performance when infected mice were tested at 30 or 50 dpi. This was compared using repeated-measures two-way ANOVA.

Of the tests, the parallel rod floor test, the SHIRPA, and the cold plate were removed from testing after the first two studies using behavioral paradigms. The parallel rod floor test was removed because the equipment had multiple malfunctions requiring repairs from the company. The SHIRPA testing was removed because initial testing did not show any differences between mice, and the total time it took to complete it made it prohibitive in combination with other tests. The cold plate test was removed because there was a large amount of variability within the control group and within all mice pre-infection. The final behavioral testing schedule is shown in **Table 7**.

Week	Mon	Tues	Wed	Thur	Fri	Sat	Sun
1			Transfer NY99 mice to ABSL3			Ear punch NY99 mice	
2				NY99: ASR/PPI			
3	NY99: Infect mice at 16 weeks old						
4			NY99 Peak Mortality				
5							
6	NY99: Last daily check TST	NY99: Rotarod screening		NY99: Rotarod	NY99: Rotarod	NY99: Rotarod	
7	NY99:		NY99: Active	NY99: Active	NY99: Active	NY99: Active	NY99: Active

Table 7: Finalized testing schedule for testing WNV infected mice.
Finalized schedule for infection and behavioral testing using NY99 WNV inoculated peripherally. This schedule was used for assessing behavioral changes, inflammatory changes, and persistence of viral RNA in mice 35 dpi.

RESULTS

It was expected that different mice would show different behavioral abnormalities, similar to the spectrum of symptoms seen in humans. The range of neurological changes could be due to differential viral spread, inflammatory responses, or susceptibility of different regions of the brain to infection and inflammation. Even in a study using an attenuated virus inoculated directly into the brain, there were two different phenotypes of mice in regards to memory function[162]. The variability in outcomes using that model, and the distribution of infection and inflammation found in human patients, indicated that damage to the brain would vary between individuals. Without knowing what factors lead to any behavioral changes, statistical analysis would need to correlate behavioral outcomes with inflammation, viral RNA persistence, and infection status. Behavioral analysis of individual mice was considered, but this would have required prohibitively large numbers of uninfected control mice to establish normal values. To solve these problems, analysis of behavioral testing consisted of performing a regression analysis to determine if infection status, as well as other factors discussed in later chapters, correlated significantly with a change in behavior, as most of the tests were continuous variables.

The cold plate test was unreliable and difficult to repeat

The cold plate test was performed initially to assess mice for hyperalgesia or cold allodynia following WNV infection. Based on previous work[280], mice were placed onto a cold plate set to 0°C for two minutes, and the time to first jump, number of times jumped, and time to first cold-based behavior (shivering or paw rubbing) during this time was recorded. This was only performed on the first group of mice tested for behavior, and testing was performed at 7, 21, 28, 40, 49, and 77 dpi. The control mice showed large variability between testing days and within individual mice, which made comparisons between infected and uninfected mice impossible (**Fig. 13**). Given these findings, mice in future experiments were not tested.

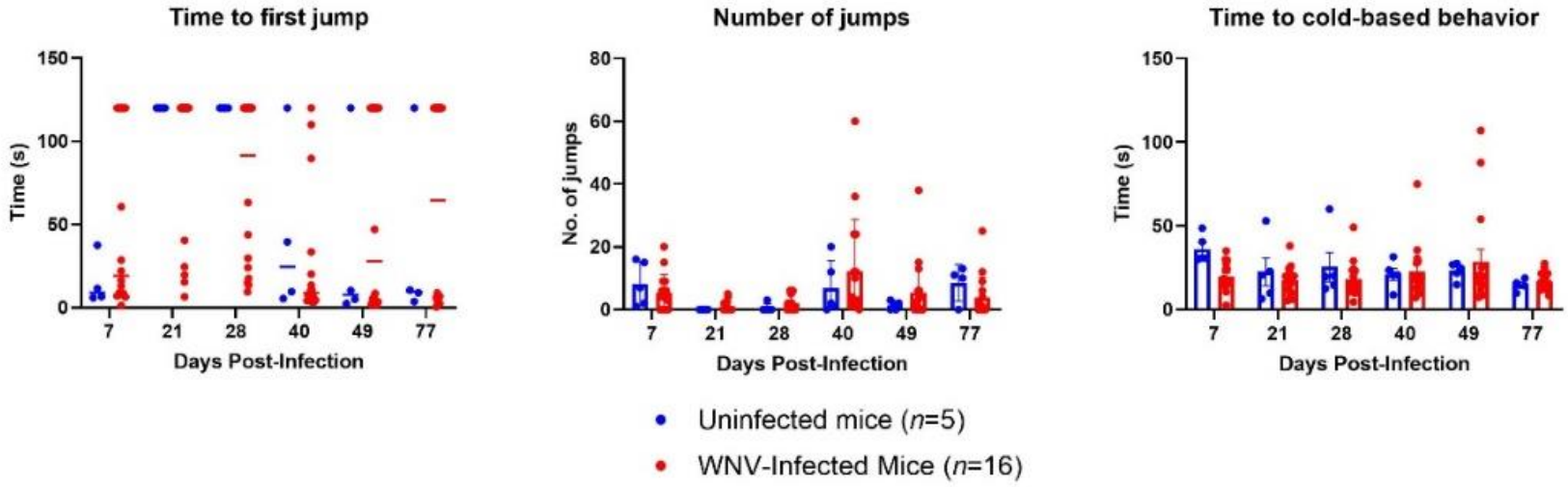


Figure 13: Results from initial cold plate testing on days 7, 21, 28, 40, 49, and 77 post-infection.
Mice were put onto a cold plate set to 0°C and observed for two minutes. The first time to jumping, nnumber of jumps, and time to first cold-based behavior were recorded on each of these days. Uninfected and WNV-infected mice showed large variability in the different measures across different days, indicating that results from this testing was difficult to reproduce.

Mice did not show depression-like behavior via the tail suspension test

The TST was performed to assess for depression in mice after infection. Mice were suspended by their tails for six minutes, and video recorded. The total time the mouse spent immobile was used as a correlate for depression. Using regression estimates, there was not a significant correlation between time spent immobile during the TST and infection status (**Table 8**). This indicated that infection did not correlate with depression. The distribution of immobility times was similar between uninfected and infected mice (**Fig. 14**). However, two of the infected mice did show longer immobility times than any of the uninfected mice, indicating a possible depressive phenotype, though this was not found to be statistically significant.

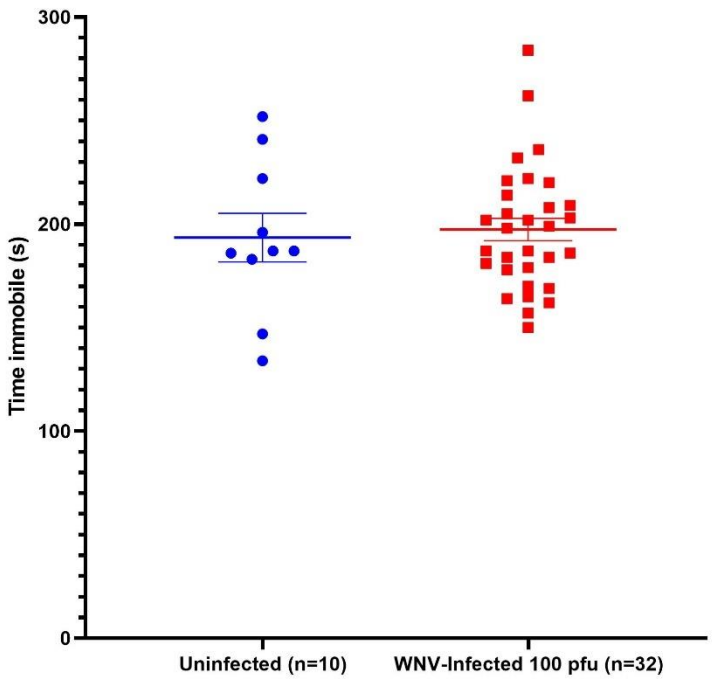


Figure 14: Effects of infection on tail suspension test. WNV-infected and uninfected mice were tested at 21 dpi. Mice were suspended by the tail for six minutes and total time immobile was recorded. Infection status does not correlate with significant changes in immobility time. Error bars are mean +/- SEM.

	TST		
Parameter	Estimate	SE	P-value
Infected (*n=31*)	3.26	14.47	0.82

Table 8: Regression estimate analysis for tail suspension test compared to infection status.

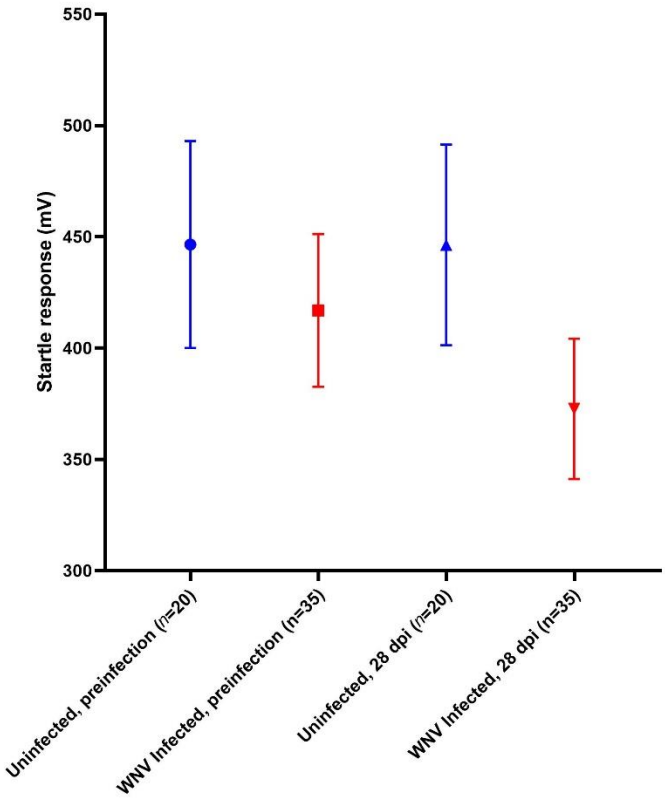


Figure 15: Effects of WNV infection on acoustic startle response.
Startle response was calculated based on the average of ten trials per mouse preinfection and at 28 dpi. Infection status does not correlate with significant changes in acoustic startle response. Values were compared using Student's t-test to compare appropriate times pre- and post-infection. Error bars are mean +/- SEM.

There was no significant difference between uninfected and WNV-infected mice in the acoustic startle response test

The ASR test did not show significant differences between infected and uninfected mice (**Fig. 15**). This would indicate that hearing loss does not occur in this mouse model following infection. It does indicate that the PPI testing would not be compromised due to hearing loss in the infected mice, so results would still be valid.

There was no difference between mice in prepulse inhibition based on infection status

PPI testing was performed to assess for sensorimotor gating in mice. The mice were exposed to sounds at 6, 12, or 18 dB above the background noise (i.e., 71, 77, or 83 dB) before receiving a 120-dB sound, and the amount of movement was measured. The total movement across ten trials for each level of noise was compared to their ASR movement, and the percent reduction in movement was recorded as their percent inhibition.

There was a high amount of variability within all mice at the +6 dB PPI testing level (71 dB). This may have been due to the low amount of auditory stimulation. The

room in which the mice were housed had a steady background level of 71-73 dB which may have reduced the mice's recognition of sounds at that level. There was also a large amount of variability at the +12 dB level, so analysis was only performed on the +18 dB response, as this has been used as a measure of sensorimotor gating in previous work, and consistently shows when changes have occurred[282–284].

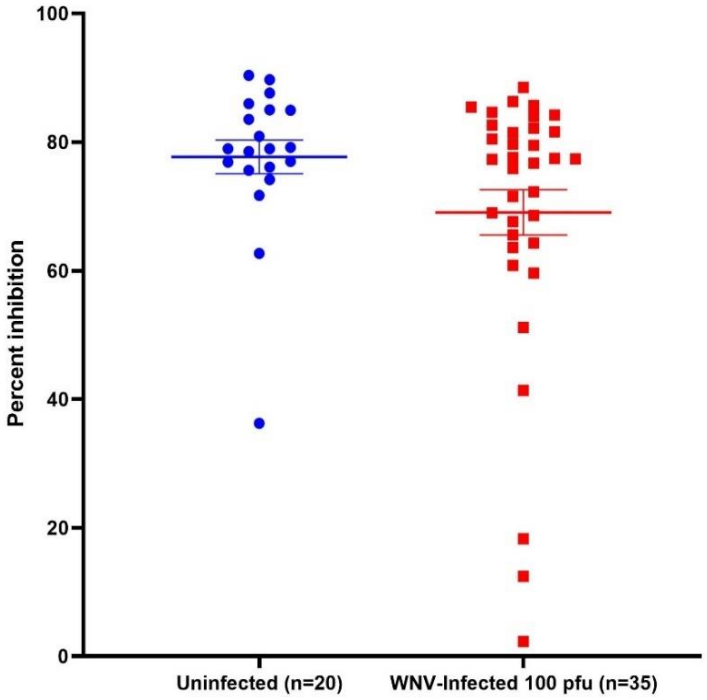


Figure 16 Effects of WNV infection on prepulse inhibition.
Percent inhibition was calculated based on acoustic startle response. Average percent inhibition across ten trials per mouse was recorded at 28 dpi. Infection status does not correlate with significant changes in prepulse inhibition. Error bars are mean +/- SEM.

Parameter	PPI 28 dpi Estimate	SE	P-value
Infected (*n*=35)	-7.70	7.51	0.31

Table 9: Regression estimate analysis for PPI testing based on infection status.

The PPI testing resulted in generally similar distributions between infected and uninfected mice, with a slightly lower average in infected mice (**Fig. 16**). While one of the uninfected mice showed <50% inhibition, five of the infected mice showed <50% inhibition with three showing <20%. While there was not a significant correlation between PPI performance and infection status (**Table 9**), this could indicate some form of damage to the neuronal circuits responsible for PPI in these specific mice.

There was no significant difference between WNV-infected and uninfected mice tested via the rotarod

Because some of the mice were not motivated to stay on the rotarod, they were initially screened using up to three trials of one minute at 4 rpm at 22 dpi. 90% (9/10) of the uninfected mice and 74% (26/35) of the infected mice that were screened passed the screening testing.

Mice were then tested three times daily for three days in a row and the time to falling was recorded for each of these trials. Control mice and infected mice showed similar performances on the first three trials, indicating that loss of motor control was not seen in this model. The control mice showed a significant increase in time on the rotarod on the fourth trial (the first trial of the second day) when compared to their initial performance. The infected mice performed generally worse

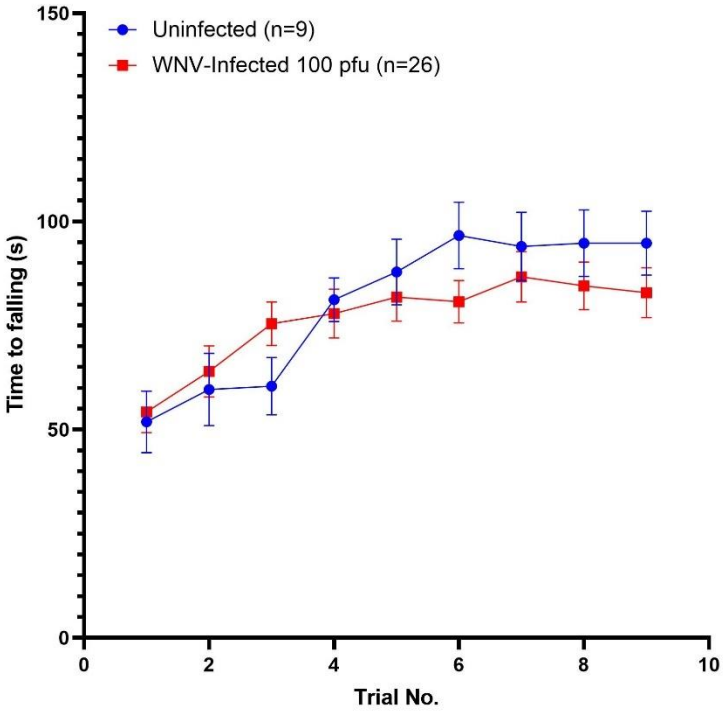


Figure 17: Effects of infection on rotarod performance. Time to falling was recorded for three trials per day across three days, from 23 dpi to 25 dpi. Infection status does not correlate with significant changes in rotarod performance on any single day. Error bars are mean +/- SEM. Analyzed by repeated-measures two-way ANOVA.

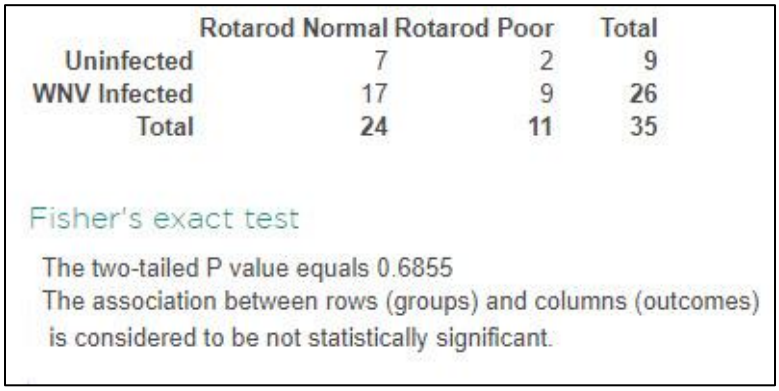
Parameter	Rotarod – trials 4-9		
	Estimate	SE	P-value
Infected (*n*=26)	10.96	12.60	0.39

Table 10: Regression estimate analysis comparison of WNV-infected mice compared to uninfected mice.

on trials 5-9 but this was not statistically significant on any single day (**Fig. 17**).

To determine if WNV infection adversely affected overall motor learning, regression estimate analysis was performed using trials 4-9 to compare uninfected and infected mouse performance (**Table 10**). These days were chosen as this was after the control mice first showed improvement on the rotarod indicating motor learning. There was not a significant difference between uninfected and infected mice via regression estimate analysis, indicating that when looking at infected mice in aggregate, there is not a significant difference in motor learning. Given that there were some low performing mice, it seemed reasonable to investigate other possible mechanisms of motor learning deficits.

To determine if a higher proportion of infected mice performed worse during the rotarod compared to uninfected mice, the bottom quartile of performance was calculated among the control mice based on trial number four. There were not enough control mice to calculate the tenth percentile, so the bottom quartile was used. Two of the uninfected mice fell below this threshold, and nine of the WNV-infected mice performed below this threshold. Using Fisher's exact test, there was no significant difference between the two groups (**Fig. 18**). This indicated that there was not a higher proportion of infected mice performing worse than the uninfected mice on trial four.



	Rotarod Normal	Rotarod Poor	Total
Uninfected	7	2	9
WNV Infected	17	9	26
Total	24	11	35

Fisher's exact test

The two-tailed P value equals 0.6855
The association between rows (groups) and columns (outcomes) is considered to be not statistically significant.

Figure 18: Rotarod performance as categorized into two groups based on trial four.
Rotarod normal mice remained on the rod for >68 sec, and Rotarod Poor mice fell in <68 sec. There is not a significant difference between WNV infected and uninfected mice in likelihood of lower performance on the rotarod.

WNV-infected mice tested via the two-way active avoidance test were more likely to fall into a lower performing group

The mice were tested for memory function using the 2WAA paradigm from 30-34 dpi. Mice were given 30 trials per day. Each trial consisted of a five second light and sound stimulus (the conditioned stimulus) followed by a five-second shock applied through the floor (the unconditioned stimulus). The mice could avoid or escape the shock by crossing from one side of the chamber to the other through the separator during the conditioned or unconditioned stimulus. The mice could learn to predict the shock and avoid it by crossing between chambers during the conditioned stimulus, which was considered an "avoidance." The number of avoidances each mouse had during each day of testing was recorded. Peak performance in uninfected mice occurred at 33 dpi, with a slight dip in performance at 34 dpi, though this was not significant.

For the 2WAA, performance between control and infected mice was not significantly different (**Fig. 19**). Some of the infected mice performed much worse individually than any of the control mice. Four of them had zero avoidances at 33 dpi, and seven had fewer than three avoidances, when none of the control mice had fewer than four avoidances. This indicated that there was likely memory dysfunction in some

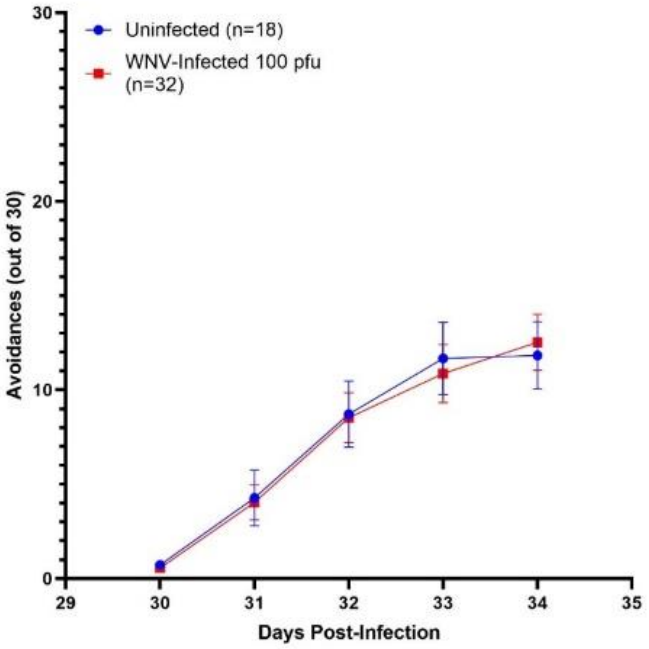


Figure 19: Effects of WNV infection on active avoidance performance
WNV-infected and uninfected mice were tested for memory function from 30 to 34 dpi. Number of avoidances out of a possible 30 are recorded for each day of testing. Infection status does not correlate with significant change in 2WAA performance on any single day. Error bars are mean +/- SEM.

mice surviving WNV infection but that this was not uniform. To determine if WNV infection increased the proportion of mice with lower 2WAA performance, the lowest tenth percentile for control mice was calculated for 33 dpi and determined to be four avoidances. This time point was chosen as it was the peak of uninfected performance (seen in **Fig. 19**). One uninfected mouse and ten infected mice fell below this threshold. The numbers of uninfected and infected mice that fell below this threshold were counted and compared via a Fisher's exact test (**Fig. 20**). This indicated that, while not significant, there was a trend toward more mice with low performance in the 2WAA test during the fourth day of testing. This was consistent across multiple days, as infected mice with low performance on 33 dpi had significantly reduced performance on 32 and 34 dpi (**Fig. 21**), indicating that this was not a single occurrence but a pattern of behavior for these mice.

	d4 Normal 2WAA	d4 Poor 2WAA	Total
Uninfected	17	1	18
Infected	22	10	32
Total	39	11	50

Fisher's exact test

The two-tailed P value equals 0.0717

Figure 20: 2WAA performance as categorized into two groups based on 33 dpi.
2WAA normal mice had ≥4 avoidances, and 2WAA Poor mice had <4 avoidances. There is not a significant difference between WNV infected and uninfected mice in likelihood of lower performance on the 2WAA, but there is a trend toward significance.

DISCUSSION

These sets of experiments showed that mouse age at time of infection played a major role in determining survival post-WNV infection, and that 100 pfu peripherally inoculated into the footpad at 16 weeks of age led to 70% survival, which was considered enough to continue with behavioral testing. Mice infected in this way showed significant

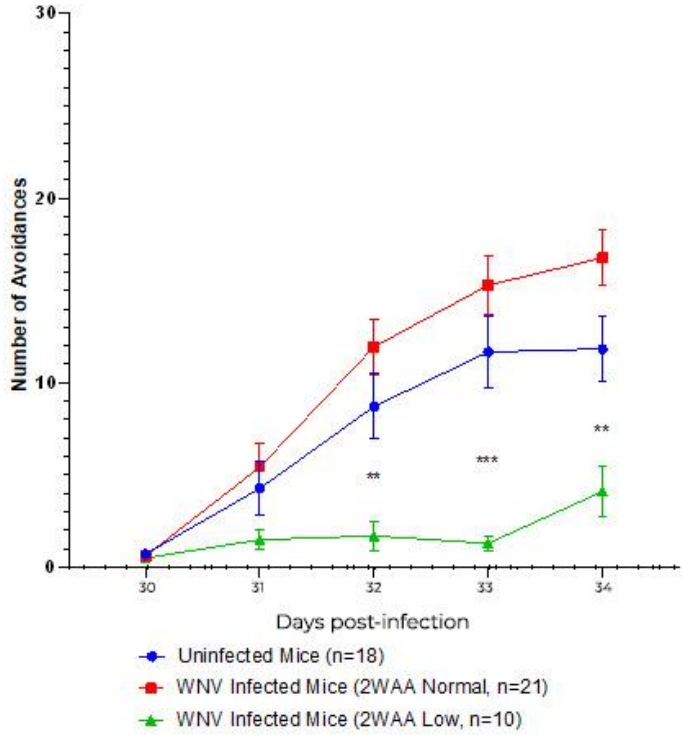


Figure 21 Two-way active avoidance results as separated by 33 dpi performance.
Infected mice that had fewer than four avoidances when tested at 33 dpi were considered "2WAA Low", and all others were considered "2WAA Normal." Infected mice that were 2WAA Low performed significantly lower than uninfected and 2WAA Normal mice when tested at 32 and 34 dpi. Analyzed via repeated measures 2-way ANOVA. ** $p<0.01$; *** $p<0.001$

weight loss midway through the acute phase of infection, and those with severe clinical signs at this point did not survive long enough to be tested for behavior.

The behavioral testing required optimization and some of the tests did not work either due to lack of reproducibility or issues with the machines. There was not a difference in memory phenotypes when mice were tested at 35 or 50 dpi. The results from the behavioral testing indicated that in this model, WNV did not induce uniform behavioral changes in infected mice after the acute phase of infection. There was a trend toward more infected mice showing depressed 2WAA performance on the fourth day of testing. Mice that had low 2WAA performance on the fourth day of testing had significantly lower performance on the third and fifth days as well.

Given the variability in human patients, the inconsistency in mouse behavior post-infection was not surprising, and indicated viral infection could lead to different outcomes. This could be due to differences in the progression of disease, immune response, initial localization of viral infection in the brain, and persistence of virus in different brain regions.

Chapter 4: Neuroinflammation

INTRODUCTION

The CNS has a specialized immune system because of its separation from the rest of the body, which is due to the existence of barriers separating the CNS from the periphery. These include the meninges, the BBB, and the blood-cerebrospinal fluid barrier. These barriers prevent the transit of most compounds or cells into the CNS except through active transport. The barriers include the meninges, the BBB, and the blood-cerebrospinal fluid barrier. Three major cell types play a role in maintaining the BBB: endothelial cells, pericytes, and astrocytes[285]. The endothelial cells have tight junctions that lack fenestrations, preventing most solutes from crossing into the brain. Pericytes and the endfeet of astrocytes contact the endothelial cells and signal to maintain the BBB and to adjust blood flow based on surrounding conditions. These barriers protect the CNS from exposure to peripheral toxins and infectious agents, but also preclude normal immune surveillance in the CNS. Cells in the peripheral blood cannot cross the BBB without alterations in its permeability, preventing immune surveillance via circulating leukocytes. The CNS lacks a strong innate immune response without the involvement of peripheral circulating cells. Because the normal innate immune cells do not normally enter into the CNS, most of the cells in the CNS have the ability to produce and respond to inflammatory cytokines[286,287].

Inflammatory agents can enter the CNS via multiple mechanisms. One way is through the areas of the CNS that are more accessible to the periphery, which can serve as points of entry for infectious agents without breakdown of the normal barriers. These include the choroid plexus, the olfactory bulb, and the circumventricular organs (CVO). The choroid plexus and CVO are in direct contact with blood flow, while the olfactory bulb has neurons projecting into the nasal cavity. Western equine encephalitis virus and

Venezuelan equine encephalitis virus, for instance, enter through the olfactory neurons[288]. The endothelial cells lining the blood vessels in the CNS can also be infected by some infectious agents, and these agents can then cross into the CNS. The barriers in the CNS can also be broken down due to signaling from cells inside or outside of the CNS, including microglia, monocytes, and lymphocytes. When this breakdown occurs, the CNS become more exposed to agents in the peripheral blood, allowing from transmission of infectious agents and toxins[285]. During inflammatory events in the CNS, including infections, peripheral cells can transit from blood vessels into the CNS through multiple mechanisms, including breaking down the BBB[289]. Infectious agents, such as rabies virus, can also travel through peripheral neurons into the CNS by using retrograde transmission[290].

When inflammation does occur, the overall response in the CNS is markedly different than in the periphery. Microglia serve as the innate immune cells of the CNS and perform regular immunosurveillance. When exposed to an inflammatory agent, the microglia become activated, changing their activity and the activity of surrounding cells including, astrocytes and neurons. Neurons in the CNS are well-differentiated and non-replicative, and therefore mostly cannot be replaced if lost. These cells tend to be more resistant to cell death for this reason. With chronic inflammation, such as from NDs or persistent viral infections, these immune responses can become pathologic, with neuronal malfunction and death[291]. While infectious agents can be a cause of inflammation in the CNS, the cause of inflammation in NDs varies and is sometimes unknown. In some cases, such as AD, misfolded proteins induce inflammation[292], while in others such as MS, autoimmune reactions to normal proteins cause the inflammation[293].

Neurodegenerative diseases and neuroinflammation

NDs are a group of diseases defined by a chronic, progressive loss of neuronal function. They include AD, PD, ALS, MS, traumatic brain injury (TBI), and HD. The clinical signs of these diseases vary based on the disease and the patient though they

commonly manifest as movement, behavioral, or cognitive disorders[294]. They are increasing in prevalence worldwide though especially in developed countries. The etiologies of these diseases vary, and many remain unknown.

NDs all show hallmarks of neuroinflammation, including activation of astrocytes and microglia, production of pro-inflammatory mediators, and peripheral immune cell infiltration into the CNS parenchyma[295]. Peripheral immune cells, including monocytes, neutrophils, and T cells, infiltrate the CNS in response to the release of inflammatory mediators. These cells cause further damage through cytotoxic activity, the release of pro-inflammatory cytokines, and breakdown of the BBB.

NDs affect specific regions of the brain, partly due to differential effects of inflammation in these regions[296]. AD affects the cortex and hippocampus, PD affects the substantia nigra of the midbrain, ALS affects motor neurons of the spinal cord and motor cortex as well as regions of the cortex, MS affects the white matter tracts of the CNS, and HD affects the striatum and basal ganglia of the forebrain[297].

Neuronal subtype can affect susceptibility to different NDs. The susceptibility of neuronal subtypes can change with age, as well[297]. AD affects large pyramidal neurons of the hippocampus and cholinergic neurons of the forebrain; PD affects pigmented dopamine neurons[298]; MS affect long, highly myelinated neurons[299]; HD affects medium spiny GABAergic neurons; and ALS affects motor neurons in the spinal cord, brainstem, and motor cortex[300]. This indicates that different neurons have different susceptibilities to specific stimuli and inflammatory mediators, which could be used to explain the susceptibility of certain neurons to virus-induced damage.

The overlap between neurodegenerative diseases and viral infections

To develop a model of WNV-induced neurological disease and compare the findings to NDs, it is important to have full understanding of the normal roles of the cells in the CNS and the roles these cells play in inflammation is important. One important area

for investigation is the pathogenesis of NDs. The symptoms of these diseases are often similar to the sequelae following neuroinvasive virus infection, including those following WNV infection. PD is characterized as motor incoordination due to loss of dopaminergic neurons in the substantia nigra, which has been reported with WNV infection as well[301]. AD commonly manifests as memory loss, which is a commonly reported sequelae following WNV infection[97].

The inflammatory mediators found to cause and exacerbate NDs are the same as those necessary for viral clearance from the CNS, including for WNV. During WNV infection, T cells cross the BBB to help reduce viral infection and eliminate infected cells. T cells crossing the BBB are also associated with long-term damage and ongoing inflammation in multiple NDs. TNF-α[166], complement proteins[302,303], IL-1β[165], IL-6, and IFN-γ[106] have all been shown to reduce mortality in the mouse model of WNV. These same cytokines have been shown to maintain and exacerbate neurodegeneration in diseases such as TBI, AD. PD, and MS[304]. Virus-induced neuroinflammation is not limited to WNV: studies have shown potential links between AD and herpes simplex (HSV) -1 infection[305–308]. Repeated HSV-1 reactivation has been shown to cause the accumulation of AD molecular markers and behavioral changes consistent with AD in a mouse model[309]. The presence of proinflammatory cytokines can also affect neurological function, as systemic administration of pro-inflammatory cytokines causes depression[310], and increased levels of these cytokines are risk factors for more severe memory loss in NDs[304].

One important aspect regarding neuroinflammation is the heterogeneity of the CNS. Although the general types of cells are similar across different regions, the subtypes of these cells vary in activity and susceptibility to disease. Different brain regions have heterogenous immune responses which affect their susceptibility to NDs and infectious diseases[287]. These responses include interferon responses[311], which play a role in determining the tropism of viruses, including WNV. These immune responses can change with age[312], leading to increased susceptibility of certain regions to NDs and viruses.

Cells of the Central Nervous System

To understand the mechanisms behind WNV-induced neurological changes and NDs, it is important to know about the cells of the CNS and their functions during health and disease. Here, three types of cells unique to the CNS are summarized, in addition to their activities during NDs and viral infections.

NEURONS

Neurons are the primary active units of the CNS and are responsible for carrying, processing, and relaying information throughout the body. They constitute a large proportion of the cells in the CNS, with estimates of up to 10^{11} neurons in the human brain[313]. Because of the varying functions of neurons, there are over a dozen subtypes of neuron that have been defined based on transcriptional activity[314], though likely there are many more. Neurons propagate action potentials and use neurotransmitters at synapses to create networks to transfer and integrate information. Neurons can change the strength of signals that they receive through a mechanism called synaptic plasticity, allowing for memory and learning. There are multiple mechanisms that cause the changes in synaptic plasticity, but most commonly it is through changing the number of ion channels in a synapse, allowing for increased reactivity of the postsynaptic neuron to a released neurotransmitter. This process is called short-term or long-term potentiation (STP or LTP), depending on the mechanism and time frame. Neurons can also develop additional dendrites to increase receptivity to other neurons[315].

Neurons are derived from neural stem cells early in development and are well-differentiated by adulthood. Neurons have varying degrees of regenerative capability, with most neurogenesis in adulthood occurring in the subventricular zone and the hippocampal dentate gyrus[316]. Because many areas of the brain do not show regenerative capabilities, when these neurons die, they cannot be replaced.

During NDs, neurons undergo several changes. One of the most prominent is the loss of neurogenesis in areas normally containing neural stem cells. In mouse models of AD, there is significant reduction in proliferation and survival of neural stem cells in the dentate gyrus, which correlates with Aβ deposition[317]. Human cases show a similar decrease in neural stem cells and neurogenesis[318,319].

Neurons are a major target for many viral infections, including WNV[155]. The different subtypes of neuron have shown varying susceptibility to viral infection and inflammation[156] primarily based on the basal levels of IFN-regulated genes. The immune system of the CNS needs to be able to control viral infections without inducing cell lysis. The non-cytolytic mechanisms that neurons have are relatively effective in controlling infections as diverse as WNV, rabies virus, MV, and varicella-zoster virus[320].

Generalized inflammation can have effects on neuronal function. Chronic inflammation can alter the functions of neurons[321]. Inflammatory cytokines can cause mitochondrial failure, mutations in mitochondrial DNA, and oxidative stress, leading to an overall reduction in neurons[322]. Neuronal excitability and plasticity also change in response to inflammatory signals[323,324], causing neurons to malfunction, which can lead to severe clinical effects. Inflammatory mediators can be toxic to neurons, leading to apoptosis of the neurons[325].

Autophagy is inhibited in neurons during NDs, and this inhibition has been shown in AD, PD, and HD. With inhibition of autophagy, autophagosomes can no longer clear misfolded or aberrant proteins[326]. The accumulation of misfolded proteins in neurons plays a role in multiple NDs including AD, PD, and TBI. **Table 11** summarizes the different proteins that, when misfolded, have been implicated in various NDs.

Table 11: Misfolded proteins and associated neurodegenerative diseases.

Protein	**Neurodegenerative Diseases**
Amyloid-beta	Alzheimer's disease[327]
	Corticobasal degeneration[328]
	Frontotemporal dementia[328]

Alpha-synuclein	Dementia with Lewy bodies
	Parkinson's disease[329]
Tau	Alzheimer's disease[330]
	Chronic traumatic encephalopathy[331]
	Corticobasal degeneration[328]
TAR DNA-binding protein 43	Amyotrophic lateral sclerosis[332]
	Frontotemporal lobar degeneration[332]

Viral infections can also inhibit autophagy in neurons. This likely assists in the virus avoiding the innate immune system, but it also can cause pathology in the CNS. HIV has been shown to inhibit autophagy. Impaired autophagy makes the neurons more sensitive to apoptotic and inflammatory signaling, and induces neuronal dysfunction and death[333]. HSV-1 blocks autophagy through inhibition of the Beclin-1 pathway[334]. The flaviviruses dengue virus (DENV) and JEV also manipulate the autophagy pathways for replication, though they activate the pathway to evade the immune system[335,336]. Manipulation of the autophagy pathway can lead to accumulation of toxic proteins, and this has been suggested as a link between NDs and viral infections[337]. Some of the proteins associated with NDs have also been associated with antiviral responses. Aβ is effective in protecting the brain from viruses including HSV-1[338,339] and influenza[340]. Aβ binds the surface glycoproteins of HSV1 and entraps the virus[341]. The production of Aβ in response to viral infections has been suggested to be an inciting event for the development of AD because the viral infection promotes Aβ deposition until it becomes pathogenic.

ND disease progression can destroy the axons and dendrites of the neurons through multiple mechanisms. Destruction of these processes reduces the ability of the neurons to function and signal to each other. In AD, synapses are eliminated after being labeled with complement proteins[342]. IL-1β, which is often overproduced in NDs, has been shown to disrupt the formation of new dendritic spines, interfering with memory formation[343]. Depression has been linked to decreased neurogenesis[344] and synaptic remodeling[345] as well, specifically in the hippocampus, prefrontal cortex, and nucleus accumbens[346]. Viral infections can also induce destruction or reduction of axons and dendritic spines. HIV has

been associated with complement-independent removal of synapses[347] and strains of influenza associated with neurological disease cause decreased dendritic spine density in the hippocampus[348,349].

STP and LTP can be influenced by inflammation induced either by NDs or viral infections. Mouse models of AD show significant reduction in LTP[350], and TNF-α[351] and IL-1β[352] have been shown to play a role in reduction of LTP in these models. Influenza virus[348], HIV[353], and rabies virus[354] have been shown to disrupt STP, LTP, and the development and maintenance of synapses, changing normal neuronal function.

Demyelination is a symptom of some NDs, most commonly MS. This can occur acutely or chronically depending on the inciting cause. Demyelination can be induced through T cells, antibodies, or death of the oligodendrocytes providing the myelin sheath[355]. Virally-induced inflammation can also cause demyelination of the neurons. Viral infections have been shown to induce demyelination as well. TMEV[356], CDV[357], and mouse hepatitis virus[358] are used as models of MS because they can induce demyelination, and demyelination has been reported in patients infected with polyoma JC virus[359], HIV[360], and MV when it manifests as subacute sclerosing panencephalitis[361].

MICROGLIA

Microglia are the innate immune and phagocytic cells of the CNS. They compose 5-15% of the cells in the brain, though this varies by brain region and animal species. In homeostasis, there are lower numbers in the cerebellum and hindbrain than found in other regions such as the cortex and hippocampus[362]. Microglia are unique amongst innate immune cells because they do not derive from the bone marrow, but initially come from the yolk sac and migrate into the developing CNS[363]. Microglia are diverse and show distinct differences in morphology, transcriptomes, and membrane properties in different regions of the brain[364].

During homeostasis, microglia perform other functions, including phagocytosis of inactive synapses during development[365], phagocytosis of apoptotic cells, and preventing inflammatory reactions through the secretion of anti-inflammatory cytokines, including transforming growth factor-β (TGF-β)[366]. Microglia monitor neuronal activity and make connections with neurons[367], which allows the microglia to change synaptic activity of the neurons[368]. Microglia produce many signaling molecules that have been shown to affect synaptic function, including cytokines, neurotransmitters, and extracellular matrix proteins, as well[369].

Due to their nature as the primary innate immune cell in the brain, microglia are mostly responsible for the initial inflammatory reactions in the brain. They express almost all TLRs[370] and so are highly reactive to infectious diseases through the recognition of pathogen-associated molecular patterns. Microglia also have receptors for damage-associated molecular patterns, which causes them to activate in response to signs of cell damage[371]. In response to different stimuli, microglia can become "activated", which refers to changes in cellular activity and phenotype. Activated microglia shorten their processes and upregulate surface markers including MHC-II, Cd11b, and ionized calcium-binding adaptor molecule-1 (Iba1), along with substantially changing their transcriptional profile[372]. Previously, activated microglia were considered to be one of two phenotypes, M1 (pro-inflammatory) and M2 (anti-inflammatory). More recent data has shown that there is a spectrum of phenotypes, indicating a more nuanced response to inflammatory stimuli[373], including some microglia that have overlapping M1 and M2 phenotypes[374]. Microglia can produce a range of pro-inflammatory cytokines, including IL-1α, complement protein C1q, and TNF-α. These cytokines signal other cells to change activity, including astrocytes[375], which can induce persistent changes in these cells.

Microglia play a major role in the initial immune response to viral infections. They are a major initial source of type-I IFNs[376] depending on the virus and are also highly responsive to IFN signaling. During HIV infection, microglia can cause pathology similar

to NDs. They release neurotoxins and cytokines that can damage neurons. These signals also reduce neuronal autophagy in HIV infection, inducing apoptosis and neuronal dysfunction[333].

Certain stimuli in NDs activate microglia, including misfolded proteins, adenosine triphosphate, or aggregates of abnormal proteins such as Aβ. Activation manifests as multiple phenotypes that can cause damage to surrounding neuronal cells. Activated microglia surround plaques in AD brains[377], and help clear Aβ peptides during early the early stages of AD. Persistent activation changes the activity of microglia and can lead them to cause pathology[378], including decreased clearance of misfolded proteins, or even their propagating misfolded proteins[379]. Microglia cannot clear plaques of Aβ, but are still recruited to regions with them, which can exacerbate the damage by causing further inflammation[380]. In many NDs, microglia cause injuries to the axons of neurons through direct contact and the release of inflammatory and neurotoxic molecules[381,382]. The prevalence of PRRs on microglia likely plays a role in their inflammatory role in NDs. It has been suggested that microglia detect abnormal proteins such as Aβ and α-synuclein, because exposure to these proteins leads to the production of similar cytokines as those induced through PRRs[383]. Similar to NDs, viral infections can cause damage through microglia-induced inflammation. While the release of pro-inflammatory cytokines is necessary for clearance of pathogens, it can also result in abnormal function. Pro-inflammatory cytokines, such as IL-1β and TNF-α, can induce neuronal apoptosis through increased glutamate production[384,385]. The pro-inflammatory cytokines that microglia release can inhibit synaptic plasticity[386], reducing the adaptability of neurons and the ability to develop memories. Excessive production of IL-1β has been implicated in multiple NDs and plays a major role in the activity of microglia in these diseases[387], and it is also associated with WNV-induced memory loss in mice[164].

Like during normal development, microglia phagocytose synapses in response to markers on the cell surface, including C1q. During NDs, this activity becomes dysregulated

and phagocytosis of healthy synapses occurs. This has been shown to be a cause of neuronal degeneration in AD[209]. This leads to neurological dysfunction, including memory loss, which can be prevented with elimination of microglia, complement proteins, or certain pro-inflammatory mediators. The phagocytic activities of microglia can also cause pathology in viral infections. Microglia phagocytose infected neurons which can help prevent spread of virus[388]. Microglia have been shown to phagocytose the myelin sheaths of neurons during mouse hepatitis virus (MHV) infection of *ex vivo* brain slices indicating that demyelination can occur in viral infections[389]. As mentioned previously, microglia are induced to phagocytose presynaptic terminals in viral infections, including in WNV[162], ZIKV[163], and HIV[347].

Microglia have been shown to play a significant role in demyelination during NDs such as MS. Microglia form focal plaques in areas of demyelination. These nodules can serve as a site allowing for pathogenic T cells to enter the CNS, and facilitate the destruction of neurons[390]. T cells have been shown to cause damage after viral infections. Viruses such as HIV and cytomegalovirus can establish persistent infections in the brain, which leads to chronic stimulation of T cells. This chronic stimulation can lead to T cells inducing persistent inflammation, which can lead to nervous system damage[391].

ASTROCYTES

Astrocytes are one of the most numerous glial cells in the CNS, accounting for up to 57% of all glial cells depending on species and brain region[392]. They represent a diverse subset of glial cells in the brain, with four described classes based on structure and anatomic location[393]. Morphologically, they are large cells with extensive fibrils that interact with other cells of the CNS. Glial fibrillary acidic protein (GFAP) is used as a marker of astrocytes, though its expression can vary depending on subtype and activation status[394,395]. Different classes of astrocytes provide different functions, but they all generally serve to support the maintenance of function and homeostasis in the brain. Astrocytes mediate

blood flow, maintain the BBB, recycle excess neurotransmitters, maintain of synapses, regulate ionic homeostasis[393], and support and recycle mitochondria for neurons. They play key roles in normal CNS function, inflammation, and neurodegeneration.

Astrocytes play essential roles in both maintaining and reducing inflammation during NDs. Like microglia, astrocytes can become activated (also known as "reactive astrocytes") in response to various stimuli. Activation changes their phenotype and activity, though the exact changes vary based on different factors. The stimuli that can induce these changes include TBI, peripheral inflammation, NDs, and infections. Astrocytes express TLRs and thus can respond to PAMPs, but the expression pattern varies based on brain region. Two major phenotypes of reactive astrocytes were initially reported: A1 and A2 astrocytes[375]. In response to stimuli such as lipopolysaccharide, microglia release complement protein C1q, and the pro-inflammatory cytokines IL-1α and TNF-α. These signals induce transcriptomic changes in astrocytes exposed to the signals, leading to a pro-inflammatory, neurotoxic form of astrocyte called an A1 astrocyte. When astrocytes are exposed to TGF-β, they take on an anti-inflammatory, neurogenic phenotype called an A2 astrocyte. In the original study, A1 astrocytes were induced when mice were given lipopolysaccharide intravenously. The paradoxical nature of astrocytes in NDs can largely be explained through the lens of the differentiation into A1 or A2 astrocytes. A1 astrocytes produce pro-inflammatory cytokines and neurotoxic molecules, exacerbating and maintaining inflammation and neurodegeneration. A2 astrocytes promote neurogenesis and reduce inflammation, leading to resolution and recovery. A1 astrocytes upregulate inflammatory signaling and pro-inflammatory cytokine release; glutamate and ATP release; and lipocalin-2 secretion. A1 astrocytes have been found in samples from patients with NDs, specifically in the regions associated with the disease. This included the substantia nigra in PD patients, the prefrontal cortex of AD patients, and demyelinating lesions in MS patients[202]. When astrocytes become activated, they can lose their glutamate reuptake function. This can lead to glutamate excitotoxicity of neurons, which causes

neurons to malfunction and undergo apoptosis due to overstimulation of the glutamate receptors[396]. This is a proposed mechanism for neuronal injury in NDs such as ALS and AD.

Astrocytes play multiple roles during viral infections, though it appears to vary based on the infecting virus type. Similar to their roles in NDs, astrocytes can be either beneficial or detrimental for resolution of damage to the CNS. In some cases such as rabies virus infection, abortively infected astrocytes serve as the major source of type-I IFNs which are crucial for viral clearance[397]. Astrocytes are often responsive to type-I IFNs, producing other cytokines important for viral clearance[398], though their reactivity depends on the region[399]. Expression of large numbers of PRRs such as TLRs and RLRs by astrocytes make them highly responsive to inflammatory stimuli. Signaling through these receptors can be crucial in clearing the virus from the CNS, but it can exacerbate neuronal damage through the production of pro-inflammatory cytokines[400]. These cytokines can both induce and restrict phagocytosis of myelin in response to viral infections[401]. Because of the close relationship between astrocytes and the BBB, astrocytes can play a role in its breakdown during acute viral infections. *In vitro*, viral infections of cultured astrocytes (including WNV) have been shown to induce matrix metalloproteases, which break down the BBB, allowing for viral entry[149]. Astrocytes can be signaled to tighten the BBB in response to IFNs, which can reduce viral infiltration of the CNS[154]. Astrocytes can also change their activity in glutamate uptake and the activity of potassium channels in response to viral infection, which can affect BBB permeability, neuronal function, and lead to glutamate toxicity[402].

These studies aimed to compare WNV infection to the inflammatory aspects of NDs and to determine if there were similar mechanisms in both cases. This would focus on screening for inflammation in multiple brain regions and comparing to behavioral outcomes to correlate inflammatory changes with neurological outcomes, with a focus on how these changes may compare to neurodegenerative diseases.

DEVELOPMENT OF TESTING

Multiple approaches were considered for neuroinflammation, including CLARITY imaging, fluorescence-activated cell-sorting, and transcriptomic analysis. Given that there was little described in the literature about long-term inflammation in the mouse model and that there were budgetary constraints, IHC was selected as the most cost-effective and adaptable testing for analyzing inflammation in the brain. Initially plans were made to perform co-staining with multiple antibodies, but there was difficulty in finding multiple, reliable antibodies that would not cross-react with primary or secondary stains. Chromogenic IHC was selected over fluorescent IHC due to ease of use and optimization, the lifetime of the slides, and the ability to identify cells and brain regions based on counterstaining.

Multiple stains were considered for use in studying neuroinflammation and ultimately Iba1 as a marker for microglia and GFAP as a marker for astrocytes. These markers are upregulated in activated microglia and astrocytes, respectively, but do stain non-activated cells of the same type. Iba1 can also stain infiltrating monocytes. However, the other options for stains were less specific or downregulated in response to activation. For example, P2Y12 is specific to microglia and not monocytes, but it is downregulated in activated microglia[403].

There were two IHC targets that were tested but were not optimized to the level of performing diagnostic staining. To investigate neuronal degeneration, staining for phosphorylated tau was performed. The tau phosphorylated at S396 is a commonly used marker for pathologic tau phosphorylation in AD and other NDs. Different levels of primary antibody were used to stain samples, but I could not acquire a positive control sample that was formalin-fixed and paraffin-embedded. I attempted staining on the samples available, but it was impossible to determine an optimal concentration that would show appropriate tau accumulation without a positive control. I tried using samples that were

paraffin-fixed and non-embedded, but the heat-mediated antigen retrieval led to inaccurate staining.

The complement protein C3 was selected as a target to characterize the subtype of activated astrocytes seen in the brain. The initial attempts aimed to co-stain C3 with GFAP, but this was determined to require too much optimization. Instead, similar to the other stains, slides adjacent to slides used for astrocyte staining were used to co-localize C3 and GFAP. Different concentrations of antibody were used to attempt to get specific signaling in the brain. Different concentrations of antibody did show varying levels of intensity, but there was significant variability across the uninfected controls regardless of antibody concentration, indicating variability in C3 expression in the normal brain. This meant that comparison between infected and uninfected brains would be impossible. It was not determined if this was due to off-target binding or if C3 was normally heterogeneously expressed. The first study describing A1 astrocytes used in-situ hybridization to find C3 mRNA rather than looking at protein expression[375], so that may be a more accurate approach.

RESULTS

WNV infection causes microgliosis and microglial nodule formation in some mice

To determine if WNV infection induced long-term inflammatory changes in the brains of mice, sections of brain from all mice euthanized at or before 35 dpi were taken and stained for Iba1. This is a marker predominantly for microglia in the brain, but it can also mark infiltrating monocytes or T cells. Reactive microglia are recognized through changes in their morphology. This includes thickening of processes, expansion of cytoplasm, and increased Iba1 expression. Microgliosis is an increase in the number of microglia in a region which is more subjective, and microglial nodules were defined as regions of densely populated microglia in a specific region.

The acute phase of infection was defined as lasting until 21 dpi. No mice required humane euthanasia after this point. The brains from mice that were euthanized during this period showed varying levels of microgliosis as measured by Iba1 staining. The majority showed severe microgliosis diffusely throughout the brain, though the regions varied from mouse to mouse.

Brain sections from mice euthanized at 35 dpi were analyzed and compared to uninfected controls to determine if there were increases in microglia or the formation of microglial nodules. Microgliosis was the most common finding, often accompanied by microglial nodule formation (**Fig. 22B, C**). There was occasional perivascular cuffing with accompanying lymphocytes though this was less common. Each brain was divided based on the anatomical landmarks used for dissection, and each region was analyzed for microgliosis or microglial nodule formation (**Table 12**). Microgliosis or nodule formation occurred in each region except the hippocampus in at least one mouse. Microglial lesions were found in 69% (24/35) of all infected mice indicating that this was a common occurrence. Lesions were most commonly found in the hindbrain, as 60% (21/35) of all

infected mice had lesions there. The midbrain was the next most common, with 49% (17/35) of infected mice having lesions in that area.

When microglial nodules or focal microgliosis were found, these were further localized to specific functional regions of the brain. An example of the distribution of microgliosis and microglial nodules compared to an uninfected control is shown in **Figure 23**. The regions varied from mouse-to-mouse. Microgliosis occurred most commonly in the pontine reticular nucleus of the hindbrain and in the multiple thalamic nuclei of the forebrain. The lesions in the midbrain were most common in the periaqueductal areas.

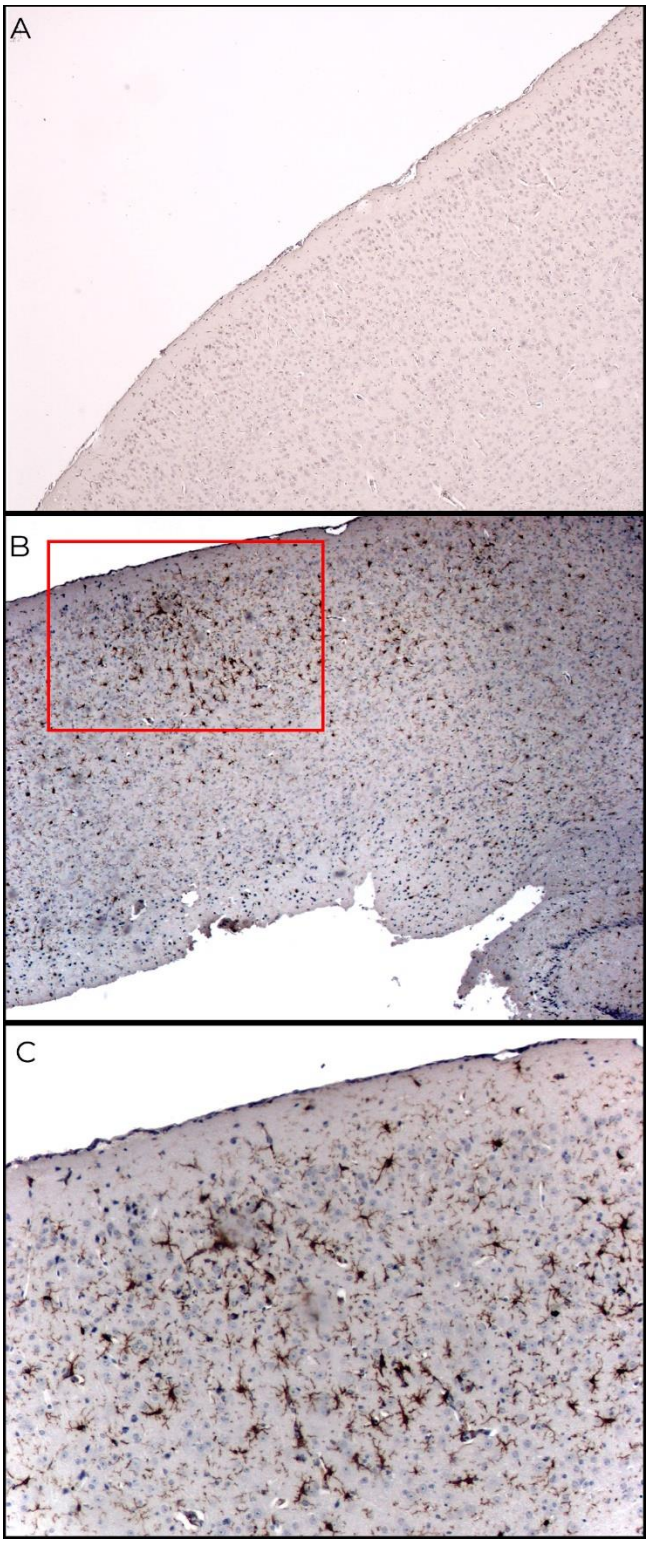


Figure 22: An example of a microglial nodule found in the rostral cortex of a mouse 35 dpi using Iba1 staining. **A**. normal microglial distribution in the cortex of an uninfected mouse (40x). **B**. the same region seen in a mouse 35 dpi with WNV. A large grouping of microglia has formed in the cortex (40x). **C.** a magnified view of the microglial nodule seen in **B (**100x**).** The microglia have enlarged and elongated processes and expanded cytoplasms indicating an activated state. They are more numerous here, and they normally are not found this close to the cortex of the brain.

	Iba1+ Lesions on IHC					
Mouse No.	Cerebellum	Hindbrain	Midbrain	Forebrain	Hippocampus	Cortex
1		+	+			+
2			+	+		
3						
4	+	+	+	+		
5	+	+	+			
6		+	+	+		
7						
8	N/D	+		+		
9	+	+		+		
10		+		+		
11	+	+	+	+		
12		+	+			
13						+
14	+			+		+
15		+	+	+		+
16	+		+	+		
17	+	+	+	+		+
18						
19						
20	+	+				
21	+	+	+			
22						+
23		+	+	+		+
24						
25						
26						
27	+	+	+	+		
28		+	+	+		
29						
30		+	+	+		
31		+	+			
32	+	+	+			
33						
34	+	+	+	+		
35		+	+	+		

Table 12: Areas of brains with microglial nodule or generalized microgliosis in mice 35 dpi.

Each row represents an individual mouse. + in a cell indicates that microgliosis or microglial nodules were detected in that anatomic region. N/D indicates not determined.

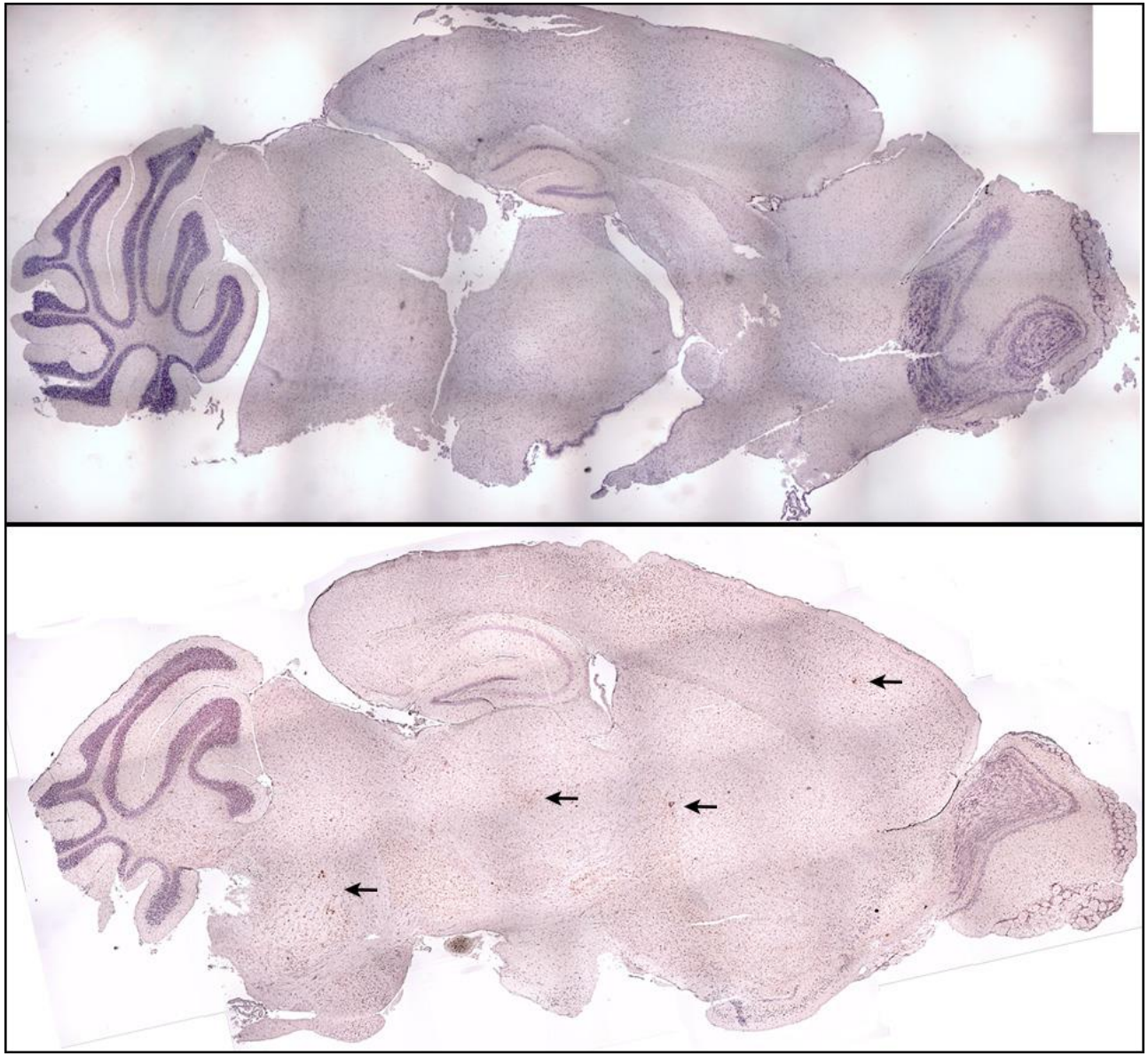


Figure 23: Comparison of microglial distribution. Microglial distribution in a control (above) and 35 dpi WNV-infected mouse (below). Stained using an anti-Iba1 antibody and DAB as the chromogen with Gill's hematoxylin as the counterstain. Note the wider distribution of microglia throughout the brain, including in the cortex, forebrain, and hindbrain. There are notable microglial nodules (marked by arrows) in the forebrain, cortex, and hindbrain.

Variability in microgliosis within individual mice

Microgliosis was found in multiple regions, and sections 1.0-1.4 mm lateral from the midline were assessed for the presence of microgliosis in each of the six anatomic regions previously discussed. This covered many of the larger functional regions of the brain, including the pons, thalamus, and most of the cortex.

Presence of microgliosis in specific regions was not consistent across multiple slides from individual animals, even when slides within 0.2 mm of each other were compared (**Fig. 24**). This could indicate an error in staining technique but given that the slides were stained using the same antigen retrieval methods, antibodies, and antibody concentration, this seems unlikely. The more likely explanation is that the microglial lesions seen are highly localized to specific regions. This means that further imaging would be necessary to fully characterize the microglial response following WNV infection.

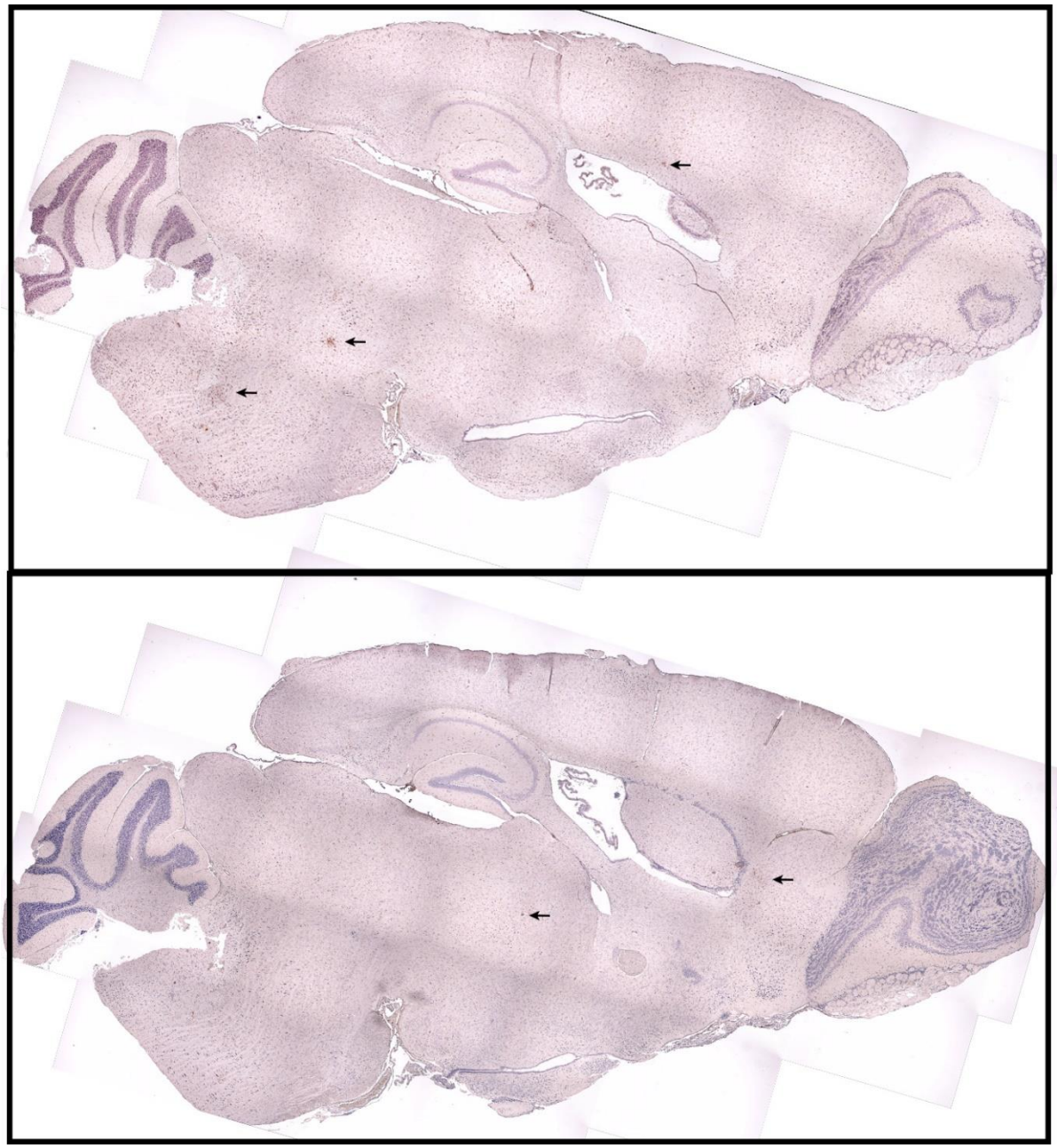


Figure 24: Sections of brain from the same mouse taken within 0.2 mm of each other. Note the presence of microgliosis in one section (top) including microglial nodules in the midbrain, forebrain, and hindbrain, and that these are lacking in the neighboring section (bottom). There are noticeable microglial lesions in the forebrain of the lower section (arrows, below) that do not occur in the above section. 40x, stained with anti-Iba1 antibody using DAB as chromogen with Gill's hematoxylin as counterstain.

WNV infection induces long-term astrocytosis in similar regions to microgliosis

To determine the extent of neuroinflammation, slides adjacent to those stained for Iba1 were stained for GFAP to investigate astrocyte activation. Astrocytosis is more difficult to determine than microgliosis, because astrocytes are more widespread than microglia, and typically have prominent processes. Astrocytosis was determined by examining the intensity of staining and looking for areas of dense astrocytes. Certain morphologic changes in astrocytes can be used to indicate reactivity, such as expanded cytoplasm, increased numbers of processes, and overlapping processes from multiple astrocytes.

There was significant difficulty in making GFAP staining repeatable as the concentration of astrocytes is already high in the brains of mice. Ultimately a relatively low concentration of primary antibody was used. This showed distinct areas of astrocytosis in some brains (**Fig 25;** magnified images are in **Fig. 26**). Astrocytosis and microgliosis did not always overlap, they were found in similar regions of the brain, including the hindbrain and midbrain. There were more examples of astrocytosis in the hippocampus as opposed to microgliosis, indicating some form of reactivity in that region.

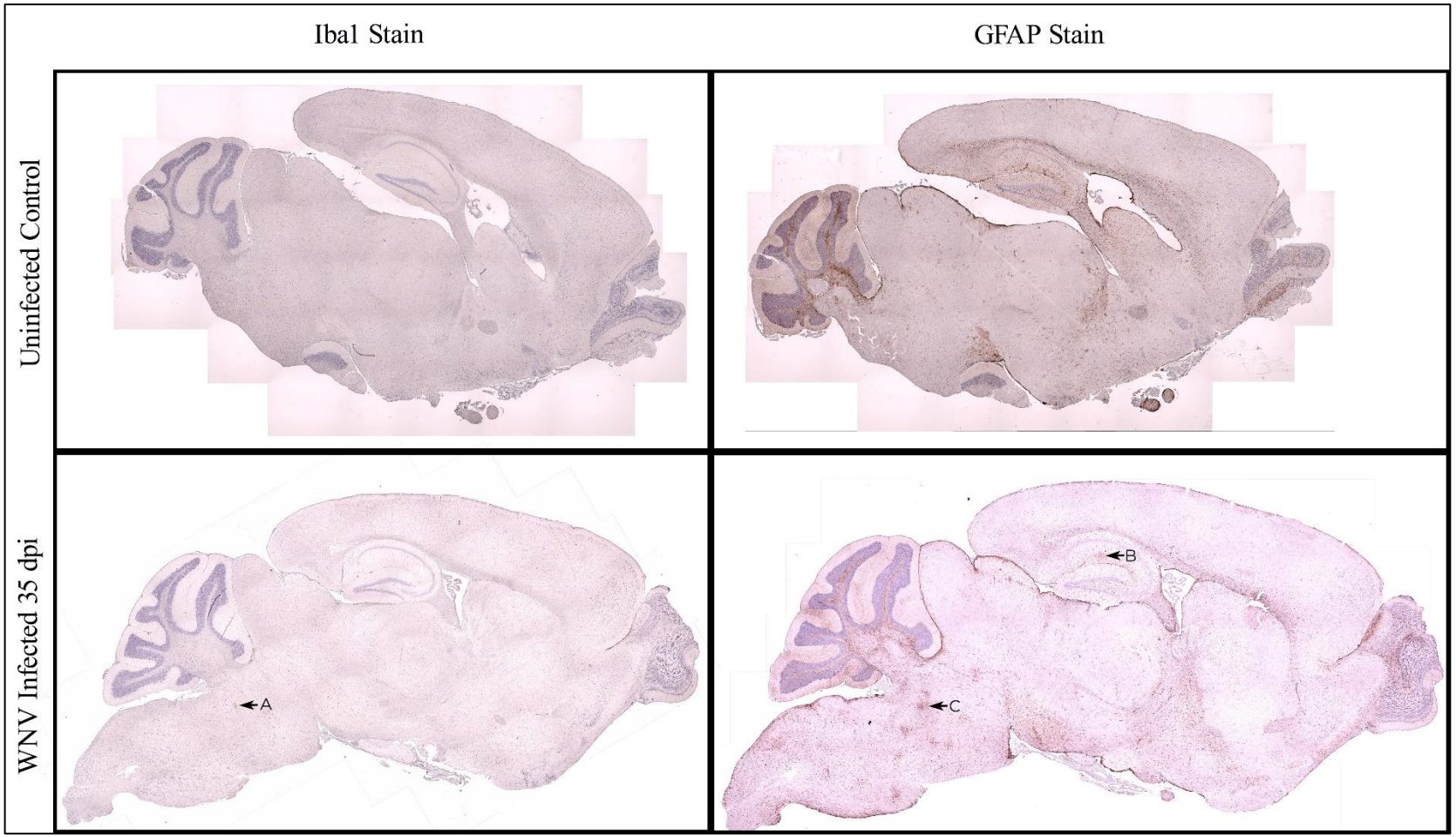


Figure 25: Comparison of GFAP and Iba1 staining in uninfected mice and mice surviving to 35 dpi with NY99 WNV. There is notable GFAP staining in the uninfected control due to the ubiquitous nature of astrocytes, while there is less staining noticeable for Iba1. In the WNV infected mouse, there are notable areas of microglial nodules in the Iba1-stained slide (arrows) and areas of reactive astrocytes in the GFAP stained slide (arrows). Magnified images for the appropriate arrows are found in Fig. 27.

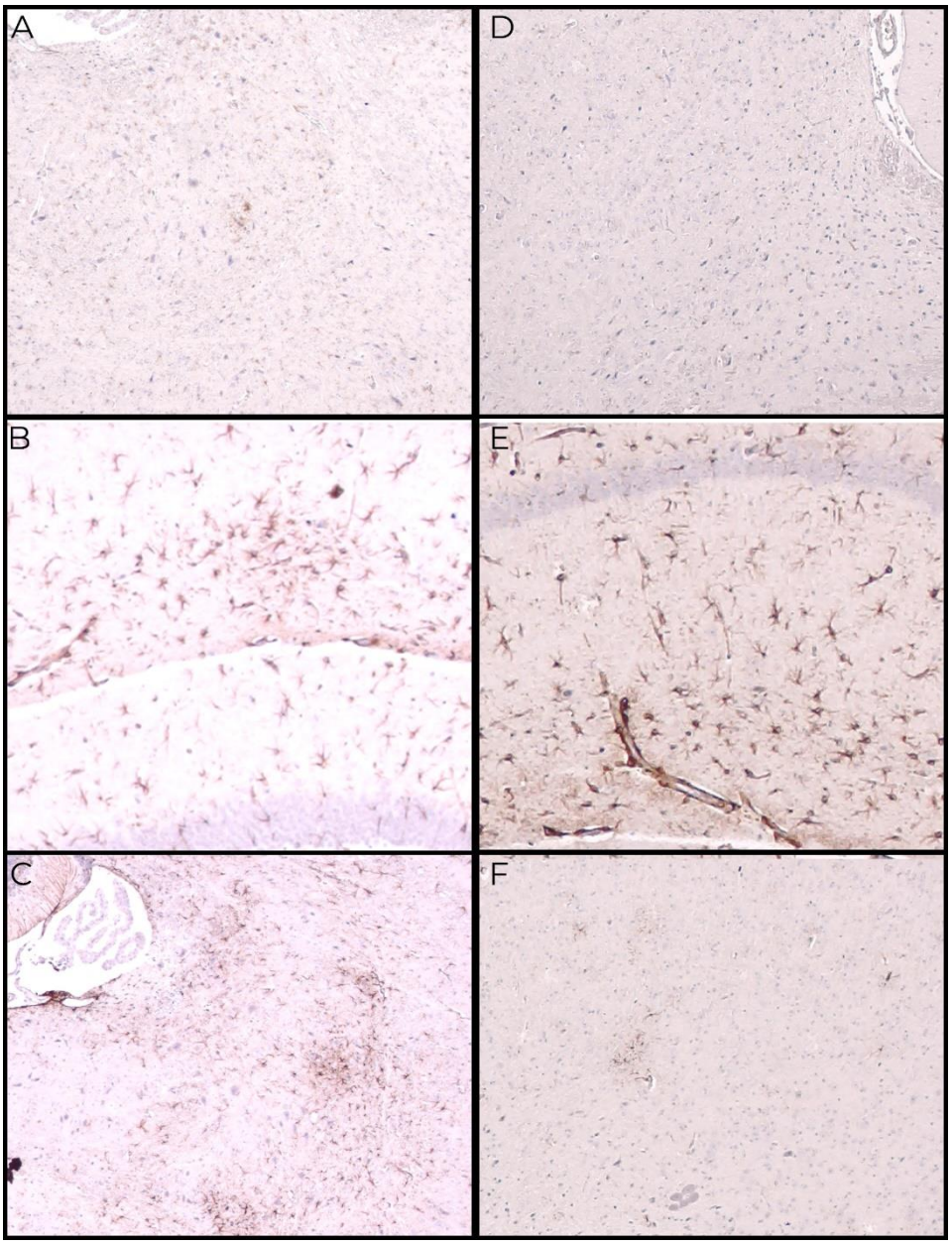


Figure 26: Magnified images of activated microglia and astrocytes from Fig. 26. **A**: An example of reactive microgliosis in the hindbrain that overlaps with reactive astrocytosis in the same region. Note the rounder cell bodies and increased number of microglia. **B**: Reactive astrocytosis in the dorsal hippocampus that occurred without microgliosis in the same region. Note the enlarged cell bodies and expanded processes. **C**: Astrocytosis in the same region as A. Note the expanded astrocytes but also the overlapping processes, indicating expansion of astrocytes. **D:** Comparative image from an uninfected mouse. Note the smaller cytoplasm indicating quiescent microglia. **E, F:** Comparative images using GFAP staining from an uninfected mouse. Note that while there are similar numbers of astrocytes, they are spaced farther apart and have less pronounced cytoplasms.

Mice with microglial lesions at 35 dpi show significantly greater weight loss in first 21 dpi

Because the hindbrain is responsible for many of the basal functions of the body including respiration, cardiac function, and the autonomic nervous system, the effects of persistent inflammatory lesions in the hindbrain were determined by comparing disease severity in mice with and without hindbrain microglial lesions. As none of the mice that survived past the first 21 dpi showed more severe clinical signs than ruffled fur, peak weight loss during this time was used as a measure (**Fig. 27**). None of the uninfected mice showed weight loss during this time and so were not compared. This showed that mice with microglial lesions in the hindbrain showed significantly greater peak weight loss than mice without microglial lesions in the hindbrain, indicating more severe disease.

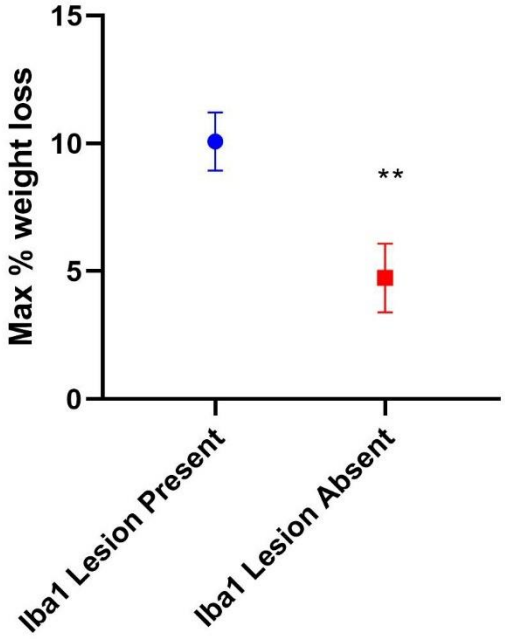


Figure 27: Comparison of peak body weight loss in mice infected with WNV, between those with and without microglial lesions in the hindbrain at 35 dpi.
Mice with microglial lesions showed significantly greater weight loss during the acute phase than those without. Analyzed via Student's t-test, ** $p<0.01$.

Microgliosis does not significantly correlate with depression-like behavior

To determine if the microglial lesions of any particular brain region could play a role in depression-like behavior among mice, a regression estimate analysis was performed to compare mice with and without microglial lesions in each region, with all uninfected

mice being included in the lesion negative group (Table 13). The analysis indicated that there was no association between depression-like behavior and microglial lesions in any of the regions.

Parameter	TST Estimate	SE	P-value
Lesions			
Cerebellum (*n*=9)	18.23	15.14	0.24
Hindbrain (*n*=17)	27.62	20.47	0.19
Midbrain (*n*=17)	-25.70	19.80	0.21
Forebrain (*n*=13)	6.25	16.33	0.71
Cortex (*n*=5)	-17.55	16.48	0.30

Table 13: Regression estimate analysis comparing microglial lesion presence to TST performance.

Presence of microgliosis in the cerebellum, midbrain, forebrain, and cortex correlates with reduced performance on rotarod

To correlate presence of microgliosis with adverse effects on motor learning, the outcomes of the rotarod testing were compared between uninfected mice, infected mice with microglial lesions in each anatomic region, and infected mice without microglial lesions in the listed region. Only one significant difference was found in these analyses (**Fig 28**): mice with microglial lesions in the cortex showed significantly lower performance on the final trial when compared to uninfected mice or infected mice.

To determine if there were significant differences that could represent the later stages of motor learning, trials 4-9 of the rotarod were compared in aggregate using regression estimate analysis. These trials were selected as trial four was the first trial in which control mice showed a significant improvement over their initial performance.

Lesions in the forebrain correlated with decreased performance, and lesions in the cerebellum and cortex trended toward correlation with decreased performance, on trials 4-9 of the rotarod test when compared using this method (**Table 14**). The forebrain contains projections from the midbrain dopaminergic neurons, including in the striatum. Damage to

this region can therefore lead to decreased motor learning[267,404].

Though not statistically significant, there was a trend toward similar findings for the cerebellum and cortex, indicating that microgliosis and inflammation in these areas could correlate with decreased motor learning. Larger cohort sizes would be needed to confirm this finding. In contrast, mice with lesions in the midbrain tended to remain on the rotarod longer than those without these lesions. The midbrain contains the ventral tegmental area that projects dopaminergic neurons to the cortex, which are crucial for motor skill learning[405]. Damage and inflammation to the midbrain therefore would likely reduce motor skill learning, but that was not the case seen here.

Parameter	Rotarod – trials 4-9		
	Estimate	SE	P-value
Infected	10.96	12.60	0.39
Lesions			
Cerebellum (*n*=9)	-16.12	9.79	0.11
Hindbrain (*n*=16)	-10.58	12.42	0.40
Midbrain (*n*=13)	19.46	11.34	0.10
Forebrain (*n*=13)	-27.29	12.11	0.03
Cortex (*n*=4)	-21.06	11.86	0.09

Table 14: Regression estimate analysis comparing microglial lesion presence to rotarod performance.

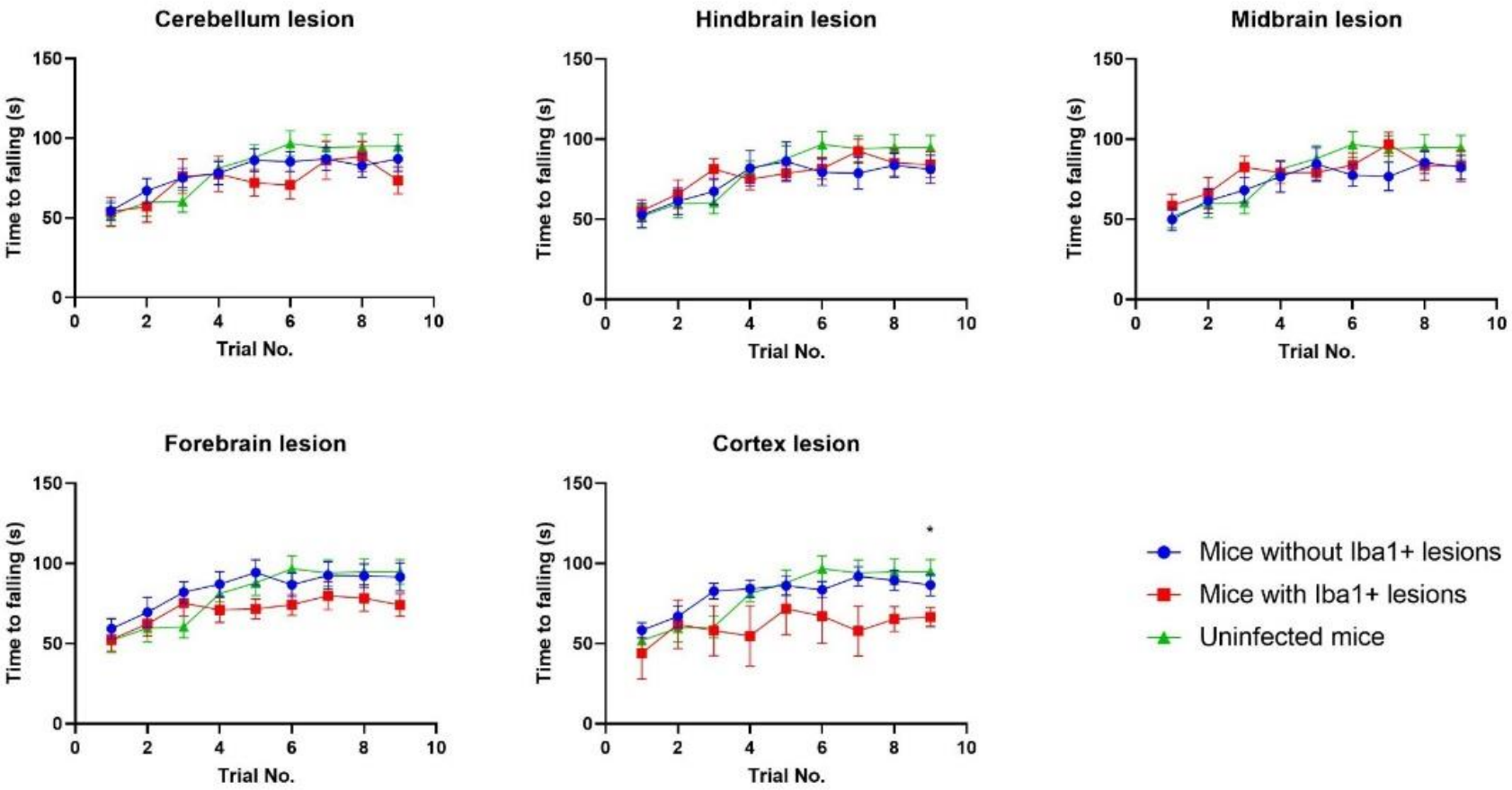


Figure 28: Rotarod performance of mice based on presence of microglial lesions in different brain regions.
Mice were tested three times per day for three days on the rotarod to measure motor coordination and motor learning. WNV-infected mice were grouped based on the presence of Iba1 lesions in each region (red), and their performance was compared to uninfected mice (green) and WNV-infected mice without Iba1 lesions in that region (blue). The only significant difference was on trial nine between uninfected mice and WNV infected mice with microglial lesions in the cortex. Analyzed by repeated measures two-way ANOVA. *: $P<0.05$.

Presence of microgliosis in the cortex trends toward decreased prepulse inhibition performance

PPI testing had not shown significant differences between uninfected and WNV-infected mice at 28 dpi, but a high proportion of the infected mice showed decreased percent inhibition when compared to the controls. To determine if the microglial lesions in any specific region correlated with decreased performance, regression estimate analysis was performed. None of the individual regions showed significant changes when comparing mice with and without lesions, but the mice with lesions in the cortex trended toward having decreased performance compared to the other mice (**Table 15**).

Parameter	PPI Estimate	SE	P-value
Lesions			
Cerebellum (*n*=12)	-0.13	8.39	0.99
Hindbrain (*n*=21)	4.07	10.37	0.70
Midbrain (*n*=19)	-9.43	9.56	0.33
Forebrain (*n*=17)	3.69	9.37	0.70
Cortex (*n*=6)	-14.96	9.16	0.11

Table 15: Regression estimate analysis comparing microglial lesions to PPI performance.

The number of mice tested using the PPI was higher than that for the rotarod, and so the difference is less likely to be due to statistical errors. There has been evidence linking cortical damage with changes in PPI[406–408], indicating a possible mechanism for immune mediated or inflammatory pathology causing deficits, but more work needs to be done to characterize this.

Microglial lesions do not correlate with changes in performance of the 2WAA test

Because a high proportion of WNV-infected mice performed poorly on the 2WAA at 32 dpi, the possible correlation between persistent microglial lesions in any region and this change in behavior was investigated. No significant changes were seen between these groups (**Fig. 29**), indicating that the microglial lesions are not strongly associated with the changes in memory function seen. To confirm this finding, regression estimate analysis was performed for the third through fifth day of 2WAA testing (32-34 dpi; **Table**

Parameter	2WAA – 32-34 dpi		
	Estimate	SE	P-value
Lesions			
Cerebellum (*n*=10)	0.96	3.88	0.81
Hindbrain (*n*=19)	-1.55	5.06	0.76
Midbrain (*n*=17)	0.85	4.32	0.84
Forebrain (*n*=15)	2.17	4.38	0.62
Cortex (*n*=6)	-3.83	4.30	0.38

Table 16: Regression estimate analysis of 2WAA performance on 32-34 dpi based on presence of microglial lesions in each region.

16). This analysis showed that there was no significant correlation between memory function and microglial lesions in this model.

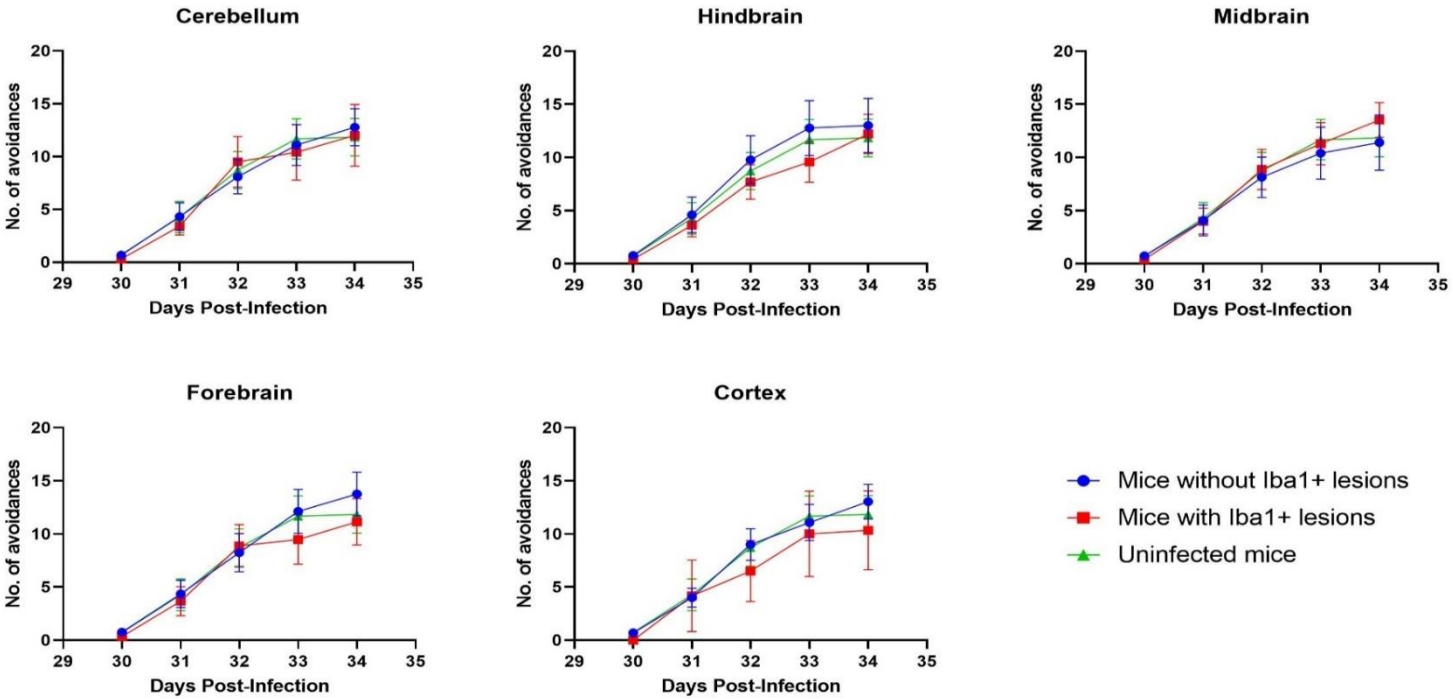


Figure 29: Two-way active avoidance performance based on presence of microglial lesions in anatomic regions of the brain.

WNV-infected mice were grouped based on the presence of Iba1 lesions in each region (red), and their performance was compared to uninfected mice (green) and WNV-infected mice without Iba1 lesions (blue) in that region. No significant differences were found when mice were separated based on these parameters. All error bars are +/- SEM. Analyzed by repeated measures two-way ANOVA.

WNV infection causes significant changes in pro-inflammatory cytokines in the hindbrain and cortex

Changes in cytokine levels in the brain have been associated with abnormal neuronal function[409], and have been correlated with neurocognitive and behavioral changes[410,411]. To see if WNV infection affected the cytokine levels of the brain, a six-plex cytokine analysis was performed on the cortex and hindbrains of a subset of mice. The cortex was chosen because it is the largest region of the brain, and multiple functions are carried out in this region. The hindbrain was because it was the most common site for inflammatory lesions and persistent viral RNA. The six-plex measured IFN-γ, IL-1β, IL-6, IL-10, IL-17A, and TNF-α. IL-17A, Il-6, and IL-10 were too low to be detected in any of the samples. The standards for IFN-γ did not work and need to be re-run.

Levels of IL-1β were significantly reduced in the cortices of infected mice when compared to uninfected mice (**Fig. 30**) but a similar effect was not found in the hindbrain. No significant changes in TNF-α were observed in either region.

Because activated microglia are a major source of pro-inflammatory cytokines, the levels of pro-inflammatory cytokines in the hindbrains and cortices of mice with and without microglial lesions in the appropriate anatomic regions were compared. The comparisons did not show a significant change (**Fig. 31**), as IL-1β was lower in the cortices of all WNV infected mice compared to uninfected controls regardless of microglial lesions, and there was no significant difference in TNF-α.

To determine if the persistence of viral RNA correlated with changes in cytokine activity, infected mice were separated based on whether viral RNA was detected in the appropriate region. The decrease in IL-1β occurred regardless of the persistence of viral RNA in this region and was not found to occur in the hindbrain (**Fig. 32**). There was a trend towards increased TNF-α in the hindbrains of mice that had persistent viral RNA in that region.

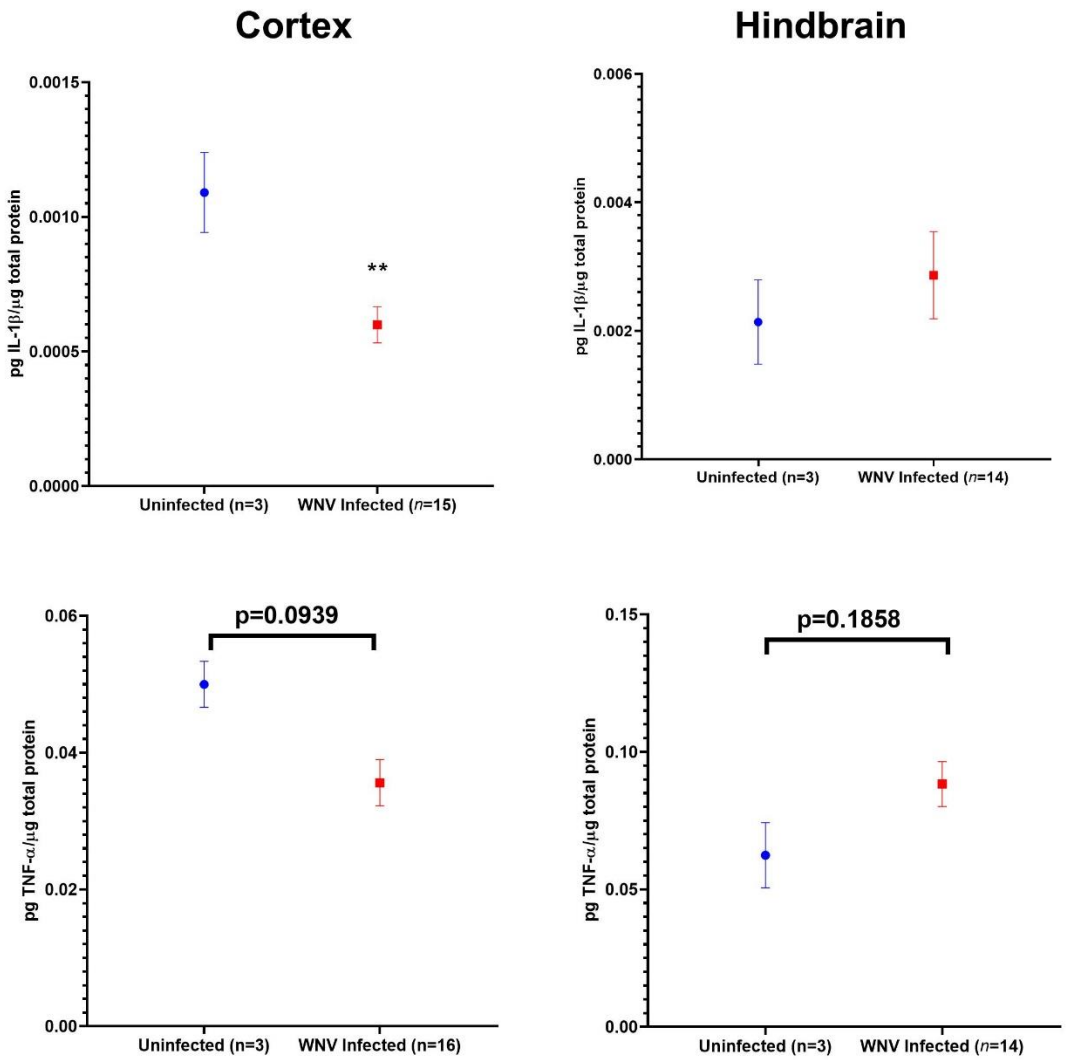


Figure 30: Comparison of pro-inflammatory cytokines in the cortex and hindbrains of uninfected and WNV-infected mice.

There is a significant decrease in IL-1β in the cortex of infected mice when compared to controls, but no other significant differences. Error bars represent mean +/- SEM. All results compared by Student's t-test.

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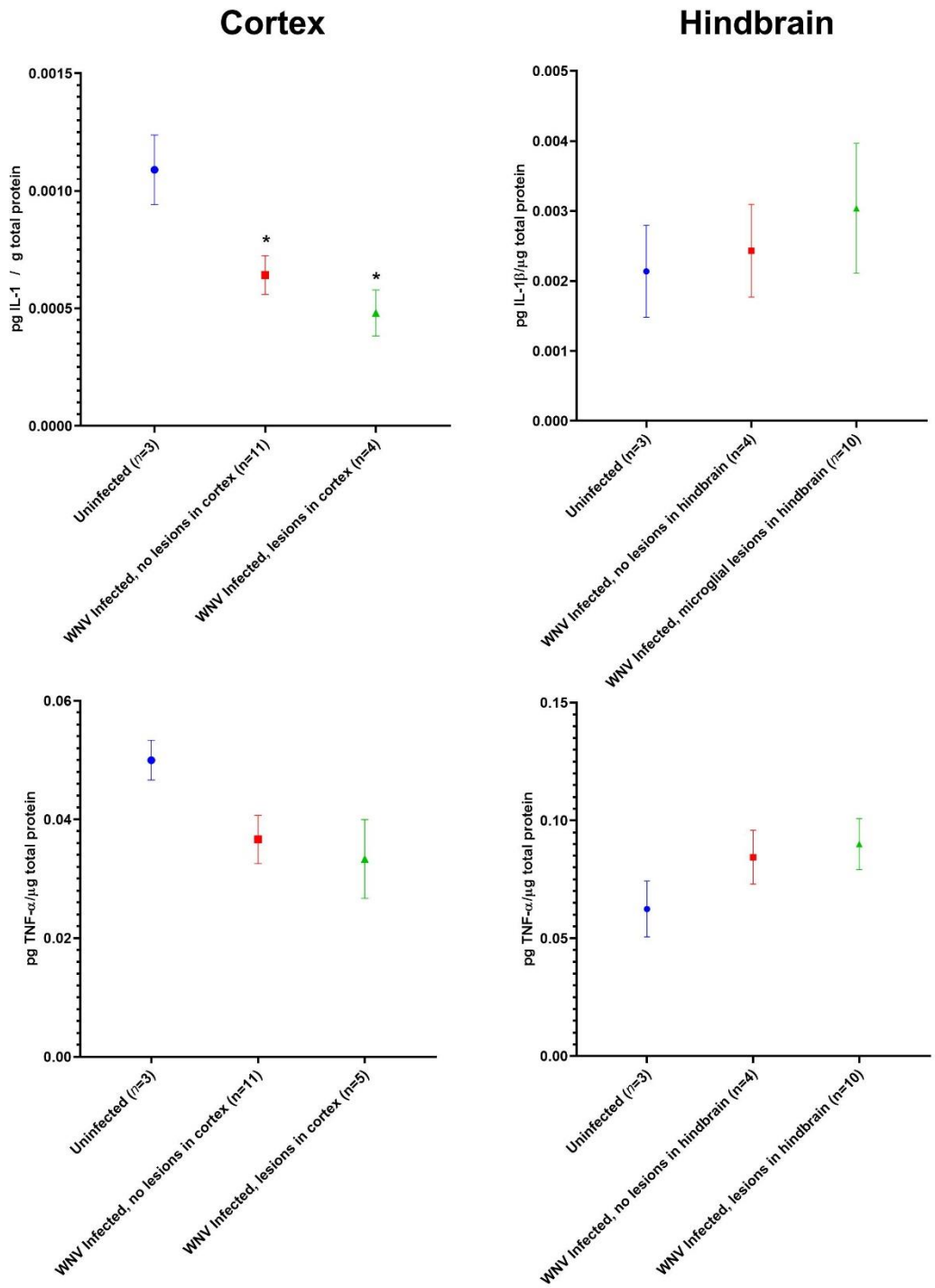

Figure 31: Measurement of pro-inflammatory cytokines based on presence of microglial lesions in the cortex and hindbrain.

IL-1β is reduced in the cortex of mice regardless of microglial nodules presence. There are no significant differences in other comparisons. All error bars represent mean +/- SEM. All results compared using one-way ANOVA. *: $p<0.05$.

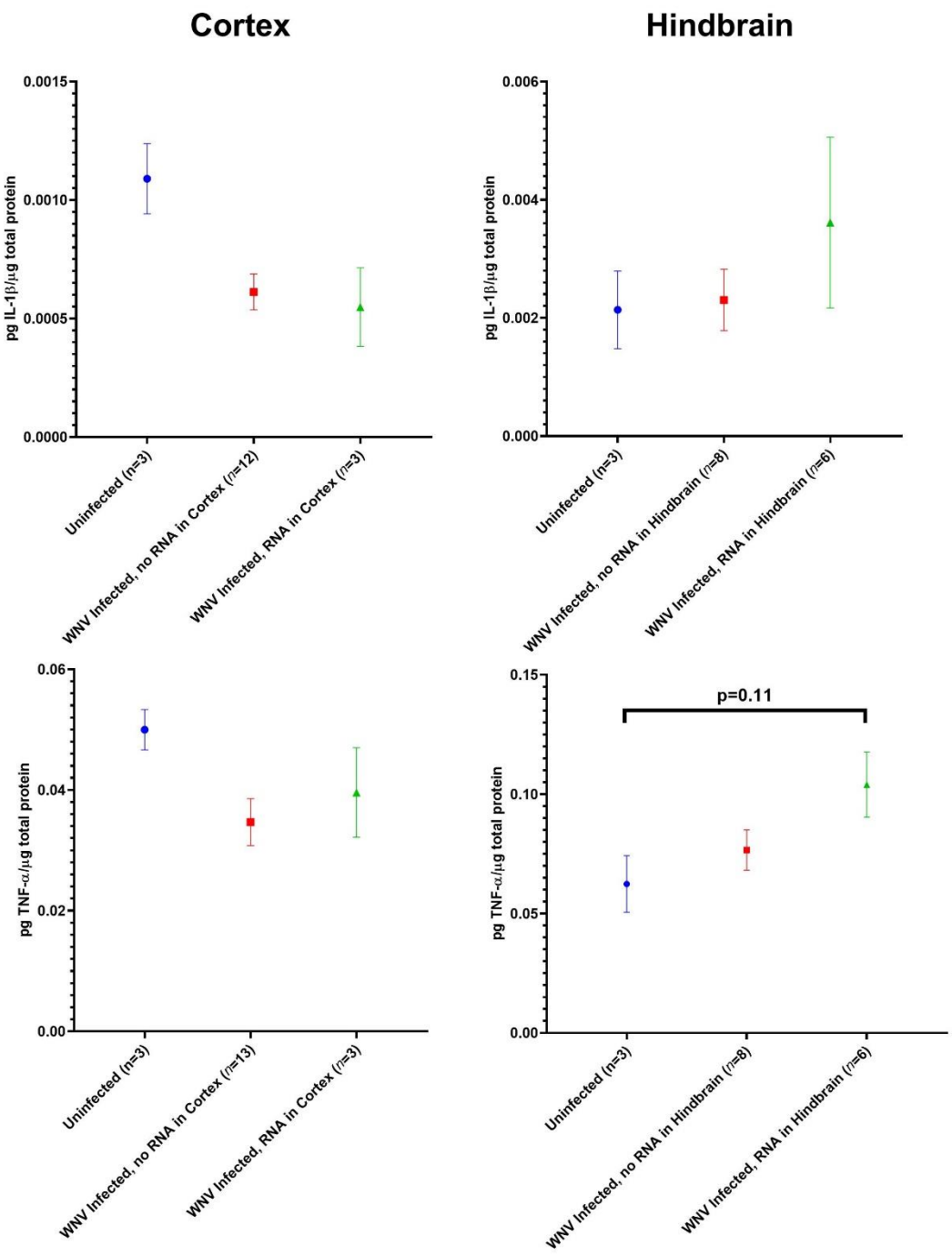

Figure 32: IL-1β and TNF-α levels in cortices and hindbrains of mice based on viral RNA persistence.

Mice were separated based on whether there was persistent viral RNA found in the appropriate region. No significant differences were found though there was a trend toward increased TNF-α in mice persistent viral RNA in the hindbrain. All results compared using one-way ANOVA.

DISCUSSION

These studies showed that WNV infection induces long-term changes in the inflammatory state of the brain, most notably with detectable microgliosis and microglial nodules in multiple brain regions. This occurred most commonly in the hindbrain, indicating that the hindbrain is a common region for inflammation long after infection. Almost all other regions did show microgliosis in at least seven mice, with the exception of the hippocampus.

These studies also show a correlation or trend toward correlation between microgliosis in certain regions and behavioral or physiological outputs. This included microgliosis in the hindbrain correlating with greater weight loss during the acute phase of infection; microgliosis of the cerebellum, midbrain, forebrain, and cortex correlating with changes in rotarod performance; and microgliosis of the cortex correlating with decreased PPI performance. These indicate that inflammation, and specifically microgliosis, after WNV correlates with the changes in these behaviors. This could indicate that inflammation is actively damaging neurons and disrupting function, or that there are underlying mechanisms that lead to both. There were no correlations seen between microgliosis and memory loss, indicating that the mechanisms leading to this outcome are either unrelated to microglia or cannot be detected via IHC staining for microglia.

These studies also showed that there are persistent changes in inflammatory cytokines post-infection in the cortex and hindbrain. IL-1β was decreased in the cortices of mice surviving WNV infection regardless of microgliosis or viral RNA persistence, while TNF-α was only elevated in the hindbrains of mice with persistent viral in the hindbrain. This indicates changes to the overall inflammatory state of the brain that should be further investigated.

Chapter 5: Viral Persistence

INTRODUCTION

Although many viruses are cleared from the host after the initial infection, some viruses, including WNV, have been shown to persist in different organs long after infection. Viruses can persist in multiple forms, including whole genomes associated with the host genome, low levels of viral replication, or quiescent viral genome persisting in cells resistant to apoptosis[412]. The CNS provides a unique opportunity for viruses to persist as it is highly resistant to apoptosis and inflammation. Many herpes viruses, including HSV-1 and 2, varicella zoster virus, and Epstein-Barr virus, have been shown to persist in the CNS. These viruses can reactivate, leading to recurrence of disease. RNA viruses such as MV, TMEV, HIV, and Ebola virus have been shown to persist in the CNS as well though the mechanisms behind these vary, and some of the mechanisms are still being determined.

Flavivirus persistence

Flaviviruses, including WNV, have been shown to persist in multiple organs including the CNS. WNV has been reported to persist in the kidneys of Syrian golden hamsters for over 200 dpi[142]. WNV can be cultured from the hamster kidneys and WNV antigen can be detected in the renal tubules at this time.

ZIKV persists in the male genital tract of mice and humans. In mice, live virus was found in spermatogonia, spermatocytes, mature sperm, and Sertoli cells for weeks post-infection, causing inflammation and cell death[413]. The ability of ZIKV to persist in the reproductive tract may be due to the immunoprivileged status of this region. The male reproductive tract is mostly separated from the peripheral immune response[414], much like the brain, meaning there may be similar mechanisms behind ZIKV and WNV persistence. Persistent viremia of ZIKV has been reported in human cases[415] and recapitulated in a rhesus macaque model[416]. In macaques, viral persistence in the CSF correlated with

upregulation mTOR, proinflammatory, and anti-apoptotic pathways[417], indicating potential mechanisms ZIKV may exploit to persist in the CNS.

DENV has been shown to persist in the CNS of patients and cause significant neurological complications as well. DENV protein and antibodies against the virus have been found more than seven years post-infection in the CNS of an immunocompetent individual[418]. The virus found in the CNS showed multiple mutations in the envelope protein, indicating possible evasion of the immune response that normally targets the E protein[419].

Mechanisms of viral persistence in the CNS

To maintain persistence in a host cell, a virus needs to avoid being detected and avoid killing the host cell. The CNS is immunoprivileged and peripheral immune cells need to cross the BBB to interact with virally infected cells, making detection more difficult. The neurons in the CNS are terminally differentiated and do not divide, and so to compensate, they tend to be more resistant to cell death[420,421]. Even in neurons, to maintain persistence, a virus must downregulate viral protein production to avoid detection or death of the host cell.

BDV establishes persistent infection in the CNS by infecting neurons and then downregulating viral replication and transcription through trimming of the 5' end of its genome[422]. This mechanism prevents overwhelming the host cell with viral protein production, and avoids activation of the innate immune system. BDV also inhibits apoptosis in host cells through its X protein, which associates with mitochondria. Rats infected with a mutant BDV lacking the X protein died within 30 days of infection, while those infected with a wild-type strain survived but had persistent viral infections in the CNS[423]. These data indicate that inhibition of apoptosis is important for the establishment of a persistent infection, but also that persistence can be in part due to reduced virulence.

HIV can infect and persist in multiple cells of the CNS, which serves as a central reservoir for the virus. There has been an increased interest in this as antiretroviral therapy drugs do not generally cross the BBB, meaning that the virus can persist in the CNS despite treatment. HIV invades the CNS early in infection and can infect microglia despite the low levels of CD4 they normally express[424,425]. Astrocytes are also susceptible to HIV infection, though this is uncommon and the astrocytes do not support high levels of viral replication[426]. HIV in the CNS seems to require adaptation to allow for more efficient infection of cells in the CNS, including requiring lower levels of CD4[427,428], and mutations in the gp120 protein that increase the viral tropism for macrophages and microglia[429].

MV has been shown to persist as RNA in the CNS of patients surviving acute infection[430]. In some cases it reactivates, leading to severe, potentially lethal diseases including subacute sclerosing panencephalitis and measles inclusion-body encephalitis[431]. MV does not normally have the receptor to bind to neurons, but it seems to be able to infect them due to multiple mutations. This means that it likely does not replicate well in neurons[432]. MV has been shown to develop additional mutations that make it more adapted to the CNS, some of which have been specifically associated with severe disease after reactivation[433]. In a mouse model, MV RNA was found to persist in the neurons of immunocompetent mice. The adaptive immune response, specifically T resident memory cells, prevented the virus from replicating. When T resident memory cells were inactivated, the virus began producing proteins again[434]. This indicates that the RNA for MV is enough to reestablish viral production, but also that viral replication is kept under control by noncytolytic mechanisms. MV produces large numbers of DI particles, particularly in cases of subacute sclerosing panencephalitis[435], indicating a similar mechanism to that of some flaviviruses for persisting in the CNS.

CDV is a paramyxovirus closely related to MV. CDV affects many mammal species including dogs, pinnipeds, and mustelids, though notably not humans[436]. CDV has been shown to persist in the CNS, though unlike MV, CDV does not produce DI

particles[437], indicating that CDV uses a different mechanism for persistence. CDV primarily infects astrocytes and neurons through noncytolytic, cell-to-cell contact[438,439]. The ability to spread cell-to-cell allows the virus to avoid immune detection which may assist in the development of persistent infection[440]. These lesions cause clinical signs similar to those seen in MS, including pain and motor incoordination. While persisting in the CNS, CDV continues to induce the formation of inflammatory, demyelinating lesions that can persist for long periods of time[441]. CDV can also induce grey matter damage, leading to severe encephalitis, years after initial infection[442].

Henipaviruses are members of the *Paramyxoviridae* family, related to MV and CDV. There are two henipaviruses known to cause neurological disease in humans: Nipah virus and Hendra virus. Nipah virus can induce relapsing encephalitis in those surviving infection[443], and it has been demonstrated to persist in the CNS of primates surviving infection[444]. Viral proteins were found in microglia and in neurons. This represents a significant difference from the related MV, which was only found to persist in neurons. Henipaviruses can inhibit the interferon response at multiple levels, including initial recognition of viral RNA, production of IFN, and the signaling downstream of IFN receptor stimulation[445]. Given how important interferon signaling is for viral clearance from the CNS, the ability to antagonize these responses likely plays a role in establishing viral persistence.

Ebola virus has been shown to persist in the CNS and lead to a viral relapse manifesting as meningoencephalitis[446], and it has been shown to persist in the ocular fluid of survivors post-infection[447]. This has been recapitulated in a rhesus macaque[448] which showed microgliosis and perivascular cuffing in the regions associated with infection. Ebola virus infection has been reported to cause meningitis and encephalitis[449], and these complications can occur later in disease due to viral spillover from the CSF[446]

The mechanisms behind flavivirus persistence are still being determined, though some mechanisms have been suggested. During viral replication, the RNA replication

complexes are sequestered in vesicles, which may allow the complexes to avoid detection by host PRRs. Although many of the viral proteins induce cell death, others have been shown to inhibit apoptosis. JEV[450] and DENV[451] both use the NS4A protein to inhibit apoptosis *in vitro* by inhibiting activation of the lipid kinase phosphatidylinositol 3-kinase (PI3K). While this has not been shown for WNV, PI3K is crucial for the control of WNV *in vitro* through the induction of the innate immune response[452]. Whether this is related to apoptosis or other pathways has not been determined.

Defective interfering particles (DIs) have been suggested as a possible mechanism for flavivirus persistence. These are viral particles missing sections of the viral genome, which makes them unable to replicate on their own. If a DI infects the same cell as a virion with the full genome, it can reproduce through the replication machinery of the other virus[453]. DIs can interfere with normal viral replication by competing for viral replication machinery in infected cells. The DIs generally outcompete the full genomes as they are shorter, which reduces viral pathogenicity[454]. Other flaviviruses, including DENV[455] and JEV[454], have been shown to produce DIs during infection. This has been suggested as a possible mechanism for persistence as the reduced replication can lead to lower levels of immune activation and cell death.

Several mutations throughout the WNV genome have been identified in WNV strains isolated from the kidneys and urine of persistently infected hamsters. These strains had amino acid changes in the E, NS1, NS2B, and NS5 proteins, and had reduced virulence in hamsters[456]. This has not been recapitulated in the CNS, though it may indicate potential mutations that could lead to decreased cellular pathogenicity in WNV. Persistent WNV has been found months after infection in rhesus macaques, and the strain isolated became non-pathogenic in mice[135]. Further work to investigate how the rhesus macaque compares to human cases needs to be done.

The different mechanisms that viruses use to persist in the CNS indicate a variety of adaptations, many of which need to be investigated for WNV. Viral RNA has been

demonstrated to persist in mice[145], hamsters[55,457], and primates[135], and has been shown in human cases. WNV spreads from cell-to-cell in the CNS[147], and has been shown to produce DI particles in birds[458], which both represent possible mechanisms of immune evasion. The production of DI particles was not protective in mice when given pre-infection, nor were the DI particles detected in mice post-infection[458].

The aims of these studies were to determine if WNV persisted in the CNS as either virus or RNA in mice and how this correlated with behavioral outcomes. During the course of these studies, a presumptive case of lethal WNND with a disease course of several months was identified at UTMB. To confirm the translatability of these studies, localization of persistent WNV RNA was tested in on CNS samples from this patient for investigation.

DEVELOPMENT OF TESTING

One aspect that these studies aimed to address was the role of persistent WNV in ongoing damage in the CNS. Previous work had established that WNV persists in the CNS of mice inoculated via footpad inoculation[145]. This was detected using qPCR for viral RNA and serial co-culturing of the brain to find live virus. In initial experiments, this same co-culture method using the brains of mice kept until 80 dpi but encountered several obstacles. The brain tissues seemed to be toxic to the Vero cells that were used for co-culture, requiring multiple attempts to keep the cells alive long enough to detect changes. Another issue was the lack of available tissue. As half of the brain was left for IHC, this left one half to be used for RNA extraction, protein analysis, and co-culture. This was not enough tissue for all three, and further prevented localization of viral persistence to specific brain regions. This procedure required a large amount of time and effort it took to perform the co-cultures.

RNAScope was considered as another method of detecting small amounts of virus in tissue samples, as it has reported sensitivity to one copy of RNA[459]. This method uses *in*

situ hybridization to detect specific RNA sequences which are amplified, allowing for fluorescent or chromogenic detection on formalin-fixed, paraffin-embedded slides[460]. The methodology was optimized, but other issues were found with the protocol. One obstacle was that removal of the brain from the BSL-3 required formalin fixation of the brain for at least 72 hours. The RNAScope protocol was originally optimized for less than 24 hours of formalin fixation, and further fixation could cause off-target binding. Given the apparently diffuse and varying distribution of inflammation in the brains of mice that survived WNV infection, and the unreliable nature of the staining after the amount of time of fixative, RNAscope did not appear to be a cost- or time-effective approach for screening to detect viral RNA in brain tissues.

To optimize the specificity of data obtained from RT-PCR and other analyses from homogenized brain samples, localization of viral RNA and inflammation was accomplished based on anatomic landmarks. Dr. Ibdanelo Cortez of Dr. Kelly Dineley's lab provided training that allowed for the performance of brain dissections without the use of sharps so that the regions could be removed in a BSL-3 setting. A total of six regions were dissected from each brain for separate analysis: the cerebellum, hindbrain, midbrain, forebrain, hippocampus, and cortex. These regions could be homogenized and used for both RNA and protein analysis.

RESULTS

WNV has been shown to persist in the CNS of mice for up to 16 months post-infection[145]. Given that other viruses show tropisms for specific brain regions, different regions of the brain were tested for viral RNA to determine tropism and if this had any impact on neurological function by using behavioral testing.

Viral RNA can be detected in multiple regions of mouse brains at 35 dpi

Some RNA viruses including MV and CDV have been shown to persist in the CNS as RNA rather than as replication-competent virions. To test for either virion or RNA persistence, qRT-PCR was performed targeting the WNV E gene using previously established primers. Mice from the last two behavioral experiments were used for determining if viral RNA persisted in each brain region. Mice from previous experiments were not used as the brains were not dissected, and many of the tissues had been used for co-culturing.

Mice were euthanized at 35 dpi and the brains were dissected into six regions: the cerebellum, hindbrain, midbrain, forebrain, hippocampus, and cortex (as described in the Methods section). RNA was extracted from each of these regions and tested for viral RNA. The RNA was quantified based on a standard curve derived from serial dilutions of plasmids containing the DNA equivalent of the WNV E protein (**Fig. 33**). The equation used for calculating the genome copies from the Cq value was:

$$log[WNV\ E\ genome] = 11.648 - (0.2964)(Cq\ value)$$

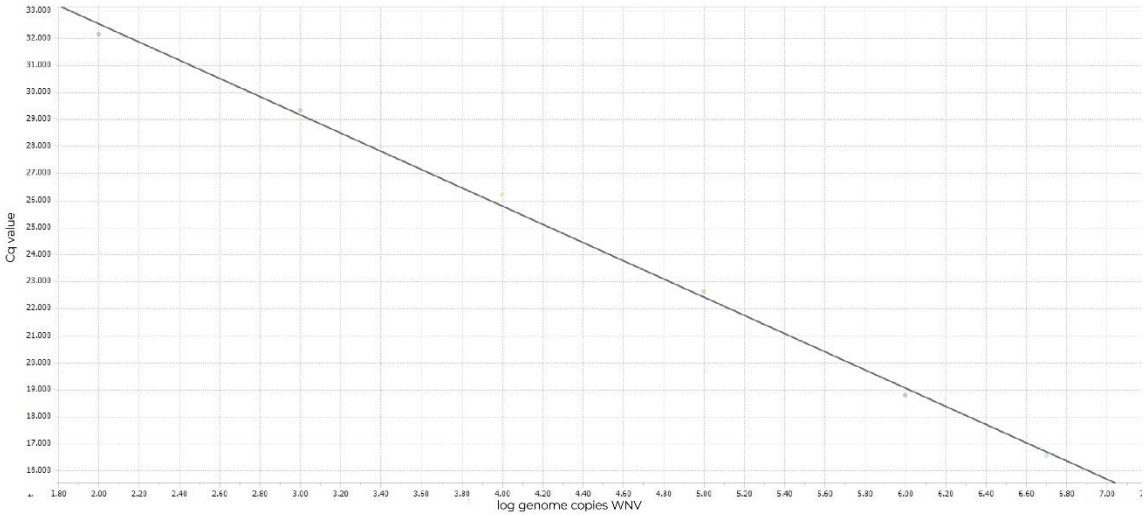

Figure 33: Standard curve used to calculate copies of WNV E genome from experimental samples.
The y-axis represents the Cq value measured on the qRT-PCR machine, and the x-axis represents the log genome copies.

WNV RNA persists in all brain regions, but most commonly the hindbrain

Viral RNA was found in mice both with and without weight loss during the first 21 dpi indicating that this was not a predictive factor for viral RNA persistence in the brain, consistent with previous findings[145]. Viral RNA was found in 66% (23/35) of mice that survived until 35 dpi. The regions and levels of RNA varied between mice. Viral RNA was found in each region of the brain at least once (**Table 17**). Viral RNA was most commonly detected in the hindbrain (12/35) and cerebellum (10/35) and least commonly detected in the cortex (5/35) and hippocampus (4/35). The levels of RNA detected ranged from 2 to 4000 genome copies, though these were not normalized to mass or baseline RNA. No region showed significantly higher or lower loads of viral RNA than others.

In summary, most mice 35 dpi have persistent viral RNA in at least one brain region. Viral RNA persisted most commonly in the hindbrain and cerebellum and least commonly in the hippocampus and cortex. These results indicate that viral RNA persistence is brain region-dependent, suggesting that there are region-specific tissue tropisms or mechanisms of clearance that differ between regions. Viral RNA persistence did not occur only in sick animals, indicating that disease severity is not predictive of viral RNA persistence.

	Genome copies of WNV RNA					
Mouse No.	Cerebellum	Hindbrain	Midbrain	Forebrain	Hippocampus	Cortex
1	0	0	0	0	0	0
2	0	0	211.9435	0	0	288.1376
3	0	0	0	37.44243	0	0
4	562.434561	574.06887	0	2.344121	98.01397442	19.9837
5	0	0	0	0	0	0
6	32.4429248	68.264671	0	54.12775	14.49999993	0
7	0	0	0	0	0	0
8	0	86.093824	284.2314	139.7707	0	18.03915
9	744.039513	7.4792836	34.73442	123.613	0	0
10	0	15.845283	0	0	0	0
11	0	0	0	0	0	0
12	0	34.972284	0	0	19.05144845	0
13	0	0	0	0	0	0
14	0	0	0	0	0	0
15	0	0	0	0	0	0
16	18.7931682	0	12.56389	1.234139	0	0
17	0	0	0	0	0	0
18	0	0	0	0	0	0
19	2278.72544	785.79272	410.8923	1175.395	4098.226904	312.7289
20	173.885753	0	0	0	0	0
21	38.2169479	78.248496	0	0	0	0
22	75.6233653	0	0	0	0	0
23	0	0	0	1143.742	0	0
24	0	0	0	0	0	0
25	0	0	0	0	0	0
26	0	0	0	0	0	0
27	0	90.925145	0	0	0	0
28	1241.35501	631.626	474.2114	0	0	373.4496
29	0	2294.3306	0	0	0	0
30	0	1422.9053	888.5053	0	0	0
31	0	0	0	0	0	0
32	0	0	0	0	0	0
33	0	0	1844.2	0	0	0
34	1375.16877	N/D	N/D	405.3218	0	0
35	0	0	0	6214.411	N/D	894.5899

Table 17: Viral RNA persistence by brain region from 35 mice at 35 dpi. Each row indicates a separate mouse, and each column represents a different brain region. Values are genome equivalent copies per region.

Persistent viral RNA in the hindbrain trends toward decreased time immobile during the TST

Parameter	TST Estimate	SE	P-value
RNA			
Cerebellum (*n*=9)	22.73	16.74	0.19
Hindbrain (*n*=10)	-35.12	18.41	0.07
Midbrain (*n*=6)	18.65	19.51	0.35
Forebrain (*n*=8)	-9.70	18.21	0.60
Hippocampus (*n*=4)	-14.53	24.87	0.56
Cortex (*n*=5)	3.66	18.86	0.85

Table 18: Regression estimate analysis comparing TST performance to viral RNA persistence.

Using regression estimate analysis, the effects of persistence of viral RNA in each region was correlated with TST activity. There were no significant differences in this data, though there was a trend toward decreased time immobile for mice with viral RNA in the hindbrain (**Table 18**). This was unexpected, as this was an indication of decreased depression-like behavior in the mice.

Persistent viral RNA in the cerebellum correlates with higher performance on rotarod

To determine if there was correlation between viral RNA persistence and motor learning, mice were separated based on the persistence of viral RNA in the six anatomic regions that were dissected during euthanasia.

Regression estimate analysis was performed for the TST, rotarod, and PPI tests. The TST and PPI tests did not show any significant correlation between viral RNA persistence in any of the regions and behavioral output. The rotarod did not show any significant findings either, though there was a trend toward increased rotarod performance on trials 4-9 in mice with viral RNA persisting in the cerebellum (**Table 19**). Trials 4-9 were selected as measures of motor learning output, because the first three days were allowing the mice to learn to perform on the rotarod.

Nine of the 35 WNV-infected mice were not tested using the rotarod because they did not pass the initial screening; only one of these mice had viral RNA in the cerebellum. Of the 26 WNV-infected mice that were tested, eleven had viral RNA in the cerebellum.

Parameter	Rotarod – trials 4-9		
	Estimate	SE	P-value
RNA			
Cerebellum (*n*=10)	18.50	9.82	0.07
Hindbrain (*n*=12)	3.58	11.95	0.77
Midbrain (*n*=7)	-7.83	11.19	0.49
Forebrain (*n*=9)	10.03	13.46	0.46
Hippocampus (*n*=4)	-21.69	15.53	0.18
Cortex (*n*=5)	-0.49	12.55	0.97

Table 19 Regression estimate analysis comparing rotarod performance to viral RNA persistence.

There was not a significant difference in performance on each individual day (**Fig 34**), but when trials 4-9 were taken in aggregate, the mice with viral RNA in the cerebellum tended to stay on the rotarod longer.

All ten of the mice with viral RNA in the cerebellum passed the rotarod screening. Of these mice, 60% (6/10) had microgliosis or microglial nodules in the cerebellum.

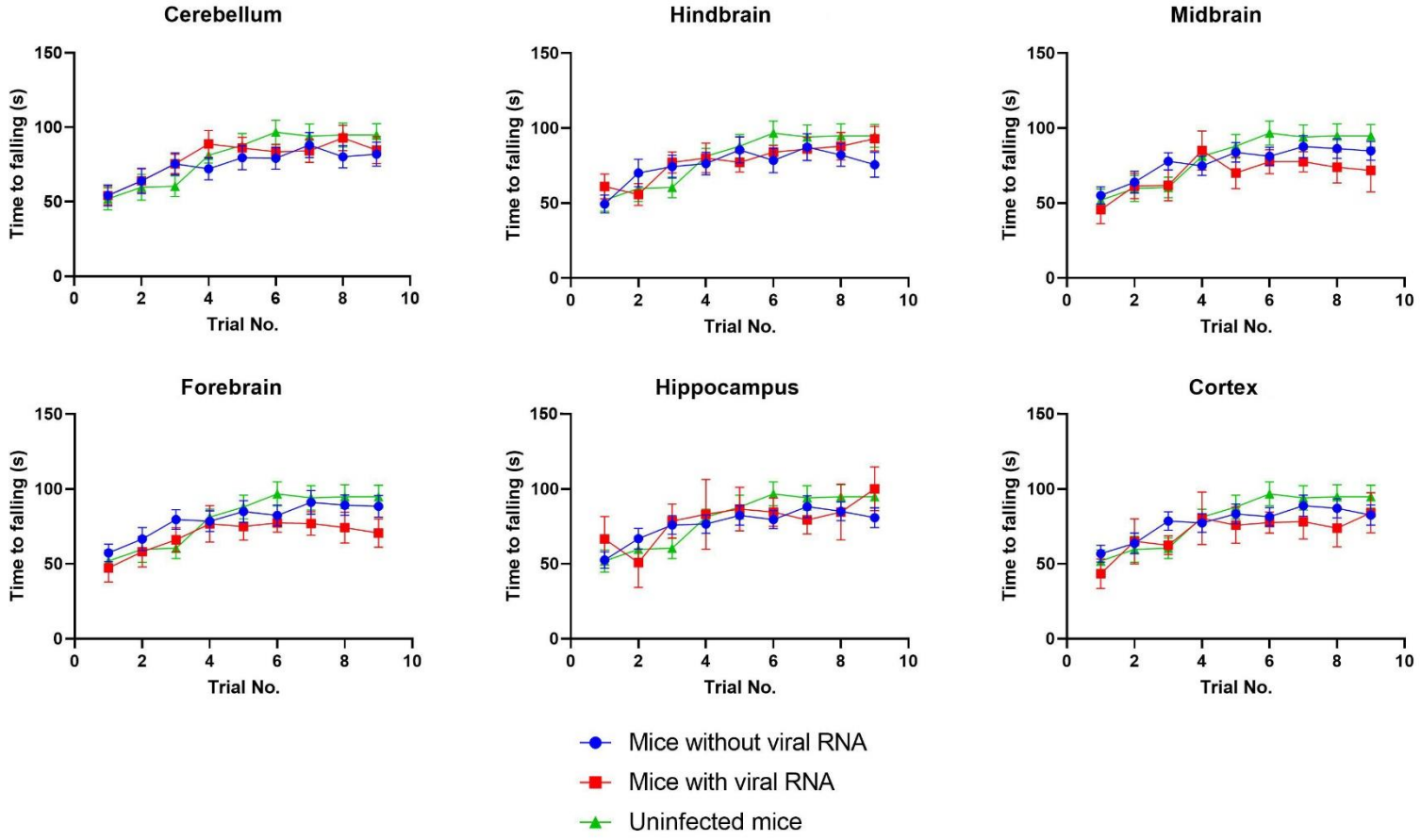

Figure 34: Assessment of rotarod performance based on persistence of viral RNA in each anatomic brain region. Mice were grouped based on infection status and whether viral RNA was detected in the associated region. No significant differences were found when comparing between groups on any single day. Analyzed by repeated two-way ANOVA.

Persistent viral RNA in the hippocampus trends toward decreased prepulse inhibition performance

The PPI results were analyzed via regression estimate analysis to determine if there was a significant difference in performance of individual animals based on viral RNA persistence in each region.

Parameter	PPI Estimate	SE	P-value
RNA			
Cerebellum (*n*=10)	6.63	9.20	0.48
Hindbrain (*n*=12)	-1.38	10.58	0.90
Midbrain (*n*=8)	-5.19	10.04	0.61
Forebrain (*n*=10)	4.67	9.39	0.62
Hippocampus (*n*=4)	-24.24	14.30	0.10
Cortex (*n*=6)	14.97	10.62	0.17

Table 20: Regression estimate analysis comparing PPI performance to viral RNA persistence.

Though there were no significant findings, there was a trend toward decreased percent inhibition in mice with viral RNA persistence in the hippocampus (**Table 20**). Four mice tested positive for viral RNA in the hippocampus, and all were positive for viral RNA in at least one other region. All except one had microglial lesions in at least two other regions, though only one had microglial lesions in the cortex, which had previously been found to trend with decreased sensorimotor gating.

Viral RNA persistence does not affect 2WAA performance

Parameter	2WAA – days 32-34		
	Estimate	SE	P-value
RNA			
Cerebellum (*n*=10)	2.25	4.12	0.59
Hindbrain (*n*=11)	-6.95	5.77	0.24
Midbrain (*n*=8)	-1.86	4.65	0.69
Forebrain (*n*=10)	-5.81	4.31	0.19
Hippocampus (*n*=4)	5.45	6.98	0.44
Cortex (*n*=6)	6.96	4.65	0.14

Table 21: Regression estimate analysis comparing 2WAA performance to viral RNA persistence.

Persistent viral RNA in any single region did not correlate with changes in any single day of performance on the 2WAA test (**Fig 35**) nor was there a significant difference when regression estimate analysis was performed using the results from 32-34 dpi in aggregate (**Table 21**). This held true when data from 32-34 dpi were considered in aggregate as well. Regression estimate analysis for 32-34 dpi indicated the same, that there was not a significant difference based on viral RNA persistence in any region. These data indicated that persistent viral RNA in any single region was not the cause of the memory loss phenotype previously noted.

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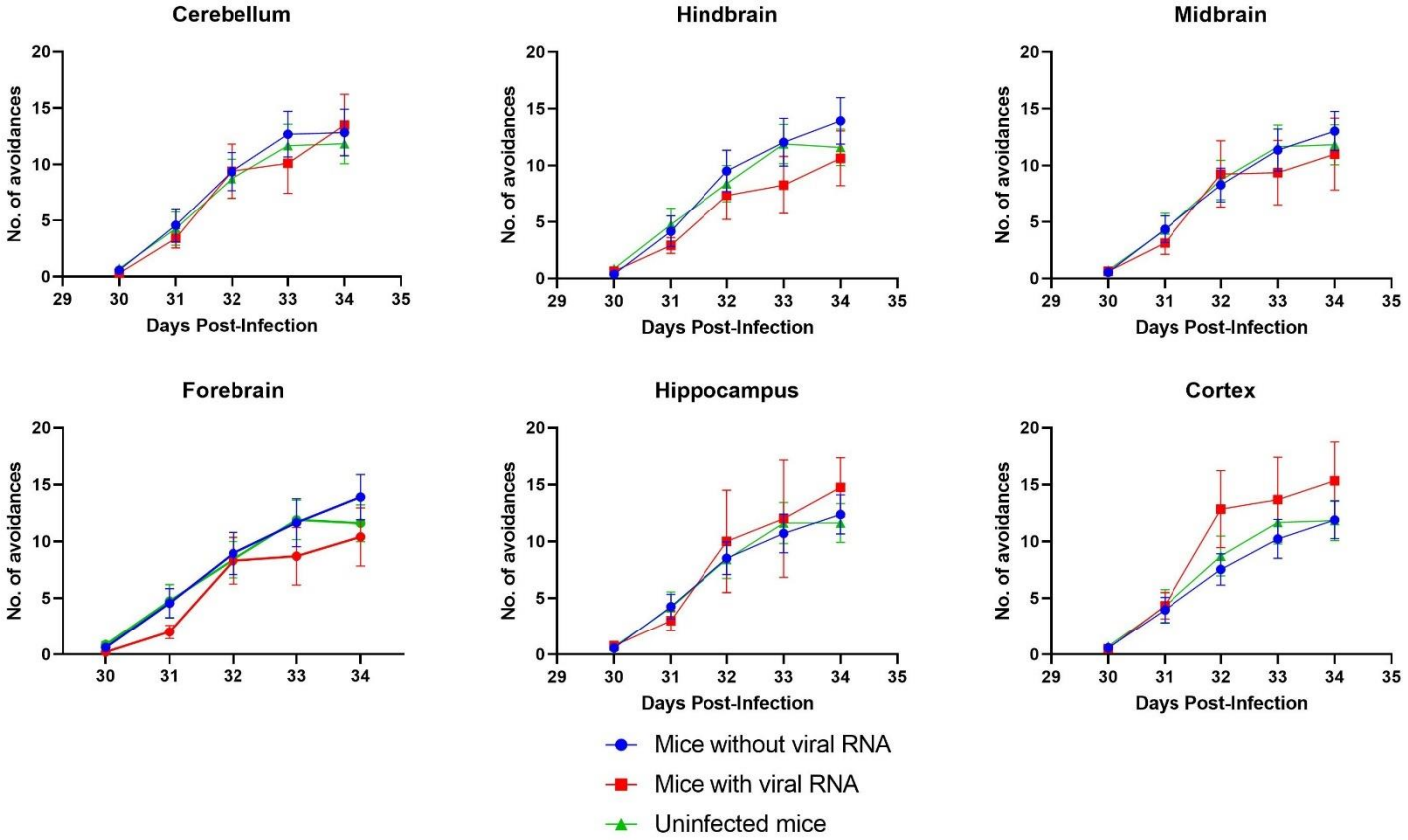

Figure 35: Performance of mice in the two-way active avoidance test based on viral RNA persistence in each region. Mice were separated into infected mice without viral RNA in the appropriate region (blue), infected mice with viral RNA in the listed region (red), or uninfected mice (green). There were no significant differences between any of the groups when analyzed using a repeated measures two-way ANOVA.

Persistent viral RNA does not always overlap with histopathological lesions

To determine if viral RNA persistence correlated with microgliosis and the formation of microglial nodules, the two parameters were compared (**Table 22**). These analyses were performed using the two different hemispheres, so definitive conclusions are hard to make. Given the observed variability in localization of inflammation and viral RNA between mice, it seems likely that viral RNA is distributed and persists differently between the two hemispheres in individual mice. There were still some conclusions to be made based on these findings, however.

The most notable finding is that infected mice could have microglial lesions with or without viral RNA persistence and vice versa. Although there was overlap (most commonly in the hindbrain), 20% (7/35) of mice showed inflammatory lesions in multiple regions without any detectable viral RNA in any region. Only one mouse had viral RNA persistence without any detected inflammatory lesions. No inflammatory lesions were detected in the hippocampus of any of the mice, although multiple mice had RNA detected in that region. The cortex was the only region besides the hippocampus that did not have any overlap between viral RNA persistence and the formation of microglial nodules.

The hindbrain most commonly had microglial lesions and RNA persistence detected, and showed the most overlap between the two as well. Of the 21 mice that had microglial lesions in the hindbrain, 47.6% (10/21) also had viral RNA. Only one with viral RNA did not have microglial lesions, indicating that viral RNA was more often found with microglial lesions, but microglial lesions could exist without viral RNA in this region.

	Cerebellum		Hindbrain		Midbrain		Forebrain		Hippocampus		Cortex	
Mouse No.	Iba1+	RNA+	Iba1+	RNA+	Iba1+	RNA+	Iba1+	RNA+	Iba1+	RNA+	Iba1+	RNA+
1	-	-	+	-	+	-	-	-	-	-	+	-
2	-	-	-	-	+	+	+	-	-	-	-	+
3	-	-	-	-	-	-	-	+	-	-	-	-
4	+	+	+	+	+	-	+	+	-	+	-	+
5	+	-	+	-	+	-	-	-	-	-	-	-
6	-	+	+	+	+	-	+	+	-	+	-	-
7	-	-		-	-	-	-	-	-	-	-	-
8	N/D	-	+	+	-	+	+	+	-	-	-	+
9	+	+	+	+	-	+	+	+	-	-	-	-
10	-	-	+	+	-	-	+	-	-	-	-	-
11	+	-	+	-	+	-	+	-	-	-	-	-
12	-	-	+	+	+	-	-	-	-	+	-	-
13	-	-	-	-	-	-	-	-	-	-	+	-
14	+	-	-	-	-	-	+	-	-	-	+	-
15	-	-	+	-	+	-	+	-	-	-	+	-
16	+	+		-	+	+	+	+	-	-	-	-
17	+	-	+	-	+	-	+	-	-	-	+	-
18	-	-		-	-	-	-	-	-	-	-	-
19	-	+		+	-	+	-	+	-	+	-	+
20	+	+	+	-	-	-	-	-	-	-	-	-
21	+	+	+	+	+	-	-	-	-	-	-	-
22	-	+		-	-	-	-	-	-	-	+	-
23	-	-	+	-	+	-	+	+	-	-	+	-
24	-	-	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-	-	-
27	+	-	+	+	+	-	+	-	-	-	-	-
28	-	+	+	+	+	+	+	-	-	-	-	+
29	-	-		+	-	-	-	-	-	-	-	-
30	-	-	+	+	+	+	+	-	-	-	-	-
31	-	-	+	-	+	-	-	-	-	-	-	-
32	+	-	+	-	+	-	-	-	-	-	-	-
33	-	-		-	-	+	-	-	-	-	-	-
34	+	+	+	N/D	+	N/D	+	+	-	-	-	-
35	-	-	+	-	+	-	+	+	-	N/D	-	+

Table 22: Overlap of viral RNA detection and microglial lesion detection in mouse brains based on region.
A comparison of Iba1+ lesions found in one hemisphere to viral RNA found in the other hemisphere. Positive squares (red, +) indicate a lesion or viral RNAwas detected in that region of the brain. Overlap is seen most commonly in the hindbrain but no overlap is found in the cortex or in the hippocampus. ND: not tested.

Detection of persistent viral RNA in a human case

As part of a clinical study to investigate persistence of WNV RNA in a human patient, RNAScope targeting the WNV genome was performed on a human sample from a fatal case of WNND[218]. The patient was a 21-year-old male who presented with a three-month history of progressive muscle weakness that necessitated intubation. The patient had normal sensory faculties but showed weakness in all four limbs with decreased reflexes. Biopsy showed decreased muscle mass due to loss of innervation. The patient developed severe respiratory distress approximately eight months after the onset of symptoms which ultimately led to death.

Sections of the cerebrum, cerebellum, brainstem, and spinal cord were taken and stained with hematoxylin and eosin, which showed meningoencephalitis, myelitis, and motor neuron degeneration. IHC was performed using polyclonal antibodies against WNV E protein, which showed viral proteins in multiple foci in the cerebrum, cerebellum, brainstem, and spinal cord. These antibodies were not used in the previous studies as they had shown significant off-target binding in the mouse brain that was not present in human tissues.

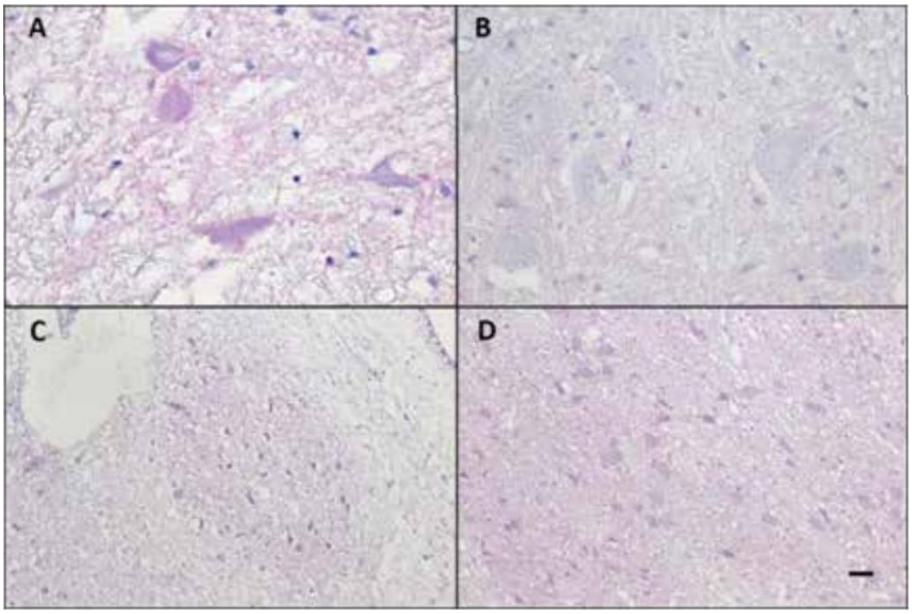

Figure 36: RNAScope staining for WNV RNA.
Areas of red indicate positive viral RNA. Source: Rodriguez et al, *Journal of Neurology & Experimental Neuroscience,* 2019

As confirmatory testing, RNAScope was performed as a more specific method to detect viral RNA persisting in the cerebrum,

cerebellum, brainstem, and spinal cord. Viral RNA was detected in neurons in each region (**Fig. 36**). These included the hypoglossal nucleus, pontine nuclei, and motor neurons of the anterior horn of the spinal cord.

DISCUSSION

These studies demonstrated that WNV RNA persists in the brains of the majority of mice surviving the acute phase of infection. The RNA persists most commonly in the hindbrain, though it was found in each region at least four times in the 35 mice tested in this study. It was least often found in the hippocampus, indicating that while the viral RNA has broad tropism for persistence, some regions are more amenable to this than others.

The effects of viral RNA persistence in different regions correlated with different behavioral outcomes. For the TST, persistent viral RNA in the hindbrain correlated with decreased time immobile. This could be associated with hyperactivity, and the hindbrain contains the pons and medulla, which regulate basal activity levels.

Viral RNA persistence correlated with increased rotarod function in the later days of testing, indicating better memory formation, recall, or execution of the motor skill needed to remain on the rotarod. The cerebellum controls fine motor movements and motor learning[461]. Specifically, it is associated with procedural memory, which is the ability to remember how to do motor actions. The rotarod, when tested across multiple days, tests procedural memory. Changes in the cerebellum have been associated with deficits in motor learning using the rotarod task[462,463]. The outcome seen in these studies, and the mechanism behind it, require further study.

Viral RNA persistence in the hippocampus trended toward correlation with decreased PPI performance. Damage to the hippocampus has previously been linked to decreased PPI outcomes[464], indicating a possible mechanism that should be further investigated.

Viral RNA and inflammatory lesions were found to overlap, but each could occur independently in different regions. This finding could represent the different distributions of viral infection in the two different hemispheres, or it could indicate that inflammation and viral RNA persistence do not have to coincide. If the latter is the case, it would mean that WNV infection induces neuroinflammation but does not maintain it. Given the findings that different regions have different cytokine responses post-infection, it seems reasonable to assume that there are regional differences that affect viral RNA persistence and the inflammatory response in those regions.

Persistent WNV RNA was detected in the brain and spinal cord of a patient over five months post-infection, indicating that viral RNA can persist in immunocompetent human patients longer than had been tested in these studies in mice. This indicates that this mouse model has translational applications.

Chapter 6: G331A Behavioral and Histopathological Studies

INTRODUCTION

Previously published work by others to develop a mouse model of survival following WNV infection had focused on IC inoculation as a method to ensure neuroinvasion and obtain consistent results. Our work showed that peripheral inoculation of a neuroinvasive WNV strain led to inflammation and viral RNA in multiple brain regions, indicating disseminated spread of the virus during early infection. Our work further showed that inflammation and viral RNA persisted over one-month post-infection, which was not reported in studies using attenuated virus inoculated intracranially. To compare how an attenuated virus and a wild-type virus spread into the brain, the ability of the attenuated virus to persist, the amount of inflammation an attenuated virus could cause, and if they caused similar behavioral outcomes, an attenuated strain of WNV our lab had previously characterized was used. In contrast to the previously reported studies using a NS5 mutant of WNV, which is generally impaired for replication compared to the WT, this experiment employed an attenuated structural protein mutant, designated G331A, with highly reduced neuroinvasive capacity.

The G331A strain of WNV was developed to study the role of the envelope gene in receptor-binding of WNV. The mutant changed a glycine to alanine in domain III of the E protein, which is a purported receptor-binding site of WNV. The G331A strain of WNV grows to lower titers *in vitro*, and shows markedly reduced lethality[465]. This is due to the elimination of neuroinvasion following peripheral inoculation and reduced neurovirulence when inoculated intracranially. The mortality in IC inoculated mice was reduced from 100% in wild-type inoculation to 50-70% following G331A inoculation[466]. G331A has been shown to develop compensating mutations in some cases leading to neurovirulence when inoculated intracranially.

The aim of this study was to assess whether direct IC inoculation of an attenuated strain of WNV resulted in neuropathogenesis leading to different phenotypes in mice surviving infection, including viral persistence, regions of inflammation, and behavioral changes.

DEVELOPMENT OF MODEL

The G331A strain of WNV was selected based on our knowledge of decreased virulence and our knowledge that it had restricted spread outside of and within the CNS. The strain had also mainly been characterized, including electron microscopy, multiple dosage studies in mice, and comparisons in heat susceptibility to wild-type WNV. This allowed us to know more about how this strain acted differently than the wild-type virus. G331A had been shown to develop compensating mutations, but these were generally uncommon in IC inoculated mice, allowing for us to ensure a large enough group of mice to survive into behavioral testing.

C57BL/6NTac mice were not chosen for this study as the G331A WNV strain had never been characterized in these mice when inoculated IC. There were consistent, reproducible results when G331A was IC inoculated into Swiss-Webster outbred mice, and so these were selected as the model for final investigation. The mice were inoculated at six-weeks-old, as this was the oldest that they could be and still allow for efficient IC inoculation through the sutures of the skull.

Because the two strains of mice likely had different normals for the behavioral tests, 15 uninfected control mice were included to approach the number of uninfected controls in the previous studies. To account for potential trauma from IC inoculation, two different cohorts of mice were used as controls. Ten of the control mice were inoculated with saline IC and five were not inoculated.

RESULTS

G331A intracranial inoculation leads to similar clinical signs and mortality in Swiss-Webster mice as observed for the NY99 WNV survival model

Of the mice inoculated with G331A, 70% survived past 21 dpi (14/20) (**Fig. 37**). This was comparable to the mortality seen in the peripherally inoculated NY99 WNV study. The timespan for death was slightly faster than with peripheral inoculation- the last mouse to be euthanized was at 11 dpi, and all other mice were euthanized between 6-8 dpi.

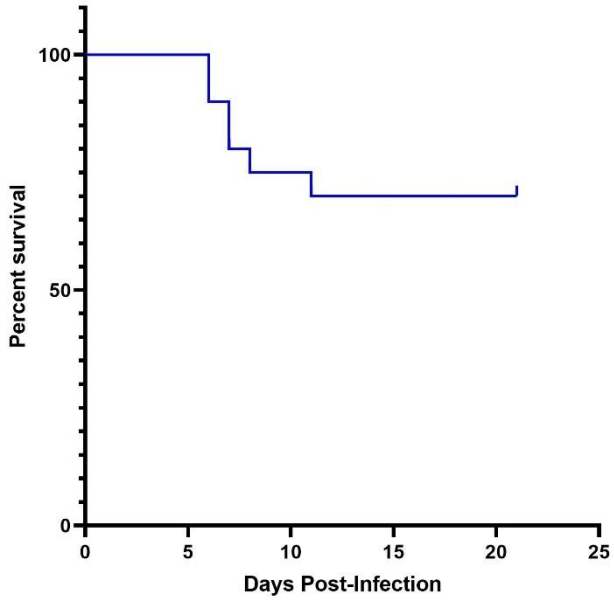

Figure 37: Survival curve for 6-week-old Swiss Webster mice inoculated intracranially with 100 pfu of the G331A strain of WNV (n=20).

Mice infected with the G331A strain of WNV that survived past 21 dpi showed significant weight loss compared to uninfected controls (**Fig. 38**). This was statistically significant at 7, 8, 12. 13, and 14 dpi. The infected mice showed an overall increase weight after 9 dpi, with most returning their starting body weight by 21 dpi.

Clinically speaking, mice inoculated with G331A WNV were more likely to recover after developing severe signs of disease than the C57BL/6NTac mice inoculated with NY99 WNV in the footpad. One mouse showed unilateral hind-limb paralysis and survived past the acute phase of disease and was kept until 35 dpi. The mouse's lowest weight was on 9 dpi at a body weight of 78.4% of her starting weight. After this, she regained weight and began acting more normal through the course of the study.

The mouse never regained use of the paralyzed limb but did show remarkable dexterity despite this. The mouse was able to climb around the cage and showed interest in food until the final day of experimentation. This mouse could not be tested using the rotarod, TST, or 2WAA due to the confounding factors the paralysis would have had.

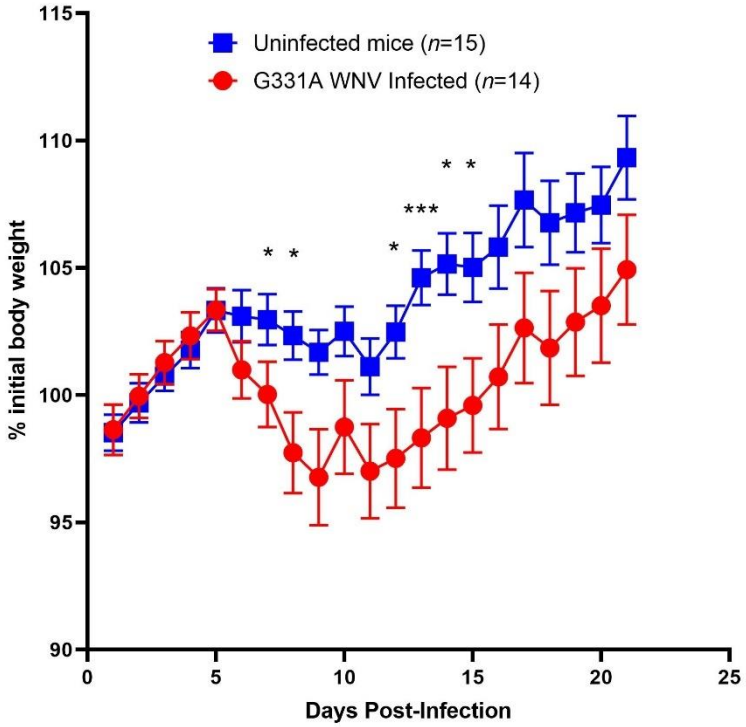

Figure 38 Comparison of body weights between uninfected and G331A-infected Swiss-Webster mice. G331A infected mice showed significant weight loss when compared to the general weight gain that uninfected mice showed. All error bars represent mean+/-SEM. Results were analyzed using repeated measures two-way ANOVA.

Swiss-Webster mice show greater variability in behavioral outcomes than C57BL/6NTac mice

One important aspect about this study that was the difference in behavioral testing between the outbred Swiss-Webster mice and the inbred C57Bl6 mice. The outbred mice showed much greater variability in their test results including behaviors that had never been noted in any of the inbred mice. This variability has been recorded in past research[467] though whether inbred mice represent neurological function as well as outbred mice has been called into question more recently[468].The work compared outbred and inbred mice found that both are appropriate for studying social behavior, but that inbred mice were more likely to show statistical significance. Our results indicate that the behavioral tests

should largely be restricted to inbred mice to better account for genetic variability. The other option would be to use large numbers of outbred mice to examine for significant functional changes.

There were no significant differences in rotarod performance based on infection status

When tested via rotarod, fewer of the uninfected mice passed the preliminary screening. The initial screening was meant to select mice motivated to remain on the rod, similarly to the NY99 WNV model mice. They were placed on the rod which rotated at 4 rpm, and were given three attempts to remain on for one minute. Only twelve of the fifteen uninfected mice passed the screening by remaining on the rod for at least one minute in one of the trials, and ten of the fourteen G331A WNV-infected mice passed. During testing, more of the outbred mice of both infected and uninfected status showed the phenotype of walking or leaping off of the rod whether it was moving or not. This indicated decreased motivation to remain on the rod. The results of testing were less consistent than

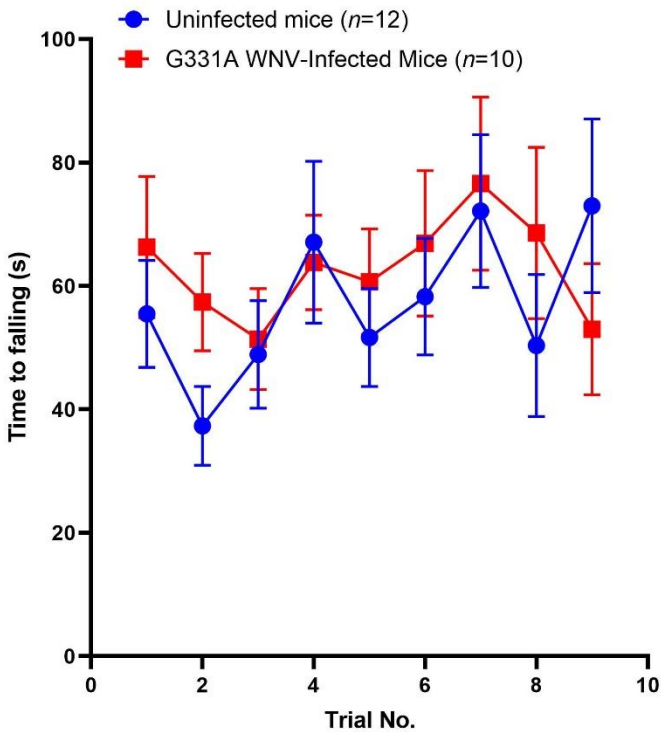

Figure 39: Rotarod performance of G331a-inoculated mice compared to uninfected mice across nine trials.
There was no significant difference between the two groups on any single trial, and neither group showed significant changes across trials. All error bars represent mean+/-SEM. Results were analyzed using repeated-measures two-way ANOVA.

those of the inbred mice. Neither the infected nor uninfected groups showed a consistent pattern of improvement, unlike the inbred strains (**Fig. 39**). There were no significant differences between trial numbers. There was no significant difference between uninfected and infected mice either. The outbred control mice showed similar time remaining on the rod to the inbred mice initially (about 57 seconds), but the outbred mice did not show steady improvement across trials like the inbred mice did.

G331A WNV infection does not cause significant changes in 2WAA testing

The 2WAA test was performed in the same manner as with the NY99 WNV infected mice. The Swiss-Webster mice showed different outcomes compared to the C57BL/6NTac mice. At least one of the infected and uninfected mice did not cross in response to the foot shock. Instead, they would jump and grab the edges of the container or separator between sections in response to the light and the foot shock. This was not expected as the ceiling of the container appeared flush with the walls. Multiple attempts were made to reduce the incidence of this including applying electrical tape or knocking the mice off of the walls using forceps, but these measures did not reduce the frequency of occurrence. Ultimately the mice stopped this behavior by 34 dpi, and these trials were not considered an avoidance for analysis purposes.

There was no significant difference in 2WAA performance between uninfected mice with or without IC inoculation, so they were grouped. Unlike the C57BL/6NTac mice, the uninfected Swiss-Webster mice continued to improve each day of testing, with their peak performance at 34 dpi, as opposed to 33 dpi. There was no significant difference between uninfected mice and WNV G331A infected mice on any day of testing (**Fig. 40**). Because fewer uninfected mice were tested, the 20th percentile was calculated and used rather than the 10th to determine if there were more G331A-infected mice that had low memory performance. When a

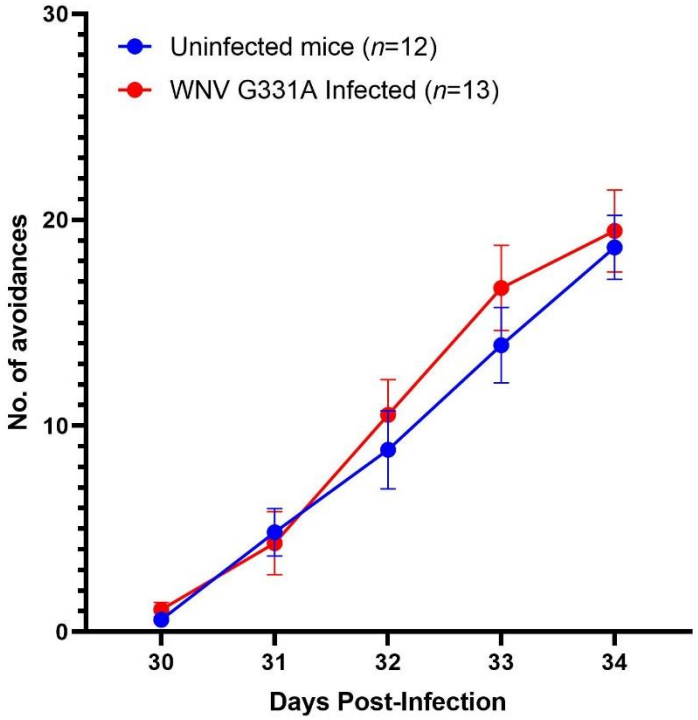

Figure 41: 2WAA performance in uninfected and G331A WNV-infected mice.
Mice were tested for five days with thirty trials per day to determine their ability to avoid foot shocks by predicting them with a sound and light cue. There was no significant difference in performance between G331A WNV infected mice and uninfected mice on any day of testing. Analyzed by repeated measures two-way ANOVA.

	2WAA Normal	2WAA Poor	Total
Uninfected	11	1	12
WNV G331A Infected	11	2	13
Total	22	3	25

Fisher's exact test

The two-tailed P value equals 1.0000
The association between rows (groups) and columns (outcomes) is considered to be not statistically significant.

Figure 40 Fisher's exact test to determine if statistically more G331A WNV infected mice fell below the 20th percentile of uninfected mouse performance based on 34 dpi.

Fisher's exact test was performed, there was not a significant difference in the proportions of uninfected and G331A WNV-infected mice falling below the 20th percentile (**Fig. 41**).

These findings indicated that IC inoculation with the G331A WNV strain was less likely to induce behavioral changes in surviving mice post-infection than NY99 WNV inoculated in the periphery. This finding should be considered critically, however, given that the Swiss-Webster mice showed more considerable variability in the uninfected mouse's behavior.

Mice euthanized during acute phase of infection showed similar levels of microglial activation as NY99 inoculated mice

For the mice that were euthanized during the acute phase, the microglial activity was similar to those seen in the previous work. Microglial activation was inconsistent in these mice. In some there was generalized microgliosis and inflammation while others appeared generally normal.

DISCUSSION

The studies performed here were aimed at comparing the behavioral effects of wild-type, NY99 WNV inoculated peripherally to the effects of an attenuated strain of WNV inoculated directly into the brain. The outcomes of these studies indicate that the attenuated strain does not induce the same level of behavioral deficits as those seen with wild-type WNV. However, it is difficult to make conclusions based on the data given that the outbred strain of mice used for these studies were less predictable and less amenable to the behavioral testing than the inbred mice previously used.

The G331A model showed similar survival rates to the NY99 model, which indicated similar severity of disease during the acute phase. Due to the limitations of the behavioral testing, further evaluation of brain tissues was not performed. Complete quantification of the viral load in each region was not performed. The brains have been

appropriately dissected and fixed, so future analysis of these tissues can be performed to determine the extent of viral persistence and inflammation.

Chapter 7: Discussion

This study shows that our model of WNV infection and long-term neurological damage following peripheral inoculation with a neuroinvasive WNV strain is reproducible and shows inflammatory, viral, and behavioral changes that can all be used as outputs for future studies. The inflammatory changes in the brain are the most consistent and most commonly associated with neurobehavioral abnormalities. This indicates that, like NDs, ongoing inflammation in the brain is associated with neurological changes measurable using behavioral testing. Persistent viral RNA appears to correlate with some neurobehavioral changes as well, though this is less common than a correlation between neurobehavioral changes and inflammation. Astrocytosis and microgliosis occur long after the initial WNV infection in the mice, indicating ongoing inflammatory processes. The inflammatory processes appear to occur independently of persistent viral RNA, though this correlation may be region-dependent. There are likely multiple mechanisms leading to these different outcomes, and the overlap between behavioral, virological, and inflammatory changes likely is more complicated than simple correlation.

ADVANTAGES OF THESE STUDIES

These studies were a multidisciplinary approach to a complicated scientific question. Rather than focusing on one aspect of the disease, the studies aimed to look at the immunological, virological, and neurological aspects of WNV infection. These studies were aimed at correlating the changes WNV infection caused in each of these to determine if there were links between the three.

These studies show many advantages over previous models used to model the long-term effects of WNV infection in humans. The footpad method of inoculation is similar to how humans are infected via intradermal inoculation of WNV by mosquitoes. This model

closely matches the normal distribution of the virus systemically, which will affect where the virus distributes in the CNS.

It was important for translatability to use a wild-type strain of WNV associated with long-term neurological changes in patients. The previously reported NS5 mutant model allowed for confirmed neuroinvasion but used a strain that did not antagonize the interferon response as efficiently as the wild-type strain[162]. The attenuation could have skewed the length and severity of the immune response to the initial viral infection, leading to changes in long-term persistence and neurological damage.

This is the first time that a battery of behavioral tests has been used to screen for neurological changes in a mouse model of WNV. Long-term WNV-induced changes in human patients manifest as a spectrum of diseases, and not all patients show all symptoms. The model presented here tests for multiple behaviors, which allows for screening of mice for deficits in one or more functions. The model shows a diversity of outcomes, including mice with memory loss, motor learning deficits, and sensorimotor gating deficits. Similar to human patients, long-term neuroinflammation was confirmed to occur in multiple brain regions in this mouse model.

The role of persistent viral RNA in behavioral changes and the localization of viral RNA in individual brain regions were also investigated. Other studies have investigated viral RNA persistence or behavior, but this is the first to use wild-type virus to compare the two. Results from the studies described here demonstrate that the virus has the potential to distribute to all regions of the brain and can persist in all of them, though with different frequency in different regions.

LIMITATIONS OF THESE STUDIES

One important note is that supportive care was not given to mice, which could have biased the selection of mice surviving infection. Human patients showing severe disease following WNV infection often receive intensive care, including breathing support,

monitoring, and fluid support, while the mice in this study did not. This difference may mean that the mice showing the most severe clinical signs were euthanized during the acute phase of disease, leaving only mice with less severe clinical signs, which are likely comparable to patients presenting with less severe WNND initially.

One of the biggest challenges in studying WNV-induced neurological changes is making a reproducible model. This model shows a spectrum of disease and damage to different brain regions, including viral RNA persisting in many different regions. The variability means that studying each of the different behaviors and regions of damage requires large numbers of animals to reliably obtain statistically relevant numbers for a study. There are consistent changes in the mice that can be observed, such as viral RNA persistence and inflammatory lesions in the brain, but the localization varies between mice. This diversity indicates that the model more closely mimics human disease than previous models relying on IC inoculation. The two models would work best in conjunction with each other. The IC inoculation model can be used to study specific mechanisms requiring an easily reproducible model, and these can be confirmed in our model to ensure that it occurs during natural spread of wild-type virus.

An additional limitation of this study is that only a few representative slides of inflammation were taken for histopathology from each animal, and only from 1.0-1.4 mm lateral from midline. The studies that were performed indicate that the inflammation can be highly localized, and images were not obtained from all the different functional regions of the brain.

AGE AND DOSAGE STUDIES (SECTION 3)

A prior study that demonstrated the persistence of WNV in the brains and spinal cords of infected mice used 1000 pfu of NY99 WNV inoculated in the same manner and using the same age, strain, and vendor of mice were originally used in these studies[145]. The studies presented here found that inoculation with 1000 pfu had a much higher mortality

rate, which precluded the use of this model for behavioral testing. The difference in mortality rates could be due to many different factors. While both studies used a single-passage virus derived from virus cloned using two plasmids, the cloning methods may have been different. It is also possible that there were quasispeciation differences, as virus used in these studies was propagated on Vero cells, and the previous study had used baby hamster kidney (BHK)-21 cells. No consensus changes were detected in the sequence of the virus used in this studied, but this does not describe the full viral population.

Significant differences in survival were associated with the age of the mice at the time of infection. This has been previously reported, though that study compared 6-10 week-old mice and 21-22-month-old mice[469]. Here, a difference in survival rates was observed between mice infected at 8 weeks versus 16 weeks of age. Mouse immune systems mature between 8 and 16 weeks of age, producing granulocytes, T cells, and B cells during this time, but maturation is not complete until approximately 26 weeks old[470,471]. The full repertoire of B and T cell receptors in the periphery are still expanding as the mouse matures[472]. The nervous system is still undergoing development at this time as well, with development of the spinal cord, hippocampus, and olfactory structures not being finished until eleven weeks of age[473,474]. Any of these developmental differences could explain the increased susceptibility in younger mice, as their immune system is not fully developed, and the nervous system is more susceptible to damage as it develops.

OUTCOMES OF BEHAVIORAL TESTING (SECTION 3)

Tail suspension test

The TST for depression-like behavior did not show a significant difference between uninfected and WNV-infected mice. Depression is one of the most commonly reported long-term symptoms of WNV, so this presents a shortcoming for this model. The only potentially significant finding related to this test was that mice with microglial lesions and viral RNA in the hindbrain showed a trend toward increased activity. This could represent

a phenotype of hyperactivity. A similar finding was made when GABAergic neurons of the midbrain and pons were destroyed, which led to mania and sleep reduction in mice[475], which could indicate a potential mechanism to be investigated.

One explanation for this may be that there are different forms of depression-like behavior, and the TST relies on a despair model. Comparison with another despair model, the forced swim test, showed differences in phenotypes between the two tests in the same mice[476]. This finding indicates that the two tests likely assess different pathways or subtypes of depression through the brain, and they may test for different mechanisms. It may be worth using the forced swim test as an alternative to the TST, but it would be difficult to perform in a BSL-3 setting, given the size and volume of water necessary for testing.

It may be worth investigating if this model does manifest other forms of depression, such as anhedonia. Anhedonia is the loss of ability to feel pleasure or the inability to react to normally pleasurable stimuli, and is a common symptom of MDD[477]. The sucrose preference test is a relatively simple behavioral test that measures anhedonia[230]. Mice are placed in a cage with two water bottles. One has normal water and the other has sucrose-laced water. The amount of water that the mouse drinks from each bottle is measured and compared to determine the level of preference the mouse shows for sucrose-laced water. Normal mice show a strong preference for the sucrose-laced water, while mice with depression show less difference in the amount of water drank between the two bottles. This test could be performed relatively easily in the ABSL-3 using larger rat cages that contain two water bottles, though mice would need to be tested individually. This would require a significant investment in time and caging, as each mouse would need to be kept in an individual cage with two different water bottles for the length of the test. The amount of time each mouse is left in these cages could be reduced, but it would still require more cages of larger size, which could present an issue. This test was not performed using these

mice because the TST was considered an accurate measurement of depressive-like behavior, but the sucrose preference test should be considered in the future.

Animal models of depression are a complicated subject, as it is difficult to replicate human depression in animals. Mice are a commonly used model due to the variety of products available for use and the ability to use genetically modified mice. Experiments studying the mechanisms of depression need to induce depression in the mice, while the studies here were designed to investigate if depression occurred. Induction of depression can be done either through the application of stressors or through biological manipulation. The validity of these models is based on whether the model shows behavioral changes like those seen in humans; whether there are biological alterations like those in humans; and whether antidepressant treatment alleviates the signs of depression[478]. The studies performed here approached the question differently by screening for depression in WNV-infected mice rather than trying to develop a model that replicated human depression. There are subtypes of depression with potentially different causes, biological changes, and behavioral outcomes between each of them[479]. Without investigating other tests of depression in this mouse model, it cannot be definitively stated that mice do not show depression-like behavior.

Prepulse inhibition

PPI testing measures sensorimotor gating, which reduces the amount of incoming information to the brain. Sensorimotor gating has been shown to be impaired in patients with HD[480], schizophrenia[481], and autism[482], and has been associated with infectious diseases including toxoplasmosis[483]. In rodent models, it has been found to be decreased in rats neonatally infected with influenza[484], cytomegalovirus[485], or herpes simplex virus-1[486]. Here, we found that while PPI was not inhibited in all mice infected with WNV, there was a trend toward a decrease in sensorimotor gating in mice with viral RNA in the hippocampus.

The hippocampus has been previously implicated in the pathways of PPI. Stimulation of the hippocampus with GABA[487] or cholinergic agonists[464] leads to decreased PPI, but lesion presence does not affect it. These results are similar to those described here, and indicate that the persistence of viral RNA may be affecting the normal activity of the neurons in that region, leading to changes in PPI. The NS5 mutant IC challenge model has shown that viral infection changes the normal function of the hippocampus without severe histologic changes[162]. These mechanisms could be playing a role in in the PPI testing outcomes observed here, and should be further investigated. It is interesting that differences were detected in the PPI in mice with viral RNA persisting in the hippocampus, but not in the 2WAA. This could be an artifact of the small number of mice that had persistent viral RNA in the hippocampus, or it could indicate susceptibility of specific regions or subpopulations of neurons within the hippocampus.

Rotarod

The rotarod is a measure of both motor coordination and motor learning, depending on the paradigm. These studies tested both. The early stages of testing assessed motor coordination, and the later stages assessed motor learning. There were no significant differences in the first three trials of testing, indicating there was likely no motor coordination deficits.

One aspect that should be addressed regarding our rotarod testing was the difficulty in keeping mice motivated. The rotarod used in these studies had a shorter fall than most rotarods used in neuroscience labs because it needed to fit inside of a BSC. In the future, using a rotarod with a greater fall distance would be recommended if possible, both to test multiple mice at once and to encourage them to remain on the rod.

Motor learning requires multiple brain regions, including the cortex, cerebellum, hippocampus, and pons[488]. Trials 4-9 of the rotarod were used as a measure of motor learning, because trial 4 was when the control mice first showed a significant improvement

over their first trial (**Fig. 17**). No correlation was observed between persistent viral RNA or microglial lesions in the hippocampus or hindbrain with performance on the rotarod. This indicated either that the inflammation or viral RNA persistence did not cause enough damage to lead to neurological changes, or that the methods of detection were not sensitive enough to detect the type of damage induced.

There was a significant difference, or a trend toward significant difference, between mice with and without microgliosis in the cortex, cerebellum, midbrain, and forebrain in their rotarod performance. The cerebellum is commonly associated with motor function including motor learning, so damage in that region can lead to deficits in motor coordination or learning[461]. The cortex contains regions for executive function, including the motor cortex, and has been linked to memory formation[489]. Damage to the motor cortex or any region necessary for memory formation or recall could lead to deficits in the later trials with the rotarod. One caveat is that we did not localize the cortical lesions further, partly because it is difficult to separate them using sagittal sections. Future work should investigate if the motor cortex is specifically damaged after WNV infection. The midbrain and forebrain contain dopaminergic neurons that emanate from the substantia nigra in the midbrain and end at the thalamus of the forebrain[490]. These neurons are important for motor execution and coordination, and damage to them or in that region could explain the deficits that were observed in rotarod testing. It is important to note that some mice showed lesions in more than one of these regions, so those mice would have been counted more than once. The other complicating factor is that widespread damage is difficult to correlate to one specific region- it is possible that multiple regions must be damaged or changed in specific ways to lead to specific outcomes.

An unexpected finding was that detection of persistent viral RNA in the cerebellum trended towards correlating with increased performance on the rotarod. The fact that mice with persistent viral RNA tended to perform better indicates that there is less functional damage to the region, so it seems more likely that the cerebellum is prone to overt

inflammation if virus is detected during the early stages of infection. Detection of viral RNA in the cerebellum indicates that the virus likely avoided host immune detection, which would decrease the amount of inflammatory damage in the region.

One important note regarding testing for motor coordination is that the mice with the most noticeable motor changes during the acute phase of infection were euthanized. All mice with profound motor weakness or paralysis needed to be euthanized before behavioral testing occurred to follow ethics guidelines, so the experimental design and euthanasia criteria selected for mice without severe motor deficits.

Two-way active avoidance

Memory testing using the model presented here indicated a trend toward higher numbers of WNV-infected mice showing decreased memory function. The mice that showed decreased memory function compared to uninfected controls also performed significantly worse than other mice on other days of testing. This finding indicates that the loss of memory was not a statistical anomaly on one day, but rather that this group of mice had decreased function on multiple days of testing. Unlike the other behavioral tests, there was no correlation with any of the changes found via IHC or testing for viral RNA.

The two-way active avoidance test relies on multiple brain centers. The circuit underlying performance includes the basal ganglia[275], hippocampus[491], amygdala[492], thalamus[493], and multiple regions of the cortex including the entorhinal cortex[494], the retrosplenial cortex[277], and the prelimbic prefrontal cortex[495]. The connection between WNV infection and memory loss has been associated with cortical atrophy in patients[96], though this was not measured during these studies. Microgliosis and viral RNA persistence were not correlated with memory loss specific to any region, though a higher proportion of WNV-infected mice showed lower performance during 2WAA testing the uninfected mice. This indicates that the metrics in these studies likely do not reflect the mechanisms behind memory loss in these mice, and further study is warranted.

The previous work using the NS5 mutant model studied changes specific to the hippocampus that included phagocytosis of synapses[162], loss of neurogenesis[164], and infiltration of T cells[163]. The studies presented here would not have detected these changes. Given that persistent viral RNA was detected in the hippocampus of multiple mice in the studies described here, it is possible that these mechanisms are occurring at the same time as the microgliosis and astrocytosis in other brain regions. Future analysis to identify the effects of peripheral inoculation of neuroinvasive WNV on cytokine levels in the hippocampus is recommended.

NEUROINFLAMMATION (SECTION 4)

Microgliosis

The most prominent finding of this section was the pronounced microgliosis and formation of microglial nodules that were detectable in mice more than one month post-infection. To our knowledge, this finding has not been reported in any previous studies, particularly using a peripheral route of inoculation. This indicates long-term, persistent inflammation induced by viral infection that can persist for over one month post-infection. Past work had shown generalized inflammation up to four months post-infection using H&E staining[145]. This included perivascular cuffing, gliosis, neuronal loss, and lymphocytic infiltration of the meninges, but did not specifically target microglia.

The presence of microgliosis and microglial nodules is indicative of an inflammatory state in the brain. Microglial nodules have been reported in other viral diseases including HIV[496] and cytomegalovirus[497]. The most common site for microgliosis observed in the current studies was the hindbrain, which is interesting because the hindbrain has lower numbers of microglia in homeostasis[362]. In some cases, the microgliosis was surrounding blood vessels, which could indicate signaling for transmigration of peripheral inflammatory cells, consistent with a persistent inflammatory state. Microglial nodules have been shown to provide an amenable environment for T cells that mediate persistent encephalitis[390]. This is worth investigating in the future, and the role of T cells in long-term WNV pathogenesis should be further studied.

One important question about the microgliosis found in these studies is whether it is protective or pathologic. Microglia are necessary for clearance and survival after WNV infection of the CNS, but their persistent activation may be pathologic. Past work has shown that chronic activation of microglia changes their activity during NDs[378]. The microglia change from being protective to being pathologic and affect neuronal activity through the release of pro-inflammatory cytokines, accumulation of misfolded proteins,

and inappropriate phagocytosis. The microglia found in these studies should be further characterized via transcriptomics and cell markers to determine if the same types of microglia are present in each of the lesions found. Further characterization of the microglia could be used to compare their activation status and activity to those in NDs and during the acute phase of WNV infection. Characterization could also determine if the microglia in different regions react differently post-infection. One indication that there is a pathogenic element to the microglial nodules we found is that the mice with these nodules in the hindbrain showed significantly greater weight loss during the first 21 dpi. This finding suggests that the mice with microglial lesions in the hindbrain had more severe disease than those without the microglial nodules. The mechanism for the persistence of these nodules was not determined, but it could be that the virus is persisting in these regions or that an uncontrolled, pathologic immune response is occurring. Because the hindbrain is responsible for basal activities including breathing, heart rate, and digestion, the inflammation in this region could significantly reduce the mouse's ability to regulate and maintain itself.

Contrary to findings from a previous study using the NS5 mutant model of WNV[162], microglial nodules were not detected in the hippocampus of any of the mice tested. The difference in findings may represent an artifact of the IC inoculation with an attenuated viral strain, or the definitions of microglial nodules may have been different between the two groups. The definition of microglial nodules was not stated in the study using the NS5 mutant IC model, and the representative images are difficult to interpret. For the current studies, microglial nodules needed to be distinct groups of Iba1+ cells, and the NS5 mutant model used H&E staining to define the nodules. Although viral RNA was sometimes found in the hippocampus, there was little to no inflammation there in the current studies.

Inflammatory lesions were most commonly found in the hindbrains of mice. Similar findings have been reported in mice lacking the innate immune molecule STING, where mice that survived WNV infection had increased T cell infiltrates in the hindbrain[498],

and virus was not detected, though that study used IHC to stain for viral antigen, which may be less sensitive than the RT-PCR used in these studies. Microglia have different phenotypes based on the region in which they reside[296]. This could explain the different responses to WNV that were observed, and the predilection for reactivity in certain regions. Cell sorting for microglia followed by transcriptomic analysis could help explain the differences between the regions and between mice with and without inflammatory lesions.

The formation of microglial nodules, as seen in some of these mice, is a common finding in multiple NDs, including MS, Rasmussen's encephalitis, and paraneoplastic syndromes. They have also been found in neuroinvasive viral infections in humans and rodent models, including neuroAIDS, HSV, and HCMV[499]. In human samples of Rasmussen's encephalitis, the microglial nodules were found to be sites supporting the translocation and activation of pathogenic T cells[390]. This was tied to TLR activation, particularly TLR3, which has previously been associated with recognition and immune response to WNV during acute disease[44,500]. Stimulation of TLR3 in microglia causes an increase in the production of pro-inflammatory cytokines that lead to neuronal dysfunction and recruitment of effector T cells.

Other viruses have been shown to induce pro-inflammatory states within the brain, leading to neurological dysfunction. Western equine encephalitis virus induces microgliosis, astrocytosis, and Parkinsonism in a small rodent model[501]. Although Western equine encephalitis virus is in a different viral family than WNV, there are likely similar mechanisms as they are both RNA viruses that induce chronic inflammation in the brain.

Astrocytosis

Astrocytes play a crucial role in NDs. When they become activated, they can help in recovery through the production of neurogenic molecules and by supporting damaged neurons. They can also cause further damage to the brain in these diseases by producing pro-inflammatory cytokines and neurotoxic molecules. These studies found persistent

astrocyte activation over one month post-WNV infection, though the astrocytosis did not always coincide with microglial activation, indicating that there are separate stimuli maintaining the activation of the two types of cells.

Astrocytes play a major role in WNV infection, though their role in long-term disease is still being studied. There is evidence of WNV infecting astrocytes in human patients[502] and in primary mouse astrocytes[158]. *In vitro,* primary astrocytes have been shown to survive WNV infection, and persistently produce virus for over two months post-infection. One caveat to the *in vitro* findings is that primary astrocytes act differently *in vitro* than *in vivo*[503]. There have not been any conclusive studies showing that WNV infects astrocytes *in vivo*, including in a mouse model, and the analyses performed in the current studies could not determine what types of cells harbored viral RNA.

Depending on how the activated microglia in the brain are acting, those found near astrocytes may induce changes in the phenotypes of the astrocytes. One area of focus was on the phenotype of the activated astrocytes post-infection, as it seemed likely that WNV would induce A1 astrocyte differentiation. TNF-α and IL-1 are both upregulated during the acute phase of WNV infection, and both are necessary for A1 astrocyte differentiation[202]. In addition, A1 astrocytes release cytokines that have been shown to be induced during the acute phase of WNV infection including IL-1β, TNF-α, and lipocalin-2[504]. These signaling molecules can act on neurons and disrupt their function, and they can act on microglia to induce further activation. Although IHC staining for C3 (a biomarker for A1 astrocytes) was attempted, the staining was highly heterogenous in control and infected brains. The past work used RNAScope to study mRNA levels of *C3*, which would require optimization for use with samples from the BSL-3.

In these studies, a specific role for astrocytes in the pathogenesis of long-term WNV-induced neurological disease was not established. However, it would make sense that they play a role and further investigation is warranted, especially given that we found the presence of astrocytosis without microgliosis and vice versa. Cytokine analysis

Cytokine analysis performed in these studies was focused on the hindbrain and cortex. The hindbrain was selected because it was the most common site to find persistent viral RNA and inflammatory lesions. The cortex was selected because it was the largest tissue collected, and regions of it can affect performance in all of the behavioral tests.

The overall decrease in IL-1β in the cortex of all infected mice when compared to controls is an interesting phenomenon. IL-1β is an idiosyncratic cytokine in the brain, because it has been reported to be both detrimental and protective in NDs. For WNV, it has definitively been shown to be protective during the acute phase of infection[165], and one study using a mouse model found that astrocyte-produced IL-1β caused decreased neurogenesis long-term in the hippocampus[164]. Neither neurogenesis nor the source of IL-1β was evaluated during the current studies, and because all the cells of the CNS can produce IL-1β, its source cannot be readily determined. One possibility is that the decrease in IL-1β in the cortex of all mice indicates an anti-inflammatory state in the larger brain after the severe inflammation that WNV induces during the acute phase of infection.

The decreased levels of IL-1β did not correlate with viral RNA persistence, indicating that it is unlikely to be virally induced. In the CNS, IL-1β is generally considered neurotoxic and restricts neurogenesis and repair. The lower levels may indicate that the cortex is in an anti-inflammatory state after the acute phase of infection, regardless of persistence of the virus. The brain likely needs to heal after the trauma of WNV infection and likely reduces inflammation to promote neurogenesis. This may be an oversimplification, as IL-1β can be both neurogenic and neurotoxic depending on the region and exposure. It has been shown to reduce neurogenesis in the hippocampus in AD models[505] and in a different mouse model of WNV[164], and it has also been shown to induce neuronal apoptosis in the substantia nigra in a mouse model of PD[506]. Another AD model showed that IL-1β reduced plaque pathology in the brain[507] and another PD model showed that IL-1β was neuroprotective, as it induced neuronal sprouting in areas with lesions[508].

The results reported here indicate that the cortex is likely in an anti-inflammatory state where it is downregulating IL-1β to preserve neurons in this region.

TNF-α can be produced by any of the cells in the CNS[509], so determining the source is difficult. Most commonly it is produced by microglia, but a statistically significant increase in TNF-α was not detected in mice with microglial lesions in the hindbrain specifically. The overall increase in TNF-α within mice with persistent viral RNA in the hindbrain indicates an ongoing inflammatory reaction. Unlike IL-1β, TNF-α is considered to be exclusively pro-inflammatory in the CNS and can be damaging to neurons by causing excitotoxicity. The fact that it is being produced indicates that there is some stimulation occurring, but not in the cortex. This indicates that the cells in the hindbrain are sensing a viral component, as there was not a significant increase when comparing hindbrains with and without lesions. This could indicate low-level viral replication or sensing of persistent viral RNA. The levels of WNV RNA in the hindbrain varied, which indicates that there is likely not ongoing replication. It is possible that there are varying levels of WNV replication in different mice, which could account for some of the differences seen. MV has been shown to stay as viral RNA in cells for long periods, which induces innate immune reactions that keep it from replicating again. A similar mechanism could explain the findings in these studies.

These general findings indicate that different brain regions respond differently to WNV infection. Using whole brains for analysis, rather than dissecting out the regions, can produce misleading data. The changes in cytokines levels should be confirmed with qPCR in the future, and was not performed at this time due to the outbreak of COVID-19. Future work should involve dissecting the brain regions into at least the six regions mentioned. This can be done relatively easily in a BSL-3 without specialized tools. Further dissection would be ideal, but requires specialized equipment and sharps that are not recommended for use in high-containment work.

WNV-induced inflammation and neurodegenerative diseases

One of the aspects of long-term WNV-induced disease that these studies aimed to address was how it compared to NDs. The findings from the current studies are inconclusive in this regard. Both forms of disease include inflammation with astrocytosis and microgliosis, but further characterization is needed to more completely connect them. The studies had initially aimed to compare the astrocyte phenotypes between the diseases, or to establish if hyperphosphorylated tau was present in the mice, but these procedures could not be optimized to a diagnostic level. There are other directions to pursue in this regard: transcriptomics will be key in comparing specific brain regions between neurodegenerative and viral diseases. It seems likely that there are overlapping mechanisms between the two, especially given the change in TNF-α in the hindbrain and the persistent microgliosis seen in the majority of the mice.

VIRAL PERSISTENCE (SECTION 5)

The first finding of this work is that WNV conclusively crosses into the brains of mice in the majority of cases. For the mice without detectable viral RNA in any brain regions, it is not possible to determine whether this means no neuroinvasion occurred, or if virus and viral nucleic were cleared prior to when the brains were tested.

Previous work indicated live virus and viral RNA can be detected in the brains and spinal cords of mice, though likely at low levels[145]. There has not been more research to localize persistent viral RNA to anatomic regions within the brain. We found that viral RNA can be detected in all regions of the brain, indicating both that the virus can spread widely initially and that the RNA can persist in multiple regions for more than one month post-infection. While the RNA could be detected in all regions, it was most commonly detected in the hindbrain, namely the pons and medulla oblongata. This finding could indicate that WNV has a specific tropism for those regions, or that these regions are less able to remove persistent viral RNA.

Different regions of the brain have different cell makeups and baseline transcriptional levels, which may lead to different regions having different susceptibility for persistent viral infection. The hindbrain normally has lower numbers of microglia than other regions which may explain some of its apparent susceptibility.

Finding viral RNA most commonly in the hindbrain was consistent with past work using macaques infected intrathalamically that found the virus most often in the pons, medulla, cerebellum, and spinal cord[147]. Another study found that the virus persisted longer in the spinal cord than in the brain[145], which is consistent with the findings reported here, as the hindbrain is the connection between the spinal cord and brain. This may be an indication of a similar or intermediate phenotype of cells between the spinal cord and hindbrain. Viral antigen is commonly found in the hindbrain in cases of human[81] and equine[510] WNV infection, though these cases were in the acute phase of infection.

Within the hindbrain, BBB permeability has been shown to be regulated by type-I IFN signaling to astrocytes[47]. That research focused on the cerebellum, while in the model described here, viral persistence was observed most commonly in the pons and medulla. The cerebellum has significantly different biology compared to the pons and medulla, and also performs different functions. Further characterization of the immune status of the pons and medulla pre- and post-infection could help clarify why a relatively high prevalence of viral RNA occurred in that region.

The closely related JEV has been shown to have a tropism for the thalamus[511] and brainstem[512], indicating that there may be a specific tropism for this region among flaviviruses. Further work should be done to determine if other related flaviviruses cause similar lesions, and to compare the pathology of JEV to WNV and its behavioral outcomes. Some work has been done, but most of this focused on using JEV as a model for PD[259,261,513].

One important factor to consider is that finding viral RNA does not correlate with finding live, replicating virus. A past study did find that persistent, replicative WNV was

relatively uncommon, but that persistent viral RNA was more common[145]. This is consistent with the current study's finding that most mice are RNA positive. The most likely explanation is that the virus persists as RNA like other RNA viruses, most notably MV, often as subgenomic particles.[434] WNV does form subgenomic RNA during its lifecycle, which plays a role in evasion of the innate immune system[514]. While it is possible that this can persist in the CNS, the current studies indicate that the most commonly found persistent viral RNA is of a different sort. The current studies used primers targeting the envelope gene of WNV, which is located in the first third of the coding region of the genome. The subgenomic RNA leaves 525 nucleotides of the 3' untranslated region, which the testing here would not detect. It would be worth investigating the mechanisms WNV uses to control replication. MV replication is controlled by the innate immune response in a chronically infected mouse model[434], and innate immunity may play a similar role in WNV. This would be consistent with the finding that TNF-α tends to be elevated in the hindbrains of mice with persistent viral RNA in the same region. Other work has shown that lack of innate immune molecules such as STING increases mortality, but also increases the inflammatory cell infiltration[498], leading to phenotypes similar to those found in this study. This indicates that the initial immune response likely plays a major role in clearance and prevention of further damage. It is difficult to determine where WNV could persist in the cell. During replication, WNV uses the endoplasmic reticulum as a membrane platform for the viral replication complex[515]. The NS4B protein of WNV initiates the formation of membrane structures in the endoplasmic reticulum, which contain both viral RNA and the replication complex. These structures could serve as reservoirs for persistent, non-replicating viral RNA, allowing the virus to persist and avoid detection by the innate immune system.

Whether WNV is able to reactivate is not yet strongly established. However, past work indicated that transient immune suppression using cyclophosphamide in mice one month post-infection led to an increase in infectious WNV without any changes in clinical

scoring or levels of WNV RNA in the brain[145]. This indicates that the virus can persist and become reproductive long after initial infection, which may explain why some patients develop new signs over time, and could be further tested using this survival model.

In these studies, the hindbrain included the pons and medulla, but excluded the cerebellum as a separate region. The pons serves as a connection to the cerebellum, and acts as a relay between the cerebellum and forebrain. Both the pons and medulla generally control vital functions such as respiration, cardiac function, sleep, and swallowing. The increased susceptibility of these vital regions would indicate that there should be serious changes in affected mice, but we did not find this in any of the behavioral tests. This is likely for two reasons: (1) that the mice that had severe disease of the hindbrain succumbed to WNV infection during the acute phase, and (2) that the neurobehavioral testing focused more on other regions of the brain. Serious damage to the hindbrain would make it difficult for an animal to survive without intensive care, so the mice that survived must have either not suffered severe damage or been able to adapt to the changes.

Viral RNA was least commonly detected in the hippocampus, which is consistent with past research finding that persistent viral RNA in the hippocampus was generally low, and did not correlate with memory loss[162]. Because the entire hippocampus was homogenized, it indicates that the hippocampus is not amenable to persistent viral RNA. This is surprising given that it is one of the only sites that has neurogenesis in adulthood but may be representative of a unique immune response in the region. Past work has shown that it is susceptible to long-term changes post-WNV infection without any form of viral persistence[145], which could indicate bystander effects from inflammation in other regions of the brain.

The viral RNA detected in the human patient indicated similar regions of persistence as the microglial lesions found in the mice, most notably the pontine nuclei. The pontine nuclei (found in the anatomic hindbrain) was one of the most common sites to find microglial nodules and was within the larger anatomic region in which we most

commonly found viral RNA. The pontine nuclei are a major connection between the cerebral cortex and the cerebellum, integrating information from the cortex to the cerebellum, meaning these regions play a crucial role in complex motor activities and motor learning[516,517].

One finding of particular note was the association between viral RNA persistence and inflammation in the hindbrain in particular. Of the mice that had viral RNA detected in the hindbrain, 83% (10/12) had microglial lesions in that region. This did not correlate in the opposite direction, however, as only 48% of the mice with microglial lesions (10/21) had persistent viral RNA. The association continued further, as mice with persistent viral RNA in the hindbrain tended to have higher levels of TNF-α than mice without persistent viral RNA, and the presence of microglial lesions is not associated. This indicates that although inflammation can persist in the hindbrain without viral RNA present, it does not produce the same levels of TNF-α. Microglia are one of the major sources of TNF-α in the CNS, though subpopulations of neurons produce it constitutively[518] and it can cross the BBB through a soluble transport system[519]. There were no mice that had both viral RNA and microglial lesions in the cortex. This could indicate that the cortex is particularly prone to maintaining the virus without any inflammation, or that it clears the virus efficiently but produces severe inflammation when it does.

G331A STUDIES (SECTION 6)

The studies using the G331A strain of WNV were designed to compare how wild-type WNV inoculated peripherally acts differently than an attenuated strain inoculated directly into the brain. This model would serve to compare and contrast different attenuated strains of WNV inoculated intracranially to determine if the mechanism of attenuation affected long-term outcomes. These studies could also be used to determine how intracranial inoculation of WNV affected the long-term outcomes and inflammatory changes of mice when compared to peripheral infection.

The acute phase of infection had two major findings. The first was that 6-week-old mice showed reduced mortality compared to the previously tested 3-to-4-week-old mice. This was similar to the findings in C57/Bl6NTac mice. Inoculation with the G331A strain led to 30% mortality, while previous studies had found that infecting mice with the G331A strain could induce up to 60% mortality. The other prominent finding was that the inflammatory lesions found in these mice were similar to those found in the peripherally inoculated model. This indicated that in severe cases of WNV infection, the route and attenuation did not seem to affect the inflammatory state of the brain. Mice that were euthanized during the acute phase were not analyzed for the previously reported compensating mutations. This could be a future goal to determine if this level of inflammation only occurred in mice with these mutations, indicating that the unmutated G331A strain is incapable of causing profound inflammation.

The behavioral aspect of the study was frustrating but educational. In the future, behavioral testing will need to be performed primarily in an inbred strain like C57BL/6NTac mice to streamline testing, reduce animal numbers, and reduce unexpected behaviors. This will require characterizing the G331A strain in C57BL/6NTac mice prior to behavioral studies to determine the proper age of mice and dose of virus.

We did not find any significant differences between uninfected and G331A-infected mice in behavioral testing outcomes. This could be due to the fact that these studies used fewer mice than the NY99 studies, but the number of mice should have been sufficient to show some difference. The more likely explanation is that the greater variability in individual performance from using outbred mice overpowered any neurological differences. It has been shown previously that the G331A strain is less pathogenic, and our results indicate this as well. Further work will be needed to determine if it persists to the same degree as NY99 WNV, and if it causes the inflammatory lesions.

GENERAL THOUGHTS

Neuroscience is still defining neuronal circuits that account for different behaviors, and each behavior requires the input of multiple brain regions. The brain is highly dynamic, despite the general lack of new cell development in most regions. Neurons change activity regularly in response to signals from other neurons and glial cells, and many of the neuronal circuits have redundant functions or can adapt to damage. These factors make it imperative that behavioral studies have stringent controls.

These studies were aimed at determining if WNV infection led to consistent viral persistence, inflammatory changes, and behavioral changes in mice. This model introduced the complication of using a virus that spreads to the brain via multiple means, and infects multiple regions, leading to inconsistent damage to the brain. In human patients, this manifests as a spectrum of sequelae, and so we needed to approach this work using that as our basic assumption. One aspect that was considered during development was evaluation of individual mice for neurological changes, and correlating these with inflammatory or viral data. One issue with this was determining what constituted an abnormal neurological finding. The behavioral tests used in these studies have been validated and shown to be sensitive in detecting changes in neurological function, but requires the use of stringent negative controls in all studies to account for variations in handling, differences in mouse populations from different vendors, and other factors that can affect behavioral outcomes. Given the variability that can be found between studies, it is impossible to establish normal values for behavioral tests with mice based on data acquired from other research. Individual mice will vary their responses from day to day as well. While the rotarod and 2WAA data look like interpretable curves, individual mice did not show the same trends. The behavioral tests also require the involvement of multiple brain regions, one or more of which could be responsible for leading to the observed behavioral changes. The variability in inflammation, viral RNA persistence, and distribution of damage among the WNV-infected mice likely accounts for the variable outcomes in these behavioral tests. Attributing the behavioral changes to individual changes is confounded by the presence of

multiple changes in mice that could explain the deficits. The neuronal pathways responsible for the studied behaviors pass through multiple regions and can be affected by multiple factors, so attributing the behavioral changes to specific lesions is difficult without doing more in-depth analysis. The ideal solution would be to establish normal values for uninfected mice and to use these as a metric for determining which mice are deficient in each behavior, similar to the analysis performed on the data from the 2WAA. The model presented here does show consistent changes in terms of inflammation and viral RNA persistence, which do correlate with some behavioral changes. Memory loss does not show a correlation with viral RNA persistence or inflammation, and further work is needed in this model to study what changes occur in different regions leading to the different behavioral outcomes.

The study using outbred mice inoculated with G331A showed some important differences. The most important finding was that outbred mice show much greater variability in testing than inbred mice. This makes testing more difficult and necessitates larger groups of mice to get statistical rigor. It also necessitates using a small group of outbred mice on any behavioral test ahead of time to ensure that they will comply with the testing. The 2WAA and rotarod both showed different behaviors that were not seen when using the C57Bl/6Tac strain.

The variability in behavioral testing using outbred mice indicates another difficulty in a potential model we had discussed. We had considered using mice from the Collaborative Cross project to model neuroinvasive viruses that lack a good mouse model. These models had been considered for multiple reasons: Collaborative Cross show changes in susceptibility to viral infections that can lead to increased survivability, and the recorded differences in the genomes of each strain can be used to determine genetic determinants of disease[520]. We had specifically discussed using Collaborative Cross mice as models of the flaviviruses of the tick-borne encephalitis complex of viruses. The mice from the Collaborative Cross project come from a series of interbred strains of mice, including

outbred and wild strains of mouse. After communicating with other researchers who have used the Collaborative Cross mice, I believe it would be nearly impossible to perform behavioral testing on these strains. Many are more active and less predictable than normal outbred mice, and so it seems highly unlikely that they would cooperate with many of the tests.

Chapter 8: Conclusions and Future Directions

This thesis presents a novel rodent model of non-lethal WNV infection that provides new insights into the pathogenesis of long-term WNV-induced neurological sequelae. The studies reported here have established that peripheral WNV infection causes long-term inflammation and changes in the morphology of astrocytes and microglia, and that microgliosis in specific regions correlates with specific behavioral changes. The inflammatory changes can occur in most of the brain regions examined, though there was tropism for the hindbrain and forebrain. This needs to be further correlated with human cases and samples but could indicate areas for future study. This model mimics human disease in that there is a spectrum of outcomes, which as seen in human cases. Persistence of viral RNA in all brain regions was also demonstrated, though it was most commonly detected in the hindbrain. The mechanisms by which the viral RNA persists, and whether low levels of infectious virus are present in these brain regions, should be further studied. Persistence of viral RNA in multiple brain regions from a severe human case of WNND months post-infection were demonstrated as well, indicating that this model reproduces at least part of the disease process.

This model provides numerous possible outcomes to measure for interventions, including measuring microgliosis, astrocytosis, cytokine levels, persistent viral RNA, or specific behavioral outcomes. Depending on the therapeutic tested, any or all of these could be used as outcomes to determine viability of the therapeutic. The microgliosis and astrocytosis are the most noticeable changes in the brain, and probably the best output, as they represent inflammation in the brain, which could be pathologic. Therapeutics targeted at reducing neuroinflammation post-WNV infection could use these as metrics. This type of study should use measurement of virus or viral RNA in the brain at the same time, in case reduction of inflammation leads to reactivation of the virus. The behavioral data could be used as outputs for treatments aimed at specific neurological dysfunction, such as the

NS5 mutant models looking specifically at memory loss. These would be the most difficult types of studies, as the behavioral outcomes were not as consistent as the inflammatory or viral data.

The disadvantage of this mouse model is that it is less predictable than other models, and it is not as amenable to using transgenic mice. Virus distribution within the CNS appears to be less predictable in human cases, so this model is closer to the actual human disease. This model is more accurate than the previously published NS5 mutant WNV model, in that it more closely mimics human disease and infection. This model allows for a normal immune response, allowing for a better understanding of viral persistence and how inflammation can potentially be pathogenic. I believe it should be used more commonly than the NS5 model, and the NS5 model should be reserved for cases when the distribution of the virus needs to be controlled or when using knockout mice that will not survive peripheral infection with wild-type WNV.

FUTURE DIRECTIONS

The work described here presents the development of a model and characterization of initial findings in this model, and there are still questions to be answered using this model. The most obvious would be further characterization of the inflammatory and histopathological changes seen in this model, with further correlation to behavioral and functional changes post-WNV infection. Further characterization of the microglia and astrocytes in the brain to determine what phenotype and activity they are performing, as well as of the cytokines produced in each brain region, would help clarify the inflammatory state that occurs. Cell sorting to allow for characterization of microglia and astrocytes in each region would also help answer many of these questions.

One direction that is worth investigating is the role of α-synuclein in virus-induced neurological sequelae, particularly with WNV. α-synuclein is a presynaptic protein that has been linked to NDs, including PD[180]. It contributes to the pathology of these diseases by

forming toxic aggregates of proto-fibrils that disrupt normal neuronal activity internally and by inducing aggregation of these fibrils in neighboring cells when it is secreted. Other viral infections, including HIV[521], hepatitis C virus[522], and Epstein-Barr virus[523], have previously been associated with an increased risk of development of PD, indicating that there may be a common pathogenic mechanism. Human cases of WNV commonly manifest PD-like symptoms, indicating that similar brain regions may be affected[53,524]. The most compelling argument that there may be a link is that in a mouse model of WNV, α-synuclein was found to be upregulated in response to neuroinvasion[182]. This appeared to be protective, as mice lacking the α-synuclein gene were more susceptible to infection. This indicates that α-synuclein likely plays an anti-viral role in the brain but becomes pathogenic in response to chronic upregulation. Further work should look into how expression of α-synuclein changes throughout the time course of disease in different regions of the brain in our mouse model. This can be compared to the inflammatory nodules and persistence of viral RNA and can be used to map inflammation and neurodegeneration.

WNV has been shown to cause the accumulation of misfolded proteins in neurons, both *in vitro* using a neuroblastoma cell line, and *in vivo* using mice[50]. More recent research has shown that the WNV capsid protein specifically induces this accumulation[51]. The C protein interacts with AMP-activated protein kinase (AMPK), and causes AMPK to be ubiquitinated and degraded. AMPK regulates the formation of autophagosomes, which are crucial for the clearance of misfolded, ubiquitinated proteins[525]. This indicates a further connection between WNV and NDs, though there has not been a connection between the specific proteins associated with NDs and WNV. There has been one recorded case of a WNV patient with a neurofibrillary tangle made of abnormal phosphorylated tau protein[170], though whether viral infection caused it was not determined. Further research into the autophagosome and its role in WNV-induced neurological symptoms is warranted.

The indoleamine pathway has been implicated in NDs and neurocognitive disorders, including HD, ALS, and PD. IDO is the rate-limiting enzyme in the kynurenine

pathway, which produces metabolites for the immune response and neurotransmission. IDO is regulated through inflammatory cytokines such as IL-1β and TNF-α, and plays a role in neuronal reactions to inflammation. Because IDO has is dysregulated in other viral infections of the brain and correlates with behavioral changes, it is worth investigating in the context of WNV infection and associated neurological changes. Analysis could be performed through RT-PCR of *IDO* levels in each brain region, enzyme-linked immunosorbent assay of IDO from each brain region, or IHC targeting the IDO protein.

The finding that WNV infection induces memory loss is consistent with previous work but was not correlated with either viral RNA persistence or inflammation in different regions of the brain. The mechanisms seen in other work, including the elimination of synapses, reduction of neurogenesis, and infiltration of T cells, should all be examined in this model. In addition, measurement of LTP should be considered. LTP is a measure of synaptic and dendritic changes in neurons, specifically of the hippocampus. LTP can detect changes that cannot be seen with histopathology, and it can indicate cellular level changes within neurons that can account for neurocognitive deficits. There were no apparent changes to the hippocampus in this model, but other research has indicated that WNV infection can cause changes to memory[162–164], so LTP should be investigated in the hippocampus of mice. This will require significant investment, as LTP measurement requires unfixed brains, and so analysis will require dedicated equipment in the BSL-3 environment.

Full imaging of infected brains is warranted to characterize the inflammatory lesions that WNV induces. Although distinct lesions were identified within particular brain regions, the full extent of these lesions was not explored because we were limited to single slices. Full Clear Lipid-Exchanged Anatomically Rigid Immunostaining-compatible Tissue hYdrogel (better known as CLARITY) imaging to stain for GFAP, Iba1, or CD3 would allow for better visualization of the lesions and would be more sensitive in detecting lesions in other areas of the brain. CLARITY is substantially more expensive and time-

consuming than IHC, but it gives a greater understanding of the damage to the brain and puts it into a three-dimensional context.

Further investigation into the mechanisms of WNV RNA persistence in the brain needs to be performed. We had difficulty determining if there was viral protein in the brain because the antibody we used was not as sensitive as the qRT-PCR. One of the easiest methods would be to perform deep sequencing of the separate regions already dissected out from the mice, or repeating the study and screening the different regions via qRT-PCR. Any positive regions could be specifically selected for deep sequencing analysis, possibly with enrichment of the viral genome to improve sensitivity. This would allow for full mapping of the viral genome in those regions and analysis of viral population and quasispecies. If the viral RNA is persisting as truncated RNA similar to MV, this analysis would clearly show truncation and what regions are truncated. WNV has been shown to form noncoding, subgenomic RNA, which plays a role in its evasion of the innate immune system[514], but this would not have been detected via the methods used in these studies. Related flaviviruses including tick-borne encephalitis virus[526,527] and Murray Valley encephalitis virus have been shown to persist *in vitro* with large deletions that allow for attenuation. Deep sequencing should be performed to determine how much of the viral genome persists and if there are consensus genome changes or shifts in quasispecies leading to a more persistent phenotype.

Another aspect of infection that should be studied is localization of virus to specific cell populations. We had aimed to do this using RNAScope and co-staining with GFAP, Iba1, or NeuN to look for overlap and determine which cells that virus was persisting in. This did not work because RNAScope was prohibitively expensive and required multiple optimization steps to be used in conjunction with other IHC targets. This method would have also required confocal microscopy to confirm co-localization of the targets, which would necessitate the use of fluorescent IHC. There are other ways to study this. Fluorescent-activated cell sorting is recommended, followed by analysis of cellular

targets[528–530]. This technique can be used to separate cells based on internal markers, including markers for WNV. These cells could then be examined for cell markers specific to different types of cell, determining which cells the virus infects and potentially what cells it persists in.

One important aspect that this model could be used for is to determine whether candidate vaccines prevent neurological damage. Many vaccine trials focus on protection against acute disease. Given the findings that many of the mice have persistent RNA or inflammation more than one month post-infection, this should be included in post-mortem analysis for vaccine efficacy. These studies show that survival of the acute phase does not preclude mice from developing inflammatory lesions in the brain or from viral RNA persisting in the brain. There was a correlation between persistent viral RNA and elevated TNF-α in the hindbrain, indicating an ongoing inflammatory response. These findings indicate that analyzing vaccines based on survival alone would miss some possible long-term changes that WNV can cause and the effects these changes may have on patient quality of life. Future vaccine candidates against WNV should analyze the brains of animal models post-infection for viral RNA persistence and inflammation to determine efficacy. These tests would be important to ensure that vaccines protect against all stages of WNV infection, and not just survival during the acute phase. If a vaccine prevents serious disease but still allows for some neuroinvasion, this could leave the patient with persistent virus in the CNS, or it may initiate a neuroinflammatory cascade even without neuroinvasion. The model presented here shows that viral persistence is not necessary for the maintenance of neuroinflammation, and this should be considered as an output for any future studies.

The studies presented here focused exclusively on the brain as the site of damage and behavioral changes. Past work has shown that WNV often affects the spinal cord in humans and rodents, and that the virus can persist there longer than in the brain[145]. Further work should be done to correlate motor changes with damage to the spinal cord, as well as to see if the findings presented here correlate with changes in the spinal cord.

Other mechanisms for behavioral changes should be investigated using this model. General changes including inflammation and viral RNA persistence were investigated, but work using the attenuated WNV and other viruses have shown many other possible mechanisms that could be investigated using this model. A study should be performed to confirm that the changes found when the NS5 mutant model is inoculated IC occur in this model to ensure accuracy of that model. The NS5 mutant IC model may represent specific outcomes in patients surviving WNV infection, and there may be other mechanisms involved. Multiple models will be needed to replicate the possible outcomes of WNV infection, and to test potential therapeutics intended to minimize or reverse neurological damage caused by the virus and/or the host inflammatory response. Further research using the NS5, G331A, and other attenuated models should be done to determine how these could serve as more reproducible models of specific aspects of WNV-induced neurological disease, with the peripheral NY99 WNV model being used as a baseline.

The findings in these studies shows correlation with the findings in human patients after WNV infection, and more should be done to compare the two. The studies here showed that WNV persists in the CNS after the acute phase of infection and that it leads to long-term inflammatory changes. These findings should be compared in human patients reporting long-term sequelae, similarly to the study correlating MRIs in patients with memory loss[96]. Our studies showed the WNV RNA can persist in multiple regions of a patient's CNS months post-infection, which should be further investigated as a possible mechanism of WNV-induced neurological sequelae.

Ultimately, I hope this model will be used going forward as a model for human disease. Much more work needs to be done in human patients to establish the translatability of rodent models, but given our own findings, I am optimistic that this model represents human disease.

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	Graduate School of Biomedical Sciences
	Department of Microbiology and Immunology
	The University of Texas Medical Branch, Galveston, TX
08/2011 to 05/2015	**Doctor of Veterinary Medicine**
	Colorado State University, Fort Collins, CO
08/2005 to 05/2009	**Bachelor of Arts**
	Department of Molecular, Cellular, and Developmental Biology
	University of Colorado, Boulder, CO

Professional Experience

Beasley/Bente Lab, *Graduate Research Assistant,* Galveston. TX **August 2015-present**

- Developed and characterized a mouse model of West Nile virus-induced neurological damage.
- Performed inoculations, clinical scoring, euthanasia, and necropsy on mouse models in biosafety level-3 settings.
- Optimized behavioral testing for use in evaluating the effects of viral infection in biocontainment.
- Performed post-mortem testing on animal tissues including immunohistochemistry, multiplex cytokine analysis, and nucleic acid quantification.
- Trained graduate students, post-doctoral fellows, and staff veterinarians in procedures inside and outside of biocontainment.
- Wrote grants in F31, R01, and R21 formats and maintained steady, independent funding for duration of project.

Bowen Lab, *Laboratory Assistant,* Fort Collins, CO **August 2012-May 2014**

- Assisted with the inoculation and preparation of rodent models for infectious diseases.
- Examined, clinically assessed, and euthanized cats and ferrets in ongoing research on rabies vaccines.
- Handled multiple animal species for work in biocontainment, including bats, goats, and horses.
- Became proficient with equipment and procedures for use in an animal biosafety level-3 lab.

Redstone Animal Hospital, *Veterinary Assistant,* Littleton, CO
January 2011-June 2011

Coal Creek Animal Hospital, *Veterinary Assistant,* Centennial, CO
May 2009-July 2010

- Instituted new records systems to improve efficiency in record-keeping and retrieving. (**Redstone)**
- Developed a program of recycling analog radiographs in order to recoup some of the costs of switching to digital. (**Redstone**)
- Monitored vital signs during anesthetic protocols, and administered drugs during anesthesia.
- Restrained animals and assisted veterinarians during exams.
- Assisted with basic medical procedures, including radiography, anesthetic induction, and medical treatments.
- Developed communication skills via answering phones, relaying medical information, and assisting clients.

CU-Boulder Dept. of Ecological and Environmental Biology, *Research Assistant,* Boulder, CO **May 2008-July 2008**

- Trapped prairie dogs for research on plague and population dynamics.
- Processed prairie dogs, including tissue collection, anesthesia administration, and blood draws.
- Extracted DNA from collected fleas for diagnosis of *Yersinia pestis*

Trainees and Supervision

Animal Biosafety Level-3 Trainees

02/2018-03/2018	Richard Chichester, DVM, PhD	Laboratory Animal Veterinarian
02/2018-05/2018	Lisa Cisneros, PhD	Post-Doctoral Researcher
07/2017-05/2018	Isolde Schuster, DVM, PhD	Post-Doctoral Researcher

Biosafety Level-3 Trainees

05/2018-06/2018	Chao Shen, PhD	Post-Doctoral Researcher
10/2019	Gabe Haila, BS	Graduate Student
10/2019-Present	Nicole Lloyd, BS	Graduate Student

Behavioral Testing in ABSL-3 Trainees

02/2019-03/2019	Emily Mantlo, BS	Graduate Student
02/2018-05/2018	Lisa Cisneros, PhD	Post-Doctoral Researcher
07/2017-05/2018	Isolde Schuster, DVM, PhD	Post-Doctoral Researcher

Graduate Student Trainees

10/2018-11/2018	Christopher Read, BS	Graduate Student
03/2019-04/2019	Gabe Haila, BS	Graduate Student
01/2019-03/2019	Nicole Lloyd, BS	Graduate Student

Undergraduate Research Trainee

06/2017-08/2017	Isabel Ott	Summer Neuroscience Undergraduate Research Program Student

Externship Experience

Texas A&M University Bench-to-Shop Program **October 2017-August 2018**
Locations: Texas A&M University College of Veterinary Medicine & Biomedical Sciences, College Station, Texas; University of Texas Medical Branch, Galveston, Texas; Onderstepoort Veterinary Institute, Pretoria, South Africa

- Represented UTMB as a veterinarian and infectious disease researcher in outreach to other universities
- Learned from experts in industry, research, and animal health about the development, testing, licensure, and production of therapeutics targeting infectious diseases.
- Examined and performed necropsies on animals infected with African swine fever, foot-and-mouth disease, and lumpy skin disease.

Centers for Disease Control and Prevention Epidemiology Elective
January-February 2014
Location: CDC Division of Vector-Borne Diseases, Fort Collins, Colorado

- Learned about epidemiology, disease control, public education, and the CDC's role during the Chikungunya outbreak of Central and South America.
- Collected, analyzed, and reported on the efficacy of the yellow fever vaccine 17D, the data for which was included in a report to the National Vaccine Advisory Committee.
- Educated travelers to the U.S. Virgin Islands about the risk of Chikungunya infection
- Collected and analyzed data on traveler awareness and preparedness for Chikungunya prevention

Smith-Kilborne Program **May 2013-June 2013**
Location: Plum Island Animal Disease Center of New York, Orient Point, New York

- Selected as the CSU representative for a federal program centered on foreign animal disease control, prevention, and intervention
- Learned from experts about foreign animal diseases, epidemiology, and public communications.
- Observed foot-and-mouth disease, African horse sickness, and Exotic Newcastle disease in live animals while on the Plum Island Animal Disease Center.

Academic and Professional Honors

09/2019-present	*T32 for Neglected Tropical Diseases,* University of Texas Medical Branch
08/2016-present	Student Member, American Association for the Advancement of Science
08/2015-present	*Presidential Scholarship*, University of Texas Medical Branch
12/2019	*Mason Guest Scholar Program Award*, University of Texas Medical Branch
08/2017-08/2019	*McLaughlin Fellow*, McLaughlin Endowment, University of Texas Medical Branch
10/2017-07/2018	*Bench to Shop Selected Attendee*, Texas A&M University
12/2017	*Steunebrink Scholarship Endowment Award*, University of Texas Medical Branch
09/2016-09/2017	*Member,* Charles Louis Davis and Samuel Wesley Thompson DVM Foundation for the Advancement of Veterinary and Comparative Pathology
05/2013	*Smith-Kilborne Program Selected Student*, Colorado State University
08/2012-08/2013	*Thomas F. Spurgeon Memorial Scholarship*, Colorado State University
01/2012, 01/2014	*Veterinary Student Day at the CDC Selected Student,* Colorado State University

Publications

Fulton, C., Dörr, M., Campbell, G., Beasley, D., Bente, D., Dineley, KT. 2020. A Mouse Model of WNV Shows Persistent Viral, Behavioral, and Inflammatory Changes in the Brain. *Brain, Behavior, and Immunity- Health. In preparation*.

Fulton, C., Beasley, D., Bente, D., Dineley, KT. Long-Term, West Nile Virus-Induced Neurological Changes: a comparison of patients and rodent models. *Brain, Behavior, & Immunity – Health*. In Press, pre-proof.

Rodriguez R, Rincon L, Yassin A, Campbell GA, Peng B-H, **Fulton, C.**, Sarria, J., Walker, D., and Fang, X. 2019. Encephalomyelitis Resulting from Chronic West Nile Virus Infection: A Case Report. *J Neurol Exp Neurosci* 5(2): 48-51.

Cherry, C. C., Beer, K. D., **Fulton, C.**, Wong, D., Buttke, D., Staples, J. E., & Ellis, E. M. (2016). Knowledge and use of prevention measures for chikungunya virus among visitors—Virgin Islands National Park, 2015. *Travel medicine and infectious disease*, 14(5), 475-480. PMCID: PMC5079757.

Lindsey, N.P., Horiuchi, K.A., **Fulton, C.**, Panella, A.J., Kosoy, O.I., Velez, J.O., Krow-Lucal, E.R., Fischer, M. and Staples, J.E., 2018. Persistence of yellow fever virus-specific neutralizing antibodies after vaccination among US travellers. *Journal of travel medicine*, 25(1), p.tay108.

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This dissertation was typed by Corey May Fulton.