Journal of NeuroVirology, 12: 129–139, 2006

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280600758341

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Temporal analyses of the neuropathogenesis of a West Nile virus infection in mice

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A West Nile virus (WNV) infection in humans can produce neurological symptoms including acute flaccid paralysis, encephalitis, meningitis and myelitis. To investigate the pathogenesis of WNV in the peripheral and the central nervous system (PNS and CNS), the authors used a murine footpad inoculation model of WNV infection. Survival curves of virus-infected animals of ages 4- to 6-weeks-old demonstrated age-dependent mortality where older animals (6-weeks-old) had a higher mortality rate compared to younger animals (4- and 5-weeks-old). The mice that survived the virus infection formed WNV-reactive antibodies, confirming viral infection and clearance. The localization of viral RNA (vRNA) and antigen in infected murine tissues was investigated using TaqMan and immunohistochemistry (IHC) respectively. During a nine day infection, vRNA levels in the spinal cord and brainstem fluctuated, suggesting early viral clearance from these tissues by days 3–4 p.i. with later re-introduction. Viral antigens detected using IHC were primarily observed in three main regions of the brain: cortex, hippocampus and brainstem. Additionally, the dorsal root ganglion neurons of the PNS stained positive for viral antigens. These data are consistent with multiple routes of neuroinvasion following a peripheral inoculation of virus and do not preclude the previous observation that virus-infected peripheral neurons can introduce virus into the CNS by a retrograde transport mechanism. *Journal of NeuroVirology* (2006) **12,** 129–139.

Keywords: central nervous system; flavivirus; immunohistochemistry; neuron; mouse; peripheral nervous system; West Nile virus

Introduction

West Nile virus (WNV) is a member of the family *Flaviviridae* of arthropod-borne positive strand RNA viruses and was first isolated from a febrile woman in Uganda in 1937 (Smithburn *et al*, 1940). The virus is maintained in a natural cycle involving a mosquito vector and avian amplifying hosts with humans as dead-end incidental host. WNV belongs to the flavivirus serocomplex group that also includes the medically important St. Louis encephalitis (SLE), Japanese encephalitis (JE), Murray Valley encephalitis (MVE), and Kunjin viruses. Its genome consists of a single-stranded positive sense RNA of approximately 11 kb in length that encodes a single open reading frame. The synthesized polyprotein is composed of both structural (envelope and capsid) and non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). All of the non-structural genes assist with viral RNA synthesis at some level (Brinton, 2002).

WNV has recently emerged as an important public health problem in the United States. Since 1999 nearly 20,000 WNV human infections have been identified. Case-patients are frequently hospitalized with encephalitis, meningitis or flaccid paralysis (CDC, 1999; Sejvar *et al*, 2003). The diagnosis of flaccid paralysis in certain individuals prompted the term "poliomyelitis-like" symptoms associated with a WNV infection (Doron *et al*, 2003; Leis *et al*, 2003). Because the North American/Israeli (NY99) strain of WNV appears to be highly neurotropic, its neuroinvasiveness is an important aspect of human

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This work was supported by the American Society for Microbiology/NCID post-doctoral fellowship program. We would like to thank Dr. Nord Zeidner for his technical assistance with the immunohistochemistry.

Received 13 January 2006; accepted 8 April 2006.

disease. To investigate routes of neuroinvasion following peripheral infection with WNV, we used an *in vivo* mouse footpad inoculation model and studied the temporal progression of viral RNA and antigens through the nervous system. Characterizing the neuropathology of the NY99 WNV is important to determine whether it is more or less neuroinvasive than other strains of WNV (Lanciotti *et al*, 2002). Since cerebral edema or encephalitis in WNV-infected individuals can result in death, understanding the mechanism of virus entry into the central nervous system (CNS) and the susceptibility of neurons to viral infection is important.

Flavivirus access to the CNS is hypothesized to occur directly via the olfactory neurons or through a blood brain barrier (BBB) breakdown (Monath *et al*, 1983). Infection of the CNS via a peripheral route such as the olfactory neurons implies a retrograde transport mechanism as the mode of neuroinvasion from the periphery to the CNS. Since the initial inoculation of the virus by the mosquito occurs in the dermal epithelium, which is highly enervated by sensory neurons, peripheral introduction of the virus to the CNS might be a possibility. Furthermore, human neuropathology data obtained from WNV-infections detected virus in distinct regions of the brain including the medulla and thalamus, which are the first connections made from the peripheral nerves to the brain. Other abnormalities have also been found in the frontal lobe, temporal lobe, parietal lobe, hippocampus and cerebellum (Sampson and Armbrustmacher, 2001). In a WNV fatal case study, findings included myositis with T-lymphocyte infiltration of nerve fibers that suggested that the virus reached the CNS via peripheral nerves (Smith *et al*, 2004). Therefore, alternate CNS entry from the peripheral nervous system (PNS) to the brainstem involving retrograde transport of virus by the neurons may be an additional route for viral neuroinvasion, given that the structures of the brain most affected by WNV have direct connections from either the sympathetic nervous system or the sensory nervous system. Additionally, a study of four fatal cases involving WNV associated poliomyelitis-like symptoms demonstrated that the sympathetic and sensory nervous system in these patients were compromised suggesting that the virus infected peripheral nerves. In this study, the authors suggest that the damage to the sympathetic ganglia may explain the instability of the autonomic nervous system in certain patients (Fratkin *et al*, 2004). Moreover, since humans do not normally have a high titer or sustained viremia, it is less likely that neuroinvasion through the BBB breakdown occurs (Biggerstaff and Petersen, 2002).

Results presented here track the viral infection from the periphery to the CNS during a nine-day course of the infection in a mouse footpad inoculation model. This study correlates the presence of viral RNA in these tissues with viral antigen expression in order to understand which neuronal sub-types amplify viral RNA in the CNS and PNS. The presence of viral antigens in the DRG neurons of the PNS suggests that these specific neurons were infected and may contribute to the rapid introduction of the virus into the CNS. The results suggest that WNV accesses the brain through multiple routes, and that virus may be cleared from some neural tissue to be later re-infected.

Materials and methods

Virus strain

The New York 1999 (NY99) strain of WNV used in these studies was obtained from the reference collection at the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention (CDC). This WNV strain was isolated from an infected Chilean flamingo in the Bronx Zoo and was passaged once in suckling mice and twice in Vero cells (Lanciotti *et al*, 1999).

Mouse footpad injection model

C57B1/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 4, 5, and 6 weeks of age. The animals were anesthetized using isofluorane and infected with WNV via a footpad injection with approximately 1200 plaque-forming units (pfu) per animal. Viral stock solution was diluted to the appropriate concentration using 1% fetal bovine serum in sterile phosphate buffered saline. For survival curves, animals were monitored during the course of the experiment and euthanized when the animals displayed clear signs of morbidity. Each treatment group consisted of 10 animals. For viral RNA (vRNA) and immunohistochemistry studies (see below) time points used for tissue harvesting were assessed based on survival curve results. Tissue samples were collected from the spinal cord, ganglia and brain on days 1 through 9.

vRNA isolation and quantitation using TaqMan analyses

vRNA was isolated from virus seed and serum samples using a QiaAmp viral RNA kit (Qiagen, Valencia, California). Tissue samples from the nervous system including brain and spinal cord were weighed and homogenized using a dounce tissue grinder in the presence of lysis buffer from the Qiagen RNeasy kit according to manufacturer's instructions. The samples were frozen at -70°C for later processing. WN virus RT-PCR primers used to determine the viral RNA content in the tissue samples were previously described and were derived from the envelope gene sequence (Lanciotti *et al*, 2000, 1999).

Five microliters of the total RNA extracted from each sample was analyzed by the TaqMan assay. The reaction included 50 pmol of each primer and lOpmol of the FAM and TAMRA labeled probe using the one-step RT-PCR master mix (PE Applied Biosystems, Foster City, California) in a 50ul reaction

volume. The samples were cycled 45 times for amplification in an ABI Prism 7700 Sequence Detection System instrument (PE Applied Biosystems) according to manufacturer's suggested protocol for TaqMan assay RT-PCR cycling conditions. A standard curve determined from the viral seed NY99 was compared to a standard plaque assay from the same samples to calculate pfu/ml or pfu/mg based on cycle threshold values (Ct). Negative controls included non-template, uninfected animal tissue and serum.

Indirect immunofluorescence detection of WNV-reactive antibodies

Animals that survived the initial infection were euthanized and blood samples were tested for the presence of WNV reactive antibodies. The blood samples were centrifuged in microfuge plasma separator tubes and plasma samples were diluted 1:2 in phosphate buffered saline (PBS). The samples were added to a slide containing acetone fixed WNV-infected Vero cells. Following incubation at 37°C, the slides were washed three times with PBS and treated with a secondary antibody anti-mouse IgG conjugated to FITC (Jackson Laboratories, West Grove, PA) diluted in PBS at 1:200. Visual analyses of the presence of fluorescence for each sample were used as an endpoint. Controls included plasma from uninfected animals and PBS alone.

Immunohistochemistry

Brain and spinal cord samples of WNV-infected mice at various time points (3–7 days post-infection, p.i.) were harvested and immersion fixed in 10% formalin (Fisher Scientific, Houston, TX). The samples were paraffin embedded and coronal sections of 5 um were mounted on glass slides. The spinal cord samples were maintained in the vertebrate and the bone was decalcified using Fisher decalcification reagents. All tissue sections were deparaffinized using two washes in xylene and hydrated with subsequent washes in 100% EtOH (2×), 95% EtOH (2×) and PBS (2×). Following hydration, the tissue sections were permeabilized with 0.05% saponin (Fisher Scientific) for 30 minutes at room temperature (RT) and rinsed 3× with PBS. The tissue sections were blocked with 2% goat serum (GS) in PBS for 20 minutes and then probed with a 1:50 dilution of polyclonal antibody against WNV for 1 h at 37°C followed by three washes with PBS. Bound antibody was detected with anti-mouse IgG conjugated to peroxidase (Jackson Immuno Research). All antibody solutions were diluted in 1% GS in PBS. The substrate used to visualize viral antigens in the tissue samples was diaminobenzadine (DAB, Vector Laboratories, Burlingame, CA) which yields a brownish precipitate or fast red that yields a red precipitate followed by counterstaining with hemotoxylin and eosin (H&E).

Analysis of the brain tissue was performed using the delineated mouse brain stereotaxic coordinates (Franklin and Paxinos, 1977). Using these coordinates, the brain was dissected into four quadrants and sectioned to represent the 93 sections of 120 um representing the entire brain of each mouse. Each slice representing each of the regions of the brain was analyzed using the stereotaxic coordinate map to determine the exact nuclei of neurons pertaining to the region with positive staining. Control serial sections of the same brain were stained with normal ascites fluid as controls for each analyzed section to determine non-specific staining.

Results

Footpad inoculation model results in a 50% animal mortality rate in 6-week-old mice and survivors had WNV-reactive antibodies

Following footpad inoculation, the animals were observed daily for signs of disease (Figure 1). Four-week-old mice were refractory to symptomatic WNV infection via footpad inoculation. Five-week-old animals died at 12 and 13 days p.i. with a 20% mortality rate ($p = .01$ student t-test compared to untreated control animals). However, 6-week-old mice were most susceptible to WNV footpad inoculation and died from 7 to 10 days post-infection (p.i.) with a 50% mortality rate ($p < 0001$). Serum was obtained from survivors 6 weeks after the initial inoculation and analyzed for WNV-reactive antibodies. The survivors of the 4-week-old age group were also tested for WNV-reactive antibodies and eight out of the ten animals were positive (80%) indicating that even though they were asymptomatic, they were infected. Eighty-eight percent of the survivors from the 5-week-old age group had reactive antibodies to WNV (7/8 were positive).

The kinetics of vRNA in the CNS displayed a rapid infection with late involvement of higher order neural structures

The presence of virus in serum and tissues was quantified using TaqMan assay probes to detect

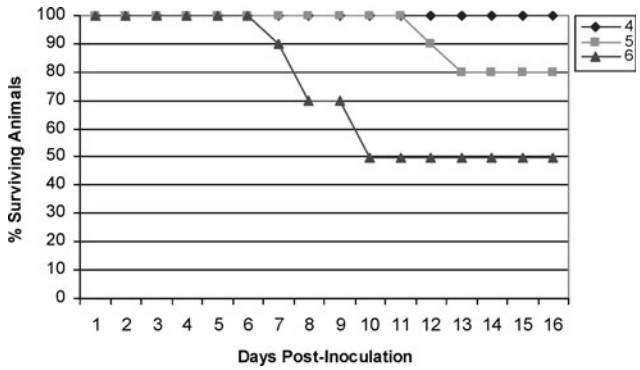

Figure 1 Survival curves of WNV infected C57B1/6J following footpad inoculation. Mice of 4, 5 and 6 weeks old were inoculated with WNV at 1200 PFU per animal and monitored for 16 days post infection. Each age group consisted of an $n = 10$ animals. (5 weeks $p = .01$, 6 weeks $p =< .0001$)

Table 1 The geometric means of the quantitative real time RT-PCR (TaqMan) measurements for vRNA in mouse tissue and serum

	Geometric Means (pfu/mg or pfu/ml)				
Days p.i.	*Spinal cord*	*Brainstem.*	*Cerebellum*	*Cortex*	*Serum*
1	1.7	10.5	1	2.2	34.99
2	88	2.1	1	0.84	959.32
3	1.1	0.99	0.35	4.6	4132.76
4	2.5	6.9	3.8	5.9	581.45
5	1.3	0.28	0.09	81.6	36.8
6	318	16.2	4.28	38.9	0
7	23080	5.27	0.58	65.6	0
9	nd	1253.7	157.1	4698.2	0

the gene encoding the structural protein E (Table 1 and Figure 2A–E). vRNA was measured from 1 to 9 days p.i. Virus was present in sera from days 1 to 5 p.i., with peak viral titer on day 3 (Table 1 and Figure 2A). In tissue on day 1 only low levels of vRNA (>2 pfu/mg) were detected except for the brainstem that contained the equivalent vRNA of 10.5 pfu/mg of tissue. At this time p.i., the average level of virus in the serum was 35 pfu/ml. On day 2 the mean virus in the serum was 959 pfu/ml and the highest level of vRNA in the tissue samples was detected in the spinal cord (88 pfu/mg) while all the other CNS structures were less than 2 pfu/mg. Day 3 marked the peak of viremia and the average virus in the serum was 4132 pfu/ml (Figure 2A). In tissues only the brain cortex expressed demonstrable but low levels of vRNA (4.6 pfu/mg) (Figure 2B–2E). On day 4, most tissues had demonstrable levels of vRNA, between 2–7 pfu/mg, and the serum contained 581 pfu/ml. Average vRNA in the serum on day 5 was 37 pfu/ml, and this day marked the end of the viremic phase. Significant tissue levels of vRNA at day 4 were identified only in the brain cortex (82 pfu/mg). On days 6 and 7 all the CNS tissues tested had elevated levels of vRNA with the highest level detected in the spinal cord. At 7 days p.i., the spinal cord reached an average

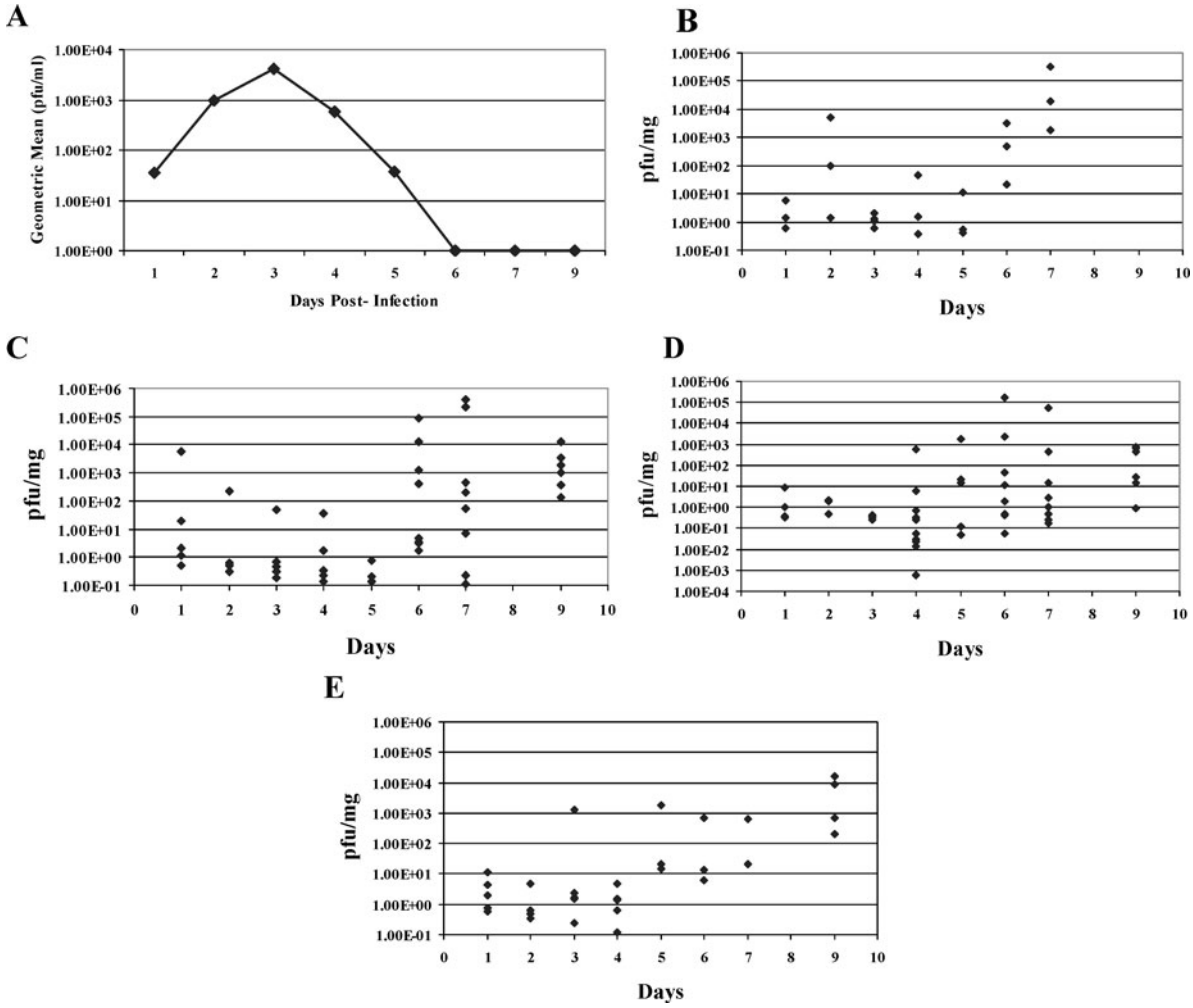

Figure 2 The presence of vRNA in the serum and CNS tissues of C57B1/6J mice infected via footpad inoculation was measured by TaqMan. Panels: **A**, serum (pfu/ml); **B**, cervical region of the spinal cord (pfu/mg); **C**, brainstem (pfu/mg); **D**, cerebellum (pfu/mg); and **E**, cortex (pfu/mg).

of 23,080 pfu/mg and the cortex, brainstem and cerebellum remained similar to the values observed after 6 days. All the samples obtained from the spinal cord were from the cervical region. Finally, on day 9 the average viral RNA in the cortex was 4698 pfu/mg, the brainstem was 1254 pfu/mg and the cerebellum was 157 pfu/mg. The peripheral nervous system was also tested for vRNA but the dissection to remove the dorsal root ganglion and sympathetic chain was variable because anatomically these structures are difficult to isolate without contamination from other tissues therefore it was not included in the final data analysis.

The kinetics of vRNA expression demonstrated that the cortex had a steady increase in vRNA over the period of the infection whereas the spinal cord and brainstem had similar fluctuations in vRNA expression with early introduction of virus in these tissues, apparent clearing of virus by days 4–5 followed by virus re-introduction on day 6. Additionally, the cervical spinal cord demonstrated the higher vRNA levels than any of the other CNS structures. The vRNA data supports a model of an early infection of the nervous system within the first 48 hours p.i. followed by virus clearance and a subsequent re-infection.

Histological analyses determined the susceptibility and association of specific brain structures with WNV infection

Paraffin embedded 5 um brain tissue sections were immunohistochemically stained for WNV antigen. The stereotaxic coordinates corresponding to the major structures of the brain was used as a guide to analyze the sections for positive staining corresponding to particular neuronal nuclei. A serial section was used as a control to identify non-specific staining. The main structures that expressed positive WNV antigen were the cortex, hippocampus, brainstem and DRG neurons of the PNS (Table 2).

Table 2 Distribution of WNV antigen in various structures of the CNS and PNS expressed as percentage of animals that had positively stained viral antigen

Nervous system structures	*% Infection*	*Days post-infection*[a]				
		3	*4*	*5*	*6*	*7*
Cortex	80	100	66	100	100	100
Piriform cortex	53	66	33	66	75	50
Entorhinal cortex	33	33	66	33	25	0
Hippocampus	81	33	66	100	75	nd
CA1	73	33	66	33	50	nd
CA2	27	0	66	0	25	nd
CA3	27	0	33	0	25	nd
DRG	20	0	33	33	25	0
Brainstem	66	0	0	66	75	50
Choroid plexus	53	33	33	100	50	33

[a]Percent of specimens positive for WNV antigen.

The PNS was positive for viral antigen expression in the dorsal root ganglion neurons approximately 20% of the time with the large neuron sub-types primarily infected on days 4–6 (Table 2). Samples were obtained from the lumbar spinal cord region. Not all animals tested positive in the DRG for viral antigen expression however when an animal tested positive a large percentage of neurons in the ganglion were infected (Figure 3). The large neuronal cells correspond to the propioception and discriminative touch and the small diameter neurons are normally involved in the pain pathways. These smaller neurons did not stain positive in this animal model.

The cortex was found to be positive in 80% of the samples tested and there was early infection of the cortex starting at 3 days p.i. (Table 2). Sub-regions within the cortex that expressed viral antigens included the entorhinal, somatosensory, piriform and ectorhinal cortex. The entorhinal cortex forms reciprocal connections with the hippocampus and various other cortical and subcortical structures and had WNV-positive stained cells from day 3–6 p.i. (Table 2 and Figure 4A). The piriform cortex has direct projections to the entorhinal cortex and stained positive at all time points tested (3–7 days) (Table 2 and Figure 4B). Occasionally, there was unilateral staining of the piriform cortex. The somatosensory region of the cortex was another region of the brain found to stain positive at all the tested time points. These neurons receive sensory input from the periphery through the medial posterior nucleus of the thalamus. One animal stained positive in the thalamic nucleus on day 6 p.i. (Figure 5A).

The hippocampus stained positive 81% of the time and the staining was mostly found in the CAl nuclear and dendritic region (Table 2 and Figure 6A, 6B). CAl region of the hippocampus was found positive at day 3 followed by the CA2 and dentate gyrus (Table 2). The involvement of CA3 occurred on day 4–6 where CAl, CA2 and CA3 were all positive for viral antigens (Table 2 and Figure 6C, 6D). The major input from the hippocampus is from projections via CAl to the subiculum which projects to the deep layers of the entorhinal cortex. The entorhinal cortex forms excitatory connections with the granule cells of the dentate gyrus of the hippocampus which connects to the pyramidal cells of the CA3 region of the hippocampus which projects to the CA1 region.

The brainstem stained positive for viral antigens 66% of the time with later involvement starting on day 5 through 7 (Table 2 and Figure 5A, 5B). The regions within the brainstem that expressed viral antigens included the medulla, pons, thalamus and trigeminal nucleus, including the sensory trigeminal nucleus and the trigeminal tracts. The thalamic nucleus is the first connection from the peripheral nervous system that receives sensory input.

The choroid plexus is an intraventricular structure composed of a single layer of ciliated, cuboidal epithelial cells and is responsible for the secretion

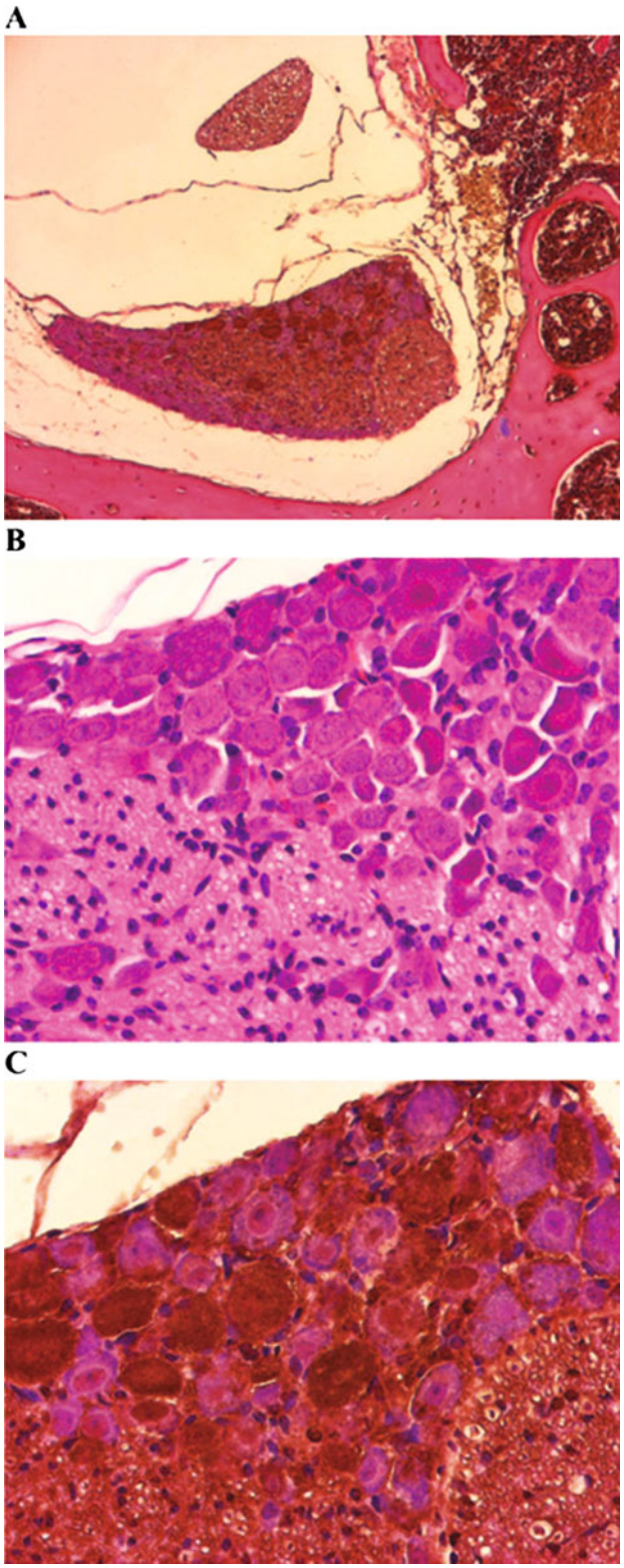

Figure 3 Immunohistochemistry of the PNS after infection with WNV demonstrating dorsal root ganglion neurons positive for viral antigen expression. **A**, dorsal root ganglion (magnification 20×); **B**, serial sagittal section incubated with normal hyperimmune ascites fluid as a negative control for non-specific staining (200×); **C**, serial sagittal section from the same tissue sample immunostained with WNV MHIAF (1:200) for detection of viral antigens (200×).

of cerebral spinal fluid. These cells stained positive 53% of the time for viral antigen however, the observed staining was mostly nuclear with less staining in the cytoplasm (Figure 7).

Consistent with the TaqMan vRNA data, the cerebellum rarely expressed viral antigens and upon expression consisted of very few cells that stained positive within each section. The cells that were found positive consisted of primarily Purkinje cells. Purkinje cells are directly connected to motor neurons of the spinal cord that control muscle contraction and movement.

Discussion

This is the first study to carefully analyze WNV replication in the central and peripheral nervous system by correlating detection of vRNA to the presence of viral antigen in specific neural tissues. The presence of vRNA in neural tissue does not necessarily prove viral replication. Therefore, the expression of viral antigens was measured by IHC as a better marker for viral replication. The IHC data also provides a temporal analysis of neuronal cell types in the brain capable of replicating viral RNA and expressing viral antigens.

The pathogenesis of a WNV infection via footpad inoculation differs from previously observed pathology following intraperitoneal infection of mice (Haahr, 1968). Following intraperitoneal inoculation of WNV NY99, the younger animals of 3–4 weeks of age were more susceptible to infection than older animals (6 weeks) with an average survival time of 8.6 days (Beasley *et al*, 2002). However, footpad inoculation is a peripheral route of infection more similar to the bite of a WNV-infected mosquito. In this model, the survival curves demonstrate that younger animals of 4 weeks old were less symptomatic than older mice of 6 weeks of age. Previous characterization of WNV footpad infection indicated a slightly higher mortality of 75% compared to our observation of 50% mortality (Diamond *et al*, 2003a). This model most closely resembles human infection because of the peripheral route of infection and the relatively low (10^3 pfu/ml) and short duration (maximum of 5 days) of the viremia.

The vRNA measured by TaqMan analyses showed that some of the tissues in the CNS expressed vRNA within the first 5 days of the infection. With encephalitic flaviviruses, the end of the viremic stage is often followed by the introduction of the virus into the CNS, however the sensitivity of TaqMan allowed us to detect low levels of virus prior to this point (Nathanson, 1980). In our studies, we observed vRNA in the brainstem at 1 day p.i. and in the spinal cord 2 days p.i. In the brainstem, the viral load diminished over the next few days to very low levels and later increased again on days 6 through 9. This observation suggests that parts of the nervous system is

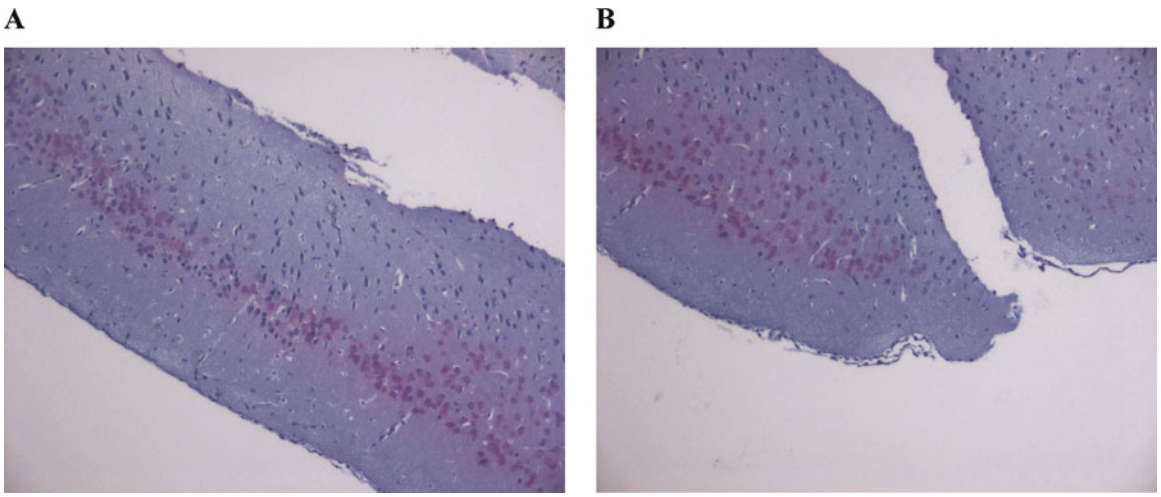

Figure 4 Immunohistochemistry of the cortex structures staining positive for WNV antigen. **A**, entorhinal cortex (100×, fast red, counterstained hemotoxylin); **B**, piriform cortex (100×).

infected early, clears the infection, and is re-infected at the later time point. A more gradual and steady increase in vRNA occurred in the cortex from day 3 to 9 p.i. There is statistical significance between the day of infection and the CNS tissue infected ($p = .003$ ANCOVA day by tissue interaction). This analysis found a correlation between the time of infection and viral load. IHC was used to determine whether vRNA measured by TaqMan correlated with viral antigen expression

Temporal analyses of brain tissue using IHC staining further elucidated the presence of viral antigen expression in specific nervous system structures. Viral antigens were expressed in large diameter dorsal root ganglion (DRG) neurons of the PNS that corresponds to the touch pressure pathway in the ascending sensory tracts and the muscle position sensory pathway (propioception). The DRG of the PNS enervates the dermal epithelial layer where the primary inoculation of the virus occurs. These DRG neurons may have been infected from the site of viral inoculation and retrograde transported the virus to the cell body. Previous studies on DRG neurons in culture indicated that these cells are susceptible to WNV and can maintain a non-cytopathic persistent infection in this neuronal cell-type (Hunsperger and Roehrig, 2005). A continual shedding of infectious virus may create a persistent and asymptomatic infection possibly explaining the observation of persistent IgM expression in humans for 500 days p.i. (Roehrig *et al*, 2003).

The large diameter DRG neurons, that expressed viral antigens, are associated with propioception and discriminative touch. These neurons send

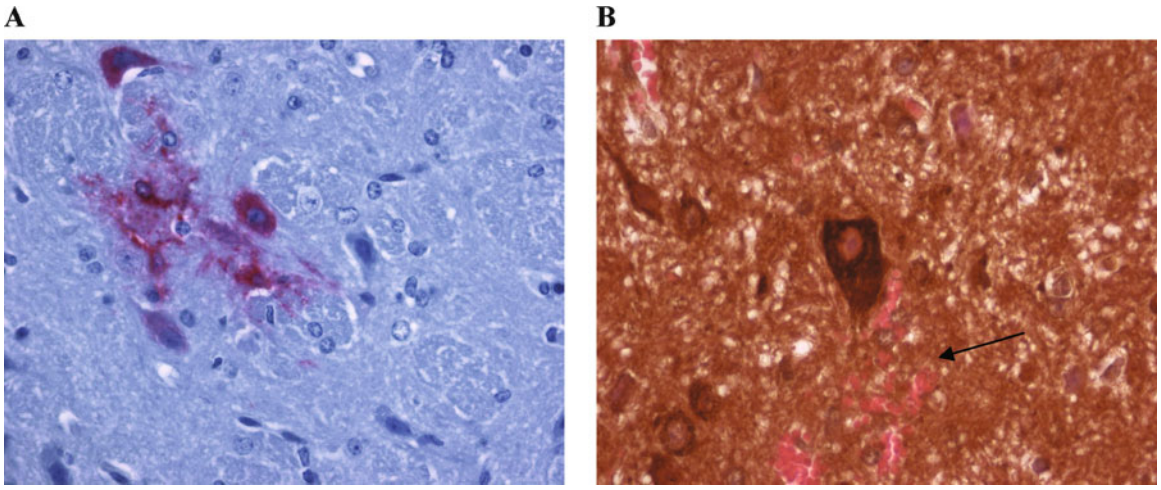

Figure 5 Immunohistochemistry of the CNS six days following a WNV infection. **A**, thalamus region of the brain with positive stained cells in red and counterstained using hemotoxyline; **B**, Infected neuron in the trigeminal nucleus of the brainstem. Arrow points to putative immune modulating cells surrounding infected cell (magnification 400×, DAB, counterstained H&E).

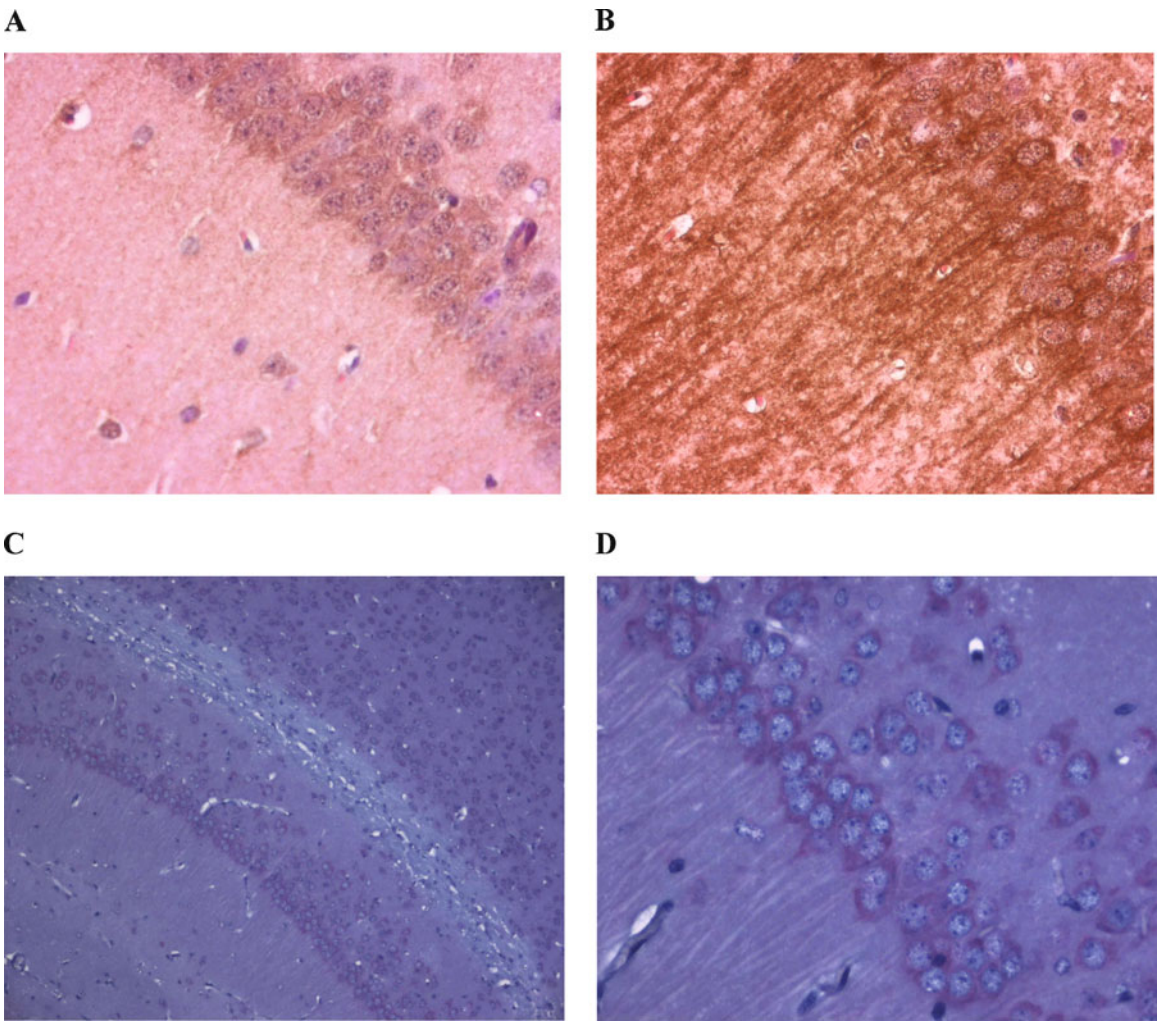

Figure 6 Immunohistochemistry of the hippocampus following WNV infection. **A**, hippocampus region of the brain treated with normal hyper immune ascites fluid as a negative control for non-specific staining; **B**, same section of hippocampus immunostained with MHIAF demonstrating cytoplasmic positive staining in the CA1 region in the neuronal cell bodies and dendrites (magnification 400×, DAB, counterstained H&E); **C**, hippocampus region staining positive for viral antigens in the cell bodies of the CA3 region (magnification 10×, Fast Red stain, counterstained hemotoxyline); **D**, same section magnification 100× of the same region demonstrating the red stained cytoplasmic region of the CA3 cell bodies of the hippocampus.

peripheral information to the somatosensory and piriform cortex where this information is processed. This sensory information ascends via tracks that synapse onto the thalamus. An animal that was positive for DRG staining also expressed viral antigens in the thalamic nucleus layer suggesting that infected peripheral neurons introduced virus to the somatosensory cortex via retrograde transport (Figure 5A).

The piriform cortex is involved in sensory information primarily from the olfactory neurons and has also been implicated in seizures (Hoffman and Haberly, 1991). Peripheral introduction of virus into the brain may involve the olfactory neurons whose connections terminate in the piriform cortex. The introduction of virus via the olfactory neurons have been hypothesized as a model for neuroinvasion for flaviviruses (Monath *et al*, 1983). The piriform cortex also communicates with the hippocampus and the sensory system which were also vulnerable to viral infection (Figure 4B).

In our studies, the hippocampus and the cortex region of the CNS appears to be consistently infected as early as 3 days p.i. Previous studies comparing flavivirus infection in rhesus monkeys and rodents determined the vulnerability of hippocampal neurons to viral infection (Nathanson and Cole, 1971). The hippocampus is an important structure of the brain for memory and learning. Together with the entorhinal cortex, these two structures are the basis of memory formation and retrieval. The entorhinal cortex receives input from the hippocampus and this connection forms an integral component of the medial temporal lobe memory system. Briefly, the entorhinal cortex inputs memory, the hippocampus is a memory addressing region and the subiculum is important for

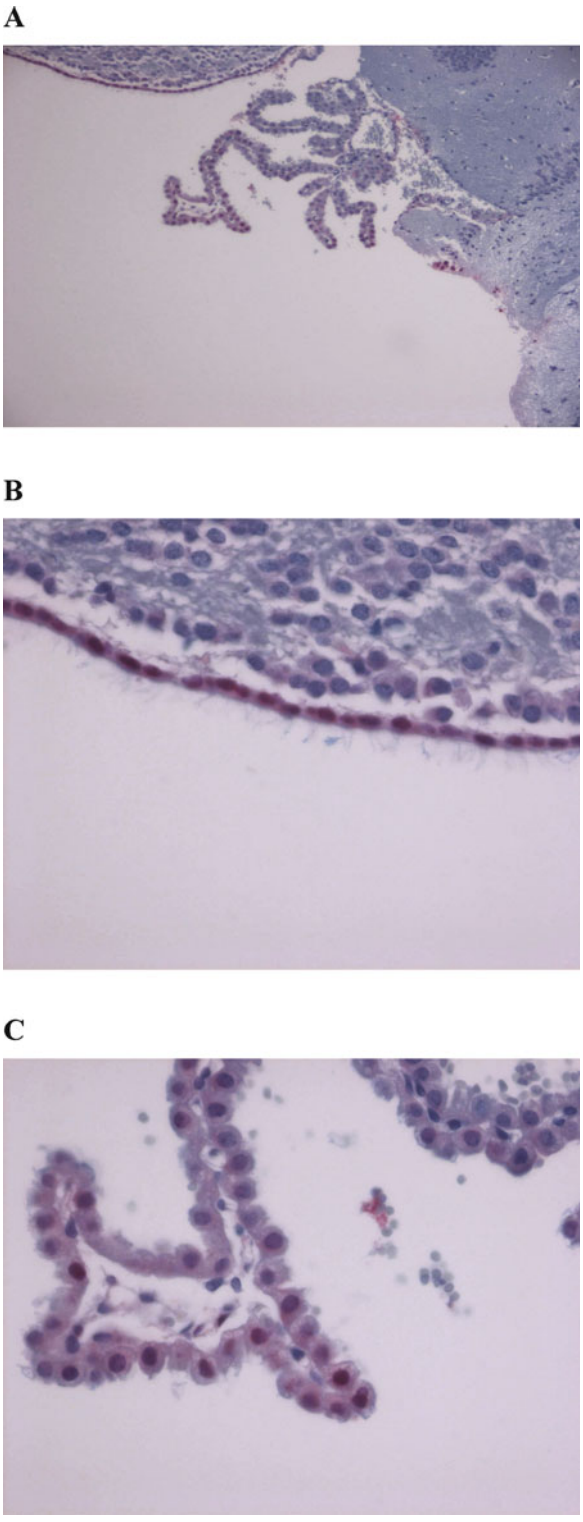

Figure 7 Immunohistochemistry of the choroid plexus region of the brain staining positive for viral antigen. **A**, low magnification showing the entire region of the choroids plexus in this coronal section (magnification 20×); **B**, higher magnification of choroids plexus cells with positive staining in the nucleus and the cytoplasm (200×); **C**, choroid plexus cells demonstrating that not all the cells stained positive and that viral antigen was found in the nucleus (200×).

memory storage. Loss of neurons from the entorhinal cortex occurs in mild Alzheimer's disease (AD) cases. Both of these structures were found to express viral antigens, sometimes simultaneously, and imply that future studies conducted in animals should measure cognitive function following a WNV infection. A study conducted with New York residents affected by the 1999 initial WNV outbreak found that these patients experienced altered or reduced cognitive function with symptoms of confusion and loss of concentration (Klee *et al*, 2004).

The vRNA detection experiments suggested that all the animals had WNV in the CNS at 9 days p.i. regardless of whether or not they had significant neurologic symptoms. Comparing these results with the survival curves, suggest that all the animals develop a CNS infection but some animals are capable of clearing the virus more readily than others. Immune modulators are most likely important contributors to whether the animals progress to encephalitis (Diamond *et al*, 2003a, 2003b; Griffin, 2003; Shrestha and Diamond, 2004; Wang *et al*, 2004). In our *in vitro* experiments, we determined that WNV infection was not lytic in neurons in culture (Hunsperger and Roehrig, 2005). Therefore, the loss of neurons may be due to microgial cell phagocytosis of infected neurons or cytokine production causing the neurons to initiate apoptosis in response to the immune modulating cells (Doron *et al*, 2003). Previous *in vitro* studies found an apoptotic cell death in CNS-like neurons in culture following a WNV infection (Shrestha *et al*, 2003). IHC results at day 6 p.i. show an infected neuron that appeared to be surrounded by immune-like cells for clearance however the immune response is not a focus of this study (Figure 5B).

The results from this study are consistent with the hypothesis that initial viral neuroinvasion may occur via the PNS. WNV infects sensory neurons in the dermal epithelium where the primary infection via the mosquito bite occurs. Viral replication and secretion occurs in draining lymph nodes and then the animal becomes viremic. Prior to peak viremia, our data shows that the virus had already invaded some CNS tissues, perhaps via the PNS. During viremia, the immune cells breach the blood-choroids plexus barrier because of increased expression of adhesion molecules permitting entrance of lymphocyte for clearance of viral infection introduced through the PNS. Introduction of virus to these structures then followed the natural progression to the hippocampus and entorhinal cortex regions. Susceptibility of these neurons to infection causes an inflammatory response initiating increased production of cerebral spinal fluid and eventual encephalitis. The difference between an animal that dies and the one that does not may be the efficiency in which the immune system is able to clear the initial CNS infection and the amount of virus introduced via the PNS. Since the majority of the animals expressed vRNA in the brain and most of

the animals were positive for neutralizing antibodies suggests that neuroinvasion normally occurred following infection. The data suggest that neuroinvasion occurs very early in the infection followed by a quiescent period between viremia and RNA expression in the brain. These results appear to be consistent with our findings of infected peripheral neurons (DRG) and early detection of viral RNA in the brainstem followed by clearance of the virus from those tissues.

In summary, our studies provide detailed information about the location of vRNA and viral antigen in specific structures of the brain and its implication in disease progression. The vRNA analyses of neural structures allowed us to correlate the temporal progression of viral antigen expression with the presence of vRNA in these tissues. Important findings include what appears to be an early introduction of virus in the spinal cord, hippocampus, and cortex. The presence of viral antigens in peripheral neurons (DRG) and choroid plexus and that viral load in the spinal cord were significantly higher compared to other CNS structures. Taken together, the results suggest a role for the peripheral nervous system in CNS infection. Also higher order structures involved in cognitive function are vulnerable to WNV and pose a public health threat.

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