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# The contribution of rodent models to the pathological assessment of flaviviral infections of the central nervous system

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## Abstract

Members of the genus *Flavivirus* are responsible for a spectrum of important neurological syndromes in humans and animals. Rodent models have been used extensively to model flavivirus neurological disease, to discover host-pathogen interactions that influence disease outcome, and as surrogates to determine the efficacy and safety of vaccines and therapeutics. In this review, we discuss the current understanding of flavivirus neuroinvasive disease and outline the host, viral and experimental factors that influence the outcome and reliability of virus infection of small-animal models.

## Introduction

The genus *Flavivirus* of the family *Flaviviridae* encompasses more than 70 recognized arboviruses and vector-unassociated, single-stranded, positive-sense RNA viruses. Encephalitic flaviviruses of relevance to humans are maintained in nature in transmission cycles involving a mosquito or tick vector and mammalian or bird amplification hosts, with humans generally serving as dead-end incidental hosts. Flaviviral genomes are approximately 11 kb in length, with a single open reading frame. As the viral polypeptide is synthesized, proteins are co- and posttranslationally processed into three structural (capsid [C], membrane [prM/M] and envelope [E]) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins. All of the nonstructural proteins have been implicated in viral RNA synthesis or protein processing.

The flaviviruses have been separated into eight antigenic complexes by cross-neutralization assays with polyclonal hyperimmune serum [22]. The most medically relevant antigenic

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complexes are the tick-borne encephalitis virus (TBEV) complex, the Japanese encephalitis virus (JEV) complex, the dengue virus (DENV) complex, and the yellow fever virus (YFV) complex. While neurological disease has been reported in humans following infection with members of each of these virus groups, not all of these viruses are neurotropic. The most medically important neurotropic flaviviruses include viruses of the JEV and TBEV complexes. For many of these viruses, small-animal models have been developed that faithfully recapitulate neurological disease syndromes reported in humans. Mice and hamsters have been widely used to assess the virulence of virus strains, test the efficacy and safety of vaccines and therapeutics, determine the tropism of viruses, and identify host and viral factors that contribute to disease. This review will encompass coverage of the ecology, pathology, and host and viral factors that contribute to neurovirulence. Given the importance of small-animal models in our evolving understanding of flavivirus-host interactions, special attention will be paid to the factors that govern accurate modeling of neurological disease, the inherent advantages and limitations of rodent models, and the future of this branch of arboviral research.

## Flavivirus antigenic complexes

### Japanese encephalitis virus antigenic complex

The majority of human arboviral encephalitis infections have been associated with Japanese encephalitis virus (JEV); however, since the mid to late 1990s, outbreaks of human encephalitis resulting from another mosquito-borne viruses of the JEV complex, West Nile virus (WNV), have been of increasing importance in both Eurasia and the western hemisphere. Two other members of the JEV complex, St. Louis encephalitis virus (SLEV) and Murray Valley encephalitis (MVEV) virus, have historically been associated with sporadic outbreaks of human encephalitis in the western hemisphere from southern Canada to Argentina and Australia, respectively. Usutu virus, an African flavivirus also in the JEV complex, has recently been associated with large epiornitics of blackbirds and raptors in Central Europe [190], with at least two cases of human encephalitis having been observed in immuno-compromised individuals either receiving a liver transplant [24] or diagnosed with B-cell lymphoma [126]; however, due to the paucity of information on neurotropic potential in immunocompetent individuals and the limited rodent studies performed with this virus [191], further mention of this agent is not provided herein.

**Japanese encephalitis virus—**Japanese encephalitis virus is the most important cause of pediatric viral encephalitis in Asia [163]. The virus is endemic in India, Nepal and areas of Eastern and Southern Asia. It has been detected as far north as Siberia in far-eastern Russia and to the west in Pakistan. Incursions into the Torres Strait Islands and the Cape York Peninsula in northeastern Australia demarcate its southeastern boundary [93, 182]. Japanese encephalitis (JE) is responsible for 30–50,000 cases of human encephalitis and approximately 10,000 deaths annually [179]. These statistics are widely considered to be underestimates of its actual incidence. JE is most common in children, principally between 3 and 6 years of age, due in part to the high seroprevalence in endemic areas [102]. However, adults are susceptible to this disease in regions in which the virus has been newly introduced [164] or where vaccine coverage is inadequate. While JEV is responsible for a high disease

burden in Asia, most infections are asymptomatic or result in a mild flu-like syndrome; only 1 in 300 infected individuals becomes ill [165].

The virus is transmitted by a variety of culicine mosquitoes, for which *Culex tritaeniorhynchus* is the principal vector in Asia [60]. *Cx. tritaeniorhynchus* mosquitoes prefer to breed in rice paddies and stagnant pools of water, bringing them into contact with ardeid wading birds, the major maintenance hosts of JEV [93]. Pigs are an important amplifying host due to their capacity to support protracted, high-level viremia, the regular replenishment of immunologically naïve animals due to intensive farming in Asia, and the predilection of *Cx. tritaeniorhynchus* to feed on these animals [102].

Symptoms of JE are varied and usually appear after a short, nonspecific febrile condition that may include headache, nausea, cough, diarrhea and rigors [164]. The virus can cause aseptic meningitis, as demonstrated by neck stiffness and Kernig's sign. Classically, JE is denoted by altered behavior, loss of cognitive function, "mask-like" facies, movement disorders (including dystonia and altered reflexes) and seizures [38, 164]. Acute flaccid paralysis occurs in some patients. While JE usually is an acute illness, there have been occasional reports of relapse during convalescence consistent with viral recrudescence [131], and viral antigen has been detected in the brainstem of a patient 151 days following the onset of illness [67]. Persistent neurological sequelae affecting cognitive and/or motor function are evident in approximately 30 % of survivors of JE [164]. The observed deficits in cognitive and motor function are likely a consequence of the profound destruction of neurons in the thalamus and brainstem during acute infection.

Adherence to cultural and religious mores regarding the handling of the deceased has greatly restricted access to cadavers following fatal JE in Asia. However, a series of articles have provided valuable insight into the tropism of JEV and the nature of the neurological pathology that it engenders in humans [51, 63, 104, 113, 154]. The type and distribution of lesions caused by JE in the brain varies depending on the time taken for the patient to succumb to infection. In acute cases in which the individual died within 1–2 weeks of the onset of symptoms, neuronal necrosis, vascular disruption and inflammation are prominent. Histological signs include vascular hemorrhage, congestion, perivascular cuffing, leukocyte infiltrates, neuronal damage, associated softening, glial cell activation and nodule formation. Inflammation of the leptomeninges is frequently observed [104]. Lesions are largely restricted to the gray matter and are most prominent in the thalamus, brain stem, cerebral cortex and anterior horns of the spinal cord. In patients that succumb to infection after protracted disease, there is less evidence of inflammation, and viral antigen is more difficult to detect in many instances. However, loss of neurons and proliferation of glial cells is more pronounced. Thickening and calcification of vessel walls is evident in some cases.

**West Nile virus—**West Nile virus (WNV) was first isolated from the blood of a febrile woman in the West Nile province of Uganda in 1937 [162]. It has a broad geographic distribution, with isolates collected from every continent except Antarctica. The WNV group comprises five phylogenetic lineages that are proposed to have emerged from an ancestral African virus [97]. Lineage 1 is subdivided into 3 clades: clade 1a (isolates from the Americas, the Middle East, Europe and Africa), clade 1b (Kunjin viruses from Australia and

Papua New Guinea), and clade 1c (isolates from India). Lineage 2 virus transmission has principally been detected in Sub-Saharan Africa and Madagascar [17]. However, recently, these viruses have been associated with epidemics of encephalitis in horses in Hungary in 2008 [73, 75] and humans in Romania [160] and Greece [124, 125] in 2010. Lineages 3 and 4 comprise isolates obtained from Central and Eastern Europe [9], but these viruses have not been associated with human illnesses. Lineage 5 is comprised of contemporary WNVs isolated in India [15].

Historically, WNV has been associated with sporadic epidemics of a mild febrile syndrome with rare cases of neurological disease. However, in the past 17 years, frequent, atypical outbreaks of disease have been reported with an increased incidence of encephalitis in humans and horses, often accompanied by high mortality in birds [23, 194]. Sequence analysis of virus isolates from cases between 1996 and 2000 confirmed that the emergent strains were lineage 1a viruses. These viruses were also shown to be more neuroinvasive in mice than were earlier isolates, in close agreement with the incidence of encephalitis and clinical presentation of the disease in humans [12]. An amino acid substitution in the viral helicase protein (NS3) was identified as having been positively selected and has been newly established on at least three separate occasions, all of which have been associated with human encephalitis outbreaks [18]. This mutation was also specifically associated with enhanced virus amplification in conjunction with a protracted viremic phase and higher mortality in American crows, an important avian amplifying host in the US [18]. Interestingly, the same helicase amino acid substitution independently emerged in the lineage 2 virus genotype isolated during the recent epidemic in Greece, in which 35 people died [125]. In addition to the apparent increase in neurological involvement observed in recent outbreaks, epidemics of atypical WNV disease have also been reported. One such outbreak involved 23 cases of encephalitis exclusively in children in Sudan in 2002 [35]. Whether local factors such as strain variation, malnutrition, high seropositivity in older individuals or concurrent pediatric infections contributed to the unusual age-distribution of this syndrome has not been determined.

Approximately 80 % of WNV infections in humans are asymptomatic [54]. The most common manifestation of disease is WNV fever that is denoted by pyrexia, headache, fatigue, malaise, anorexia, nausea, myalgia and occasionally a rash. In approximately 1 in 150 infected people, a neurological syndrome develops. Roughly 40 % of neurologic disease cases present with meningitis, and the remaining 60 %, with encephalitis [32]. The symptoms of West Nile neurologic disease (WNND) are fever, nuchal rigidity, headache, fatigue, muscle weakness, cerebrospinal pleocytosis and altered mental status (reviewed in refs. [32, 152, 189]). Unlike the closely related JE, WNND is most prevalent in the elderly and rarely induces seizures. People with diabetes, hypertension and genetic or acquired immunodeficiencies are also predisposed to neurological disease [23]. Acute flaccid paralysis observed in some patients has been proposed to result from the destruction of motor neurons and spinal cord anterior horn cells [153].

Individual recovery times from WNND are highly variable, and persistent neurological sequelae are common. The incidence of prolonged convalescence depends on the initial nature of disease presentation. In a case study of 228 hospitalized patients in Colorado in

2003, individuals recovering from WN encephalitis were more likely to have sequelae at the time of discharge than people recovering from meningitis [14]. Considerable variation exists between studies in the incidence of neurologic sequelae, in part as a consequence of the use of different metrics for assessing persistent symptoms. In a report tracing the recovery of 15 patients following WNND, 27 % of the enrolled subjects reported symptoms 8 months after infection [152]. In a separate study outlining persistent sequelae in 15 individuals one year after discharge, 63 % of respondents indicated that they had not fully recovered. Fatigue, mental deficits, muscle weakness, ambulatory problems and insomnia were common among respondents with protracted recovery times.

Examination of post-mortem tissues from immuno-competent individuals who succumbed to WNND demonstrated that pathological changes in the central nervous system (CNS) were concentrated in the brainstem and the anterior horn of the spinal cord [1, 56, 121]. In a study investigating histological changes in 23 adults who died following WNND, lymphocytic infiltrates, microglial nodules and neuronal destruction were observed at these sites in each specimen [56]. Discrete inflammatory foci were most prominent in the gray matter of the medulla, pons and midbrain. Sparsely distributed viral antigen was observed in neurons in the pons, medulla and midbrain as well as in the cerebellum and cerebral cortex [56]. Antigen-positive cells were rare in the brains of individuals who died more than two weeks after the onset of disease, indicating that immune clearance of infected cells had occurred. In contrast to the marked tropism of the virus in immunocompetent individuals, viral antigen was detected throughout the brains of immunosuppressed people [56, 70], illustrating the important role of host immunity in stemming the dissemination of WNV in the brain following neuroinvasion.

**St. Louis encephalitis virus—**St. Louis encephalitis virus (SLEV) was first isolated from brain sections of deceased patients during an encephalitis outbreak in the Mississippi Valley in St. Louis, Missouri, in 1933 [89]. SLEV has subsequently been associated with periodic outbreaks of human encephalitis in urban and rural areas of the western hemisphere from southern Canada to Argentina [87]. The virus circulates in enzootic habitats between culicine mosquito vectors and their bird hosts, most typically columbiform and passeriform birds, which develop viremia of sufficient magnitude to infect additional mosquitoes [134, 137]. Although humans can become infected with SLEV and exhibit severe clinical symptoms, viremia is believed to be insufficient to infect subsequently feeding mosquito vectors [107]. Outbreaks of encephalitis resulting from SLEV infection are typically associated with periods of rainfall followed by warm, dry intervals that favor virus amplification in poikilothermic mosquito vectors and provide optimal breeding conditions for *Culex* species mosquitoes [135, 138]. Such outbreaks have been pronounced in close proximity to enzootic habitats, most notably in California [136], Florida [99], Texas [26] and Louisiana [64]. A constellation of ecological factors, including temperature and rainfall [33] in addition to bird and human seroprevalence rates, are undoubtedly associated with the potential for the initiation of outbreaks [137].

The vast majority of human infections with SLEV are subclinical, with an estimated rate of illness between 0.2 and 6 % and mortality rates for symptomatic individuals ranging from 5 to 20 %. Severe disease is more commonly associated with infections of the elderly [105,

178]. Other clinical factors have been associated with the progression of more-severe SLEV-associated disease presentation including hypertension, arteriosclerosis, asthma and diabetes [49]. Clinically apparent disease can range from encephalitis/meningoencephalitis to fever and nausea with minimal associated CNS manifestations [20]. Imaging studies of several human SLE cases have independently demonstrated the potential importance of pathology in the substantia nigra with clinical presentation in humans [25, 188]. Symptoms can abate spontaneously and clinically defined cases have been associated with sequelae that include memory loss and incoordination. Post-infection encephalitis also has been documented following mild illness, implying the potential for immune-modulated neurological disease in humans [122].

Lethal outcome from SLEV infection generally occurs within two weeks of symptom onset, associated with direct damage to the CNS; however, lethal infections that have progressed beyond the two-week interval have been associated with underlying disease complications. Analyses of autopsy specimens from individuals who have succumbed to SLEV infection have uniformly demonstrated meningitis associated with lymphocytic infiltration, most notably in the midbrain and pons regions [20]. Although most prominent in the gray matter regions of the brain, perivascular cuffing has also been observed throughout the cerebral white matter. The substantia nigra has been most frequently observed to exhibit pathological manifestations [49, 139].

**Murray Valley encephalitis virus—**Murray Valley encephalitis virus (MVEV) is responsible for sporadic outbreaks of encephalomyelitis in Australia that has been described variously as “Australian X disease”, “Australian encephalitis” and “Murray Valley encephalitis (MVE)”. The virus is endemic in Papua New Guinea [48], the Kimberley region of Western Australia, and possibly in areas in the Northern Territory, where the virus exists in a natural zoonotic cycle between ardeid wading birds, such as the rufous night heron, and culicine mosquitoes, principally *Culex annulirostris* [44]. Large epidemics extending as far south as Victoria and New South Wales in the Southeast of Australia have historically been associated with unseasonably high rainfall. These conditions provide waterlogged areas that attract the ardeid wading birds from endemic areas [92].

MVEV was first isolated from the brain of a woman who succumbed to infection in Victoria, Australia, during an epidemic of what at the time was called “Australian X disease”, in 1951 [47]. Australian X disease was first reported in 1916 with epidemics of the syndrome also occurring in the southern and eastern regions of the country in 1917, 1918, 1922, 1925, 1951, 1974 and 2011 [3]. The clinical: subclinical infection rate is low (1 in every 500–1,000 infections); however, once neurological disease is observed, the case-fatality rate is high (71 % mortality across the 1916–1921 outbreaks and 42 % in 1951). MVE affects all age groups but is particularly prominent in children and males. Children under the age of 15 comprised 71 % of all cases between 1917 and 1918, and 62 % of cases in 1951. In 1974, the disease syndrome was renamed “Australian encephalitis”. Later it was found that some cases of Australian encephalitis were attributable to Kunjin virus, a subtype of WNV.

A comprehensive review of clinical symptoms from the outbreaks of Australian encephalitis between 1917 and 1951 was provided by S. G. Anderson [3]. Headache and drowsiness are the most common symptoms of MVE, with irritability, malaise, confusion, limb and neck pain, and limb weakness. Vomiting is common in children, and fever, nuchal rigidity, involuntary movements and convulsions were observed frequently in comatose patients. The average survival time for patients who succumbed to Australian encephalitis was 4–6 days after the onset of symptoms [3].

The histological hallmarks of encephalitis varied depending on the length of time that the person survived. As has been observed for JE, inflammatory changes were increasingly evident the longer the patient survived neurological infection. Pathological changes were most prominent in the gray matter, with inflammatory lesions, perivascular cuffing, neuronal damage, neuronophagia and a marked loss of Purkinje cells in the cerebellum. Some perivascular cuffing was also evident in the white matter and meninges. Inflammatory signs were more pronounced in the brain of one individual who died 34 days after the onset of symptoms. Histological examination revealed greater neuronal loss, microglial and astrocyte proliferation and degeneration in the basal ganglia and pons. Brain tissue from a 5-month-old child who died 60 days after disease onset exhibited more-pronounced glial cell proliferation, degeneration of the thalamus and cerebella folia, and calcification in the basal nuclei [3].

## Tick-borne encephalitis virus antigenic complex

The tick-borne encephalitis (TBE) antigenic complex comprises 13 members. Tick-borne encephalitis virus (TBEV), Kyasanur Forest virus, Langat virus, Omsk hemorrhagic fever virus and Powassan virus are the most commonly studied viruses within this complex. The majority of these viruses circulate in Europe and parts of Asia. The vector for this complex is primarily ticks of the genus *Ixodes*, and the main vertebrate hosts and reservoirs are small mammals. TBEV is the most prevalent arbovirus in Europe and many parts of Asia and has been divided into European, Siberian and Far Eastern subtypes. Despite the fact that there is an effective vaccine available for TBEV, there are still approximately 2,000 human TBE cases in Central Europe and up to 10,000 cases in Russia and the Far East annually [74, 169]. As the preponderance of data on small-animal models of flaviviral tick-borne arboviral encephalitis have focused on TBEV, we have restricted our discussions in this review to models involving study of this specific agent.

**Tick-borne encephalitis virus—**The clinical outcome of TBE is dependent on the subtype. For example, the response following infection with the European subtype is typically asymptomatic; however, of the symptomatic cases, 72 % present in a biphasic manner; a viremic phase with signs and symptoms of fever, fatigue, general malaise, headache and body ache followed by an asymptomatic period of one or more weeks preceding the onset of meningoencephalitis [84]. Fatalities range from 1 to 2 % for the European subtype, and approximately 10–20 % of the neurological cases develop neuropsychiatric sequelae. Interestingly, for the Far Eastern subtype, the disease is primarily monophasic, with a high incidence of CNS involvement and a fatality rate of approximately 30 % [40]. In severe forms of the disease, the patient can develop fever, headache, anorexia,

nausea, vomiting, photophobia, stiff neck and neurological dysfunctions that include paresis, paralysis of the arms, and convulsions.

## Viral determinants of flavivirus encephalitis

Neuropathogenesis comprises two important yet distinct factors: neuroinvasiveness and neurovirulence. Neuroinvasiveness is the capacity of the virus to invade the CNS, whereas neurovirulence is defined as the ability of a virus to cause disease in the CNS. Members of the JEV and TBEV complexes have evolved a variety of mechanisms that facilitate their entry into the CNS. It is unclear whether the neurovirulence of flaviviruses is an ancestral trait retained by many of these viruses or whether selection for increased vector transmission has resulted in the adoption of this characteristic by various members of the family. Although many genetic determinants of altered neurovirulence and neurotropism have been described for members of the JEV and TBEV complexes, specific natural genetic determinants that have been identified to impart these phenotypes, rather than engendering attenuation, are discussed briefly below.

The flavivirus envelope glycoprotein (E) is believed to be an important determinant for neurotropism. The E protein functions in viral attachment, viral entry and viral assembly [58]. The glycosylation status of the E protein of WNV correlates with murine neuroinvasive phenotypes, with reduced viral entry into the CNS being evident for non-glycosylated and/or mutagenized viruses. The reduced neuroinvasive properties have been hypothesized to result from altered tropism, increased sensitivity to low-pH conditions in endocytic vesicles and a reduced affinity for receptors such as DC-SIGN for infection of monocytes/macrophages [13, 31, 94, 115, 155].

Historically, genotype III (GIII) was the predominant genotype of JEV circulating in Asia and was considered to cause more-severe disease than the other genotypes. Recently, GI has largely replaced GIII in Asia and has been linked to human encephalitis [186]. Sequence analysis of viral strains isolated from an outbreak in 300 American soldiers that resulted in 17 deaths in 1949–1950 showed similarity to the Bennett strain, which had an 11-nucleotide (nt) deletion after the stop codon in the 3′UTR. This same deletion was also found in most GI and GII isolates and may be an important viral determinant of severe disease following JEV infection [151].

NS1’, an isoform of the NS1 protein generated by a frameshift event that results in translational extension, was found to be unique to JEV-complex viruses [45, 46]. Ablation of frameshifting by disrupting base pairing in a pseudoknot predicted to dictate polymerase slippage was demonstrated to reduce murine neuroinvasiveness of Kunjin virus, indicating the involvement of this extension protein as a flavivirus virulence determinant [101]. Interestingly, NS1 has been associated with disregulation of the host complement cascade [7] as well as TLR3-mediated innate immune responses [192]. Whether the contribution of NS1’ to facilitating neuroinvasion emanates from a similar antagonism of host innate immunity remains to be determined.

Small subgenomic flavivirus RNA (sfRNA) is generated during infection with a variety of flaviviruses as a result of the resistance of conserved RNA secondary structures in the

3′UTR to 5′-exonuclease activity. Abrogation of the formation of sfRNA through mutagenesis of the West Nile virus 3′ UTR promoted a reduction in murine neuroinvasive phenotypes [130].

## Host determinants of flavivirus encephalitis

The most common host risk factor identified for severe illness is age. The association of age with the incidence of severe disease has been documented in both clinical and animal studies. Younger individuals have a higher susceptibility to neuroinvasive disease, implying that factors such as increased susceptibility of the developing nervous system to neuroinvasive disease or to reduced immune responsiveness of younger individuals could lead to increased neuroinvasiveness. For example in WNV, younger patients are more susceptible to aseptic meningitis, but elderly patients are at greater risk of developing encephalitis with poor prognosis. In addition, JE is largely a pediatric condition in which encephalitis is primarily observed in individuals from 0 to 9 years old; however, this epidemiological finding is highly biased by the high sero-prevalence rates of older individuals in areas endemic for JEV. Human epidemiological studies support this finding, and this is recapitulated in many of the flaviviral encephalitis animal models.

For the elderly, the risk is most likely related to a compromised immune system or pre-existing medical conditions that increase susceptibility of the CNS to infection. Pre-existing systemic conditions such as auto-immune diseases, diabetes and obesity are risk factors for severe disease in WNV patients [116]. In humans, both the innate and the adaptive immune responses show waning activity as individuals age [85, 123]. Hence, the senescence of the immune system is most likely the main contributor to advanced age as a risk factor for flaviviral infections. A recent study of WNV-infected human dendritic cells showed a weaker immune response to type I interferon (IFN) production in older donors than that in their younger counterparts [133]. Additionally, Piazzi et al. determined that changes in memory T-cell phenotype and differentiations were associated with WNV disease severity [129].

The three main genetic host risk factors that have been associated with severe flaviviral neurological disease include HLA-A and HLA-B, CCR5 and oligoadenylate synthetase (OAS). OAS is a member of the IFN-regulated gene family, and the OAS gene cluster consist of three genes, OAS1, OAS2 and OAS3, mapped to locus q24.1 of chromosome 12 in humans. These three genes encode various forms of the 2′5′OAS protein, with differing cellular compartmentalization, protein conformation and activation thresholds [65]. The OASL gene is located at another locus of chromosome 12 (q24.1) and encodes two proteins that do not have enzymatic activity. The OAS gene cluster activates an important mediator of viral RNA degradation, RNAse L. In humans, sequencing of the OAS gene cluster in 33 individuals with severe WNV infections did not reveal deletions or nonsense mutations; however, 23 single nucleotide polymorphisms (SNPs) in OAS genes were identified that occurred at a higher frequency in symptomatic individuals. In another study, an allele of OAS1 (‘A’ allele at SNP rs10774671) that enhanced mRNA splicing was more common in WNV-infected individuals than in uninfected controls [76]. That study also tested the function of the SNP on viral replication in an *ex vivo* model using primary human lymphoid

tissue and determined that virus replication varied among the donors and was most efficient among individuals who were homozygous for the 'A' allele [80]. In studies of TBE in humans, the same 23 SNPs were analyzed for 142 TBE patients in Russia. In that study, there were differences in genotype, allele and haplotype frequencies for three OAS2 SNPs and two OAS3 SNPs between TBEV-infected and control groups, indicating an association between these five OAS SNPs and the outcome of disease severity [11].

Mutations in the chemokine receptor CCR5 have been directly associated with more-severe disease following flavivirus infection [53]. CCR5 knockout (KO) mice display increased WNV titers in the CNS as well as a significant increase in mortality [52]. Subsequent human studies demonstrated an association between symptomatic WNV disease and a complete loss of CCR5 function through the acquisition of a mutation called CCR5Δ32 [81]. This 32-base-pair deletion in the CCR5 gene yields a truncated form of the protein, eliminating its surface expression. Lim et al. [82] reported that CCR5Δ32 mutants were not associated with an increase in human susceptibility to WNV infection as had been observed in mice, but rather was present in individuals with an early clinical presentation of the disease and with higher frequencies of symptoms. Those individuals who were CCR5Δ32 homozygous had a higher frequency of multisystemic symptoms such as lymphadenopathy, neurological deficits and gastrointestinal complications. Similar results were obtained for TBEV in which individuals possessing the CCR5Δ32 allele were found to have a statistically higher frequency of symptomatic TBEV infection than subjects who were homozygous for WT CCR5 [68].

Human leukocyte antigen (HLA), the human equivalent of MHC, functions in the presentation of foreign antigens to circulating leukocytes. HLA-A and HLA-B alleles can either increase or decrease susceptibility to a flavivirus infection. Most of the research on the association between HLA expression and flaviviral disease has been performed with dengue viruses. A few studies have indirectly implicated HLA alleles in modulating the severity of WNV infections [34, 66]. HLA-A2 transgenic mice showed enhanced survival following WNV challenge. In that study, the transgenic mice had lower viral loads in the brain as well as higher survival rates [66].

The Toll-like receptor 3 (TLR-3) gene has been studied as a genetic risk factor for TBEV infection. TLR-3 modulates the immune response by recognition of dsRNA. The presence of dsRNA activates TLR-3 and induces the production of IFN type I and the induction of the pro-inflammatory cytokine TNF-$\alpha$. A study performed on 128 TBE patients from Lithuania reported that a functional TLR-3 is a risk factor for severe TBEV infection [69]. Similarly, elevated expression of TLR-3 in WNV-infected macrophages harvested from older individuals has been associated with increased vascular permeability of the blood-brain barrier (BBB) due to increased cytokine production [71].

## Mechanisms responsible for flaviviral encephalitis

One of the central questions in the search for therapeutic targets for the treatment of flaviviral neurological syndromes is whether these diseases are induced by virus-mediated cytolysis, immunopathological responses to virus infection, or a combination of both. While the mechanisms underlying flavivirus neuropathology remain to be clearly defined, a

growing body of work undertaken using mouse and hamster models have indicated that there is considerable variation in the presentation and nature of different flaviviral encephalitides, even between those syndromes elicited by closely related viruses of the same antigenic complex.

Despite the close genetic relationship between WNV and MVEV, the causes of these neurological syndromes appear to be distinct. Transgenic mice have been employed to demonstrate that leukocyte infiltration of the CNS and the expression of inflammatory cytokines are essential for host survival during WN encephalitis. Among the cells and immune modulators that have been implicated in protection from WNV neurological disease are $CD8^+$ [156] and $CD4^+$ T lymphocytes [161], B cells [43], neutrophils [8], monocytes [83], type 1 and 2 IFNs [141, 157], TNF-$\alpha$ [158] and complement factors [100]. In particular, $CD8^+$ T cells are required for clearance of WNV from the brain following viral neuroinvasion [156]. In contrast to the protective role played by the host innate and adaptive immune responses during WNV infection, MVEV neuroinvasion and neuropathology appear to be mediated by immuno-pathological responses to virus infection. Depletion of neutrophils through the administration of neutrophil-specific, cytolytic monoclonal antibodies resulted in reduced mortality rates in mice [4]. Similarly, mice genetically deficient in the $CD8^+$ T cell cytolytic effector proteins fas, granzyme A or B or combinations of fas, granzymes and perforin also exhibited significantly reduced mortality rates upon infection with MVEV [79, 114].

Japanese encephalitis virus, a member of the same antigenic complex as WNV and MVEV, appears to induce neurological disease through a combination of virus-induced neuronal apoptosis, host inflammatory responses and autoimmunity. However, the precise contribution of each of these mechanisms to disease is unclear. While apoptosis of neurons has been observed in neuronal progenitor cells *in vitro* [78] and in mice [170] following JEV infection, it remains to be established whether viral infection of neurons or immunopathological responses to neuronal infection are responsible for programmed cell death *in vivo*. In contrast to the protective role played by monocytic cells during WNV infection, several *in vitro* studies have implicated microglial cell activation and the subsequent induction of inflammatory cytokines in neuronal destruction resulting from JE [27, 118]. In agreement with this hypothesis, inhibition of TNF-receptor-associated death domain (TRADD) expression – a critical factor in the TNF-$\alpha$-dependent signaling cascade – resulted in decreased activation of neuronal apoptosis and microglial cell activation *in vitro* [171]. In mice, depletion of this protein was correlated with reduced expression of caspase 3, pro-inflammatory cytokines and pro-apoptotic factors as well as increased survival in mice, implying a role for immunopathology in the severity of JE [170, 171].

Autoantibodies to neural antigens induced during JEV infection, including myelin basic protein and neurofilament proteins, were reported in 25 patients from a cohort of 72 JE patients [36]. In that study, the presence of antibodies to neurofilament proteins was correlated with fatal outcome. Autoantibodies specific for $\beta$2-glycoprotein 1 were detected in a patient who suffered cerebral ischemia [28]. However, the extent to which autoimmune responses contribute to JE remains to be determined.

Immunopathology also appears to be associated with the severity of TBE, but as is the case for JE, the relative contributions of virus-mediated cytolysis and immune mechanisms to disease severity require further investigation. Prominent inflammation was noted in post-mortem brain tissue from 26 human cases of TBE with CTLs closely associated with TBEV-infected neurons undergoing apoptosis [50]. SCID and CD8 (-/-) mice were used to demonstrate that mice deficient in mature CD8 T lymphocytes exhibit longer mean survival times following infection with TBEV than immunocompetent mice derived from the same genetic background [140]. Furthermore, in that study, adoptive transfer of $CD8^+$ T cells to TBEV-infected SCID mice hastened mortality. Inhibition of nitric oxide production also delayed mortality in TBEV-infected mice, highlighting the role played by various components of the immune response in the potentiation of neurological manifestations [72]. Failure to reduce mouse mortality through the selective depletion of individual immune factors may reflect an essential role of direct, virus-mediated neuronal destruction in TBE, or alternatively, it may imply the requirement for the cumulative effects of a combination of host immune factors for severe disease. Interestingly, administration of IL-12, a cytokine that up-regulates IFN-$\gamma$ and enhances CTL function, exacerbated TBEV infection when co-administered with a therapeutic monoclonal antibody [128]. In contrast, the same treatment significantly reduced mortality in SLEV-infected mice, again highlighting the marked differences in the mechanisms contributing to neurological disease elicited by infection with different members of the genus.

## Mechanisms promoting flavivirus neuroinvasion

There is also considerable variation in the mechanisms employed by different neurotropic flaviviruses to gain access to the CNS. Mouse models have permitted temporal analysis of the progressive dissemination of virus infection within the CNS. Such studies demonstrated that SLEV [106] and MVEV [4, 98] enter the brain via the olfactory lobes. In both cases, following peripheral inoculation, infection in the brain was first detected in the olfactory bulb, and virus progressively trafficked through the remainder of the CNS in a rostral-to-caudal direction.

Conversely, JEV appears to enter the brain via a hematogenous route. Viral antigen and lesions are broadly distributed throughout the brains of humans and experimentally infected animals [37, 63]. In one study, viral antigen was absent in the olfactory bulb of individuals that exhibited disseminated CNS infections, indicating that infection of olfactory nerves is not a major route of JEV neuroinvasion. In agreement with this statement, only 40 % of macaques infected intranasally with JEV had viral antigen in the olfactory bulb, despite the presence of virus in other regions of the brain [117].

Although it has been shown that JEV can infect across brain microvascular endothelial cells *in vitro* [39], viral antigen is rarely detected in these cells in infected humans or in experimentally infected animals. Since the function and integrity of the endothelium of the BBB is supported by interactions with glial cells and neurons, it is possible that monoculture systems do not faithfully recapitulate processes that occur *in vivo*. It has been suggested that the occasional detection of virus in cerebral endothelium in human cadavers results from phagocytosis [37]. Conversely, the limited sensitivity of immunohistochemistry and *in situ*

hybridization assays employed to assess endothelial cell infection in vertebrate hosts may preclude detection of low-level replication in these cells. Electron microscopy was utilized to demonstrate that JEV can migrate across cerebral microvascular endothelial cells and pericytes by transcytosis [86]. This process may seed virus in the CNS at several sites, permitting establishment of neuronal infection.

Numerous mechanisms have been proposed to explain how WNV enters the CNS, including (1) infection of olfactory nerves [62], (2) disruption of the BBB by vasoactive cytokines permitting free viral egress [187], (3) infection across the BBB [39], (4) a Trojan horse mechanism whereby infected myeloid cells carry the virus into the CNS, and (5) retrograde axonal transport [142]. A growing body of work supports the contention that axonal transport is the principal route of neuroinvasion. Direct inoculation of the sciatic nerve was used to demonstrate that WNV can utilize retrograde axonal transport to gain entry to the spinal cord [142]. In that study, hamsters developed acute flaccid paralysis as a result of virus-mediated destruction of neurons in the anterior horn of the lumbosacral region of the spinal cord. The viral distribution and disease presentation elicited by this experimental model mirrored human infection. Transection of the nerve above the inoculation site prevented the onset of paralysis in these hamsters. In a separate study in which mice were inoculated with WNV in the footpad, virus was found to infect peripheral nerves that innervate the injection site, and the subsequent distribution of virus in the brains of these mice was consistent with neuroinvasion at the sites in which peripheral nerve roots are located in the brain [62].

## The application of mice to model flavivirus encephalitis

Mice represent the most extensively utilized animal model of flavivirus encephalitis, in large part due to the high degree of susceptibility of most laboratory strains to flaviviral encephalitis, the similarities in disease presentation and virus tropism between rodents and humans, and the amenability of this model for the utilization of large numbers of animals. Modeling flaviviral disease in rodents affords several advantages over the examination of postmortem human tissues from infected individuals. In many cases, religious and cultural concerns have greatly restricted access to tissue samples from cadavers. Moreover, the distribution of virus and histopathological changes in the brain vary markedly depending on the time taken for different individuals to succumb to infection [104] and their immune status [6]. Additionally, post-mortem human specimens only provide a snapshot of pathological markers and virus distribution in the terminal phase of disease. Conversely, by sacrificing experimentally infected mice at regular intervals post-infection, a number of investigators have elegantly demonstrated the evolving process of flavivirus dissemination in vertebrate hosts and the concomitant pathological changes that occur. Likewise, it is difficult to study flavivirus neurological disease in non-human primates because infection is typically mild.

As a result of their sensitivity to many types of flavivirus encephalitis, mice are also used to compare the pathogenicity of different strains of viruses, to measure vaccine virus attenuation empirically and to investigate the efficacy of therapeutic agents. However, several factors strongly influence how faithfully mice recapitulate human disease. Provided

below is a summary of the major factors that govern the development of flaviviral disease in murine models.

## Genetic factors regulating the susceptibility of mice to encephalitis

Most laboratory strains of mice are highly susceptible to various forms of flaviviral disease [91, 144]. Resistance was mapped as an autosomal dominant trait to the *flv* (flavivirus resistance) locus of chromosome 5 [145, 181]. Detailed mapping studies implicated the 2′-5′ oligoadenylate synthetase 1b (OAS1b) gene in the control of host resistance to flaviviral infection in mice [127]. Members of the OAS family of enzymes are expressed as part of the type 1 interferon cascade and mediate the synthesis of 2′-5′ oligoadenylates from ATP [61]. These polynucleotide sequences bind to and activate RNase L, an enzyme that is responsible for the destruction of viral double-stranded RNA.

A variety of susceptible laboratory mouse strains were shown to have inherited a single point mutation in OAS1b that introduces a premature stop codon resulting in truncation of the protein [21]. It has not been established whether this truncation ablates or simply ameliorates the function of this enzyme. *In vitro* and *in vivo* studies have clearly established the functional importance of OAS1b in resistance to flavivirus infection. WNV replication was depressed in mouse neuroblastoma cells that overexpressed WT OAS1b compared to cells expressing the mutant allele [88]. The introduction of a functional OAS1b gene into susceptible mice by genetic knock-in increased resistance to yellow fever virus [148].

In addition to the primary role that OAS mutations play in mediating susceptibility to flaviviral infection in an array of laboratory mouse strains, more subtle genetic variations exist that also contribute to the degree of host resistance to viral challenge [96, 103]. However, the loci responsible for these interspecies variations in virus resistance remain to be identified.

## The role of age in the susceptibility of mice to flaviviral encephalitis

Mice demonstrate age-dependent susceptibility to lethal infection with a variety of neurotropic viruses. Juvenile mice are highly susceptible to encephalomyelitis elicited by members of both the JEV and TBEV antigenic complexes. Likewise, old mice (>18 months old) are predisposed to neurological disease [19]. Conversely, neurological syndromes resulting from infection with many of these viruses is rare in adult mice or can only be induced with a large infecting dose. Several host factors have been implicated in age-related susceptibility to different viral pathogens. A number of viruses, including JEV [120] and Sindbis virus [183] exhibit a marked tropism for immature neurons. This predisposes young animals with developing central nervous systems to neurological disease. Additionally, in some cases, the distribution and expression level of receptors for a virus is elevated in juvenile mice [132]. In other cases, virus replication is elevated in extraneural tissues of young animals compared to adults, driving the production of higher systemic viremia [77], leading to neuroinvasion.

Age-dependent differences in the nature of the immune response to viral challenge have been implicated in the severity of disease in young and aged mice. Both over-stimulation

and attenuated expression of pro-inflammatory cytokines in suckling or weanling mice have been associated with poor disease outcome [10, 29, 177]. Likewise, differences in the function of immune cells between animals of different ages can influence the development of viral disease [59].

It has been proposed that differences in the integrity of the BBB between newborn, juvenile and adult mice also contribute to differences in the severity of disease in these age groups [180]. While the architecture of the BBB is different between neonatal and adult mice, recent studies have demonstrated that the BBB is functional from birth [41, 146]. Also, there is some evidence that the BBB of newborn mice is refractory to large macromolecules such as virions [108]. However, it has been shown that leakage of the BBB is more readily induced in younger mice in the presence of elevated levels of vasoactive cytokines [5]. In addition, the BBB of young mice is more permissive to the trafficking of cytokines [175] that are expressed at elevated levels during peripheral virus replication, a state that may serve to activate resident brain cells, drive immune cell infiltration of the CNS and alter the integrity of the BBB. These characteristics of the developing BBB may contribute to an age-dependent increase in barrier permeability in response to an inflammatory insult elicited by flavivirus infection.

Viral determinants also may contribute to the age-dependent resistance of mice to virus infection. While in most cases neurological disease following infection with the alphavirus Sindbis virus is evident only in suckling or weanling mice, adaptive mutations in the E2 gene have been associated with elevated neurovirulent phenotypes in adult mice [90]. Similarly, the neuroinvasiveness and neurovirulence of US strains of lineage 1 WNV is comparable between juvenile and adult mice, while other lineage 1 isolates of WNV – including Kunjin virus and the Egypt 101 strain – exhibit strong age bias in their propensity to elicit neurologic disease [12, 42].

## The role of gender in the susceptibility of mice to flavivirus encephalitis

There is a limited amount of data on the role of gender as a predisposing factor to flaviviral encephalitis. However, male mice exhibited statistically higher mortality rates than female mice following infection with a limited number of JEV [96] and SLEV strains [2]. Whether other strains of these viruses or other neurotropic flaviviruses exhibit gender-dependent susceptibility to encephalitis remains to be determined.

## The contribution of the route of inoculation and virus dose to flaviviral encephalitis

The route of inoculation is a major determinant of the course of infection in animal models of disease. Inoculation of virus into extraneural sites is commonly performed to assess the neuroinvasive potential of a flavivirus. Direct intracranial inoculation of virus, commonly used to determine the neurovirulence of virus strains, engenders a more rapid onset of disease symptoms and mortality than does inoculation into extraneural sites [55, 57]. Due to the shorter time course of infection following intracranial inoculation, immunopathological changes are less prominent than they are after peripheral virus challenge.

Studies comparing subcutaneous inoculation and mosquito transmission of WNV have demonstrated that mosquito saliva can promote the generation of higher viremia early in

infection in mice and newly hatched birds, reduce the time taken for the virus to enter the CNS, and elicit higher mortality rates [149, 166, 167]. Mosquito saliva has been shown to modulate immune cell infiltration at the site of inoculation and reduce the expression of Th1 cytokines and TLR3 [150, 174]. However, the adoption of this more natural route of inoculation for virulence studies in vertebrate animals is curtailed by the inherent variability of the infecting dose delivered by individual mosquitoes, the inability to increase the titer of the inoculum, and the potential for selection of mutations in the virus by mosquito passage. Since capillary-feeding mosquitoes discharge virus-laden saliva into the dermis during probing as well as into the blood during feeding, most studies utilize subcutaneous injection to experimentally mimic the natural route of infection. Subcutaneous co-inoculation of virus with mosquito saliva has also been employed by some groups to imitate natural infection [168].

Different routes of peripheral inoculation have been employed to assess distinct aspects of flaviviral infection. Although intraperitoneal inoculation represents a more ‘artificial’ route of virus delivery than subcutaneous injection, it has been shown to promote higher mortality rates for some viruses in mouse models, and for that reason it has historically been used by some groups to assess the neuroinvasive potential of virus isolates and vaccine strains [19, 62, 119]. For other flaviviruses, the route of inoculation has a more limited effect on disease progression. Indeed, no difference was observed in the mortality rate or the magnitude of virus-specific antibody responses following JEV injection of 1- and 6-month-old mice by the subcutaneous, intradermal, intravenous and intraperitoneal routes [55]. Such differences in the effect of the route of inoculation on virus infection may reflect differences in the cell populations that support peripheral amplification of each virus.

The administration of viruses to mucous membranes of various rodent models – such as the nose, eyes, mouth and vagina – have been performed to model potential modes of transmission for flaviviruses in humans. In particular, intranasal inoculation has been utilized to evaluate the potential for aerosol transmission of arboviruses. There is tremendous variation even among closely related flaviviruses in their capacity to infect across epithelium. Saint Louis encephalitis virus competently infects mice following intranasal administration [106], whereas WNV and JEV are poorly infectious by this route of inoculation [12].

The size of the infectious dose can also strongly affect the course of flaviviral infection and neuroinvasion. The shorter survival time of mice challenged by the intracranial route as compared with peripheral inoculation indicates that replication in peripheral tissues is necessary before the virus can enter the CNS. Peripheral challenge of mice with viral doses of MVEV or WNV ranging from $10^2$ to $10^6$ infectious virus particles produce similar mortality rates and average survival times; however, if the infecting dose is elevated to $10^7$ infectious units of virus or greater, the survival time of mice is similar to that observed in animals challenged by the intracranial route [79, 114]. These observations imply that high-titer inoculums can promote direct neuroinvasion without the need for prior replication in peripheral tissues.

## Genetic knock-out (KO) and congenic mouse strains in the study of flavivirus encephalitis

Since the development of the first gene KO mouse in 1989 [195], in excess of 1,000 genetically modified mouse strains have been generated. More than any other development in animal modeling, the advent of genetic KO and knock-in mouse lines has permitted the discovery of host factors that govern protection and pathological changes associated with virus infection. Over the past decade, genetic KO mice have been employed extensively to investigate the role of selected genes and host cell populations in flavivirus neuroinvasion, neuropathology and the induction of protective host immune responses. Much of this work has concentrated on WN encephalitis, the emergence of which in the United States coincided with the rapid proliferation in the number of transgenic mouse lines available to research institutions as a result of the concerted efforts of independent investigators and consortia including the NIH-funded KO Mouse Program. To date, more than 70 studies have been published applying KO mouse strains to assess the role of host factors in WNV infection.

While surgical, chemical and antibody-mediated depletion have been utilized to study the role of a variety of immune cells and cytokines during flavivirus infection in mouse models [4], the variety of targets that can be studied by these approaches is comparatively limited. Additionally, complete inhibition of the function of specific cell types and proteins is not always possible by selective depletion. By contrast, gene KO mouse models are readily amenable to studying the roles in infection played by intracellular signaling proteins, transcription factors and effector proteins that are difficult to deplete by conventional strategies.

Despite the tremendous advantages afforded by KO mouse models, several limitations have been observed that restrict the application of this approach for characterizing host genes that influence virus infection. The principal problem with this approach is that the elimination of some genes is lethal in mice. Additionally, depletion of certain genes can elicit dramatically different phenotypes in mice and humans or at different stages in the development of each. Yet other immune cells and factors have biphasic roles during flavivirus infection, contributing to protection during peripheral infection while having deleterious effects when the virus enters the CNS [187]. Most importantly, as with other mouse models, factors such as age, virus strain, inoculum dose and route of administration strongly influence the outcome of studies with KO mouse strains. Such variations have contributed to conflicting reports on the role of TNF-$\alpha$ receptor 1 and Toll-like receptor 3 (TLR3) in WNV neuroinvasion and encephalitis. One study [187] reported that mice with a genetic deficiency in TNF-$\alpha$ receptor 1 or TLR3 exhibited reduced BBB permeability and lower mortality rates following WNV infection. Conversely, two subsequent studies using mice lacking TNF-$\alpha$ receptor 1 or TLR3 reported that these factors were essential to protection from lethal WNV challenge and mortality rates in TNF-$\alpha$-receptor-1- or TLR3-deficient mice infected with WNV were significantly higher than those observed in wild-type mice [30, 158]. These examples highlight the need for caution when interpreting data obtained using genetic KO animals.

## Hamster model for flavivirus neuroinvasive disease

Although fewer immunological reagents exist for studying the neural pathogenesis of flaviviruses in non-murine models, advantages of the use of alternative models include secondary validation of pathogenic manifestations, exhibition of pathological manifestations for certain flaviviruses that are more representative of human disease, and a larger body size for which certain surgical procedures have proven easier to perform than when using mouse models. Although a number of non-murine models of flavivirus infection have been established, many of these systems have been developed to assess vaccine and therapeutic efficacy or ecological factors associated with increased potential for transmission of disease to humans. The vast majority of studies investigating neuropathogenesis in non-murine models have been performed with Syrian golden hamsters (*Mesocricetus auratus*) [143, 147, 159, 173, 176, 184, 193]. Although the hamster model has been used extensively for the assessment of immunological correlates of protection [16, 95], the study of the efficacy of vaccine [172] and therapeutic monoclonal antibodies and antivirals [110], and the modeling of persistent renal infection and renal shedding of WNV [176], this section will focus solely on the development and use of the hamster model for the study of mechanisms of flaviviral neuropathology and neuroinvasion.

Following the introduction of WNV into North America in 1999, an intraperitoneal inoculation WNV hamster model was established that demonstrated moderate viremia for a period of 5–6 days, after which CNS involvement, including incoordination, muscle weakness and lethargy, was observed, similar to murine models of WNV disease [193]. Histopathological analyses indicated involvement of the cerebral cortex, hippocampus, basal ganglia and brain stem and indicated that WNV antigen staining was correlated with pathological lesions. Neuronal apoptotic death was observed prior to perivascular cuffing and microglial infiltration, indicative of direct infection, and neuronal death was a prerequisite for secondary inflammatory responses [193]; however, alterations in BBB permeability were not demonstrated to be associated with neuroinvasion of WNV in this model [111]. In contrast, experimental inoculation of Syrian hamsters with SLEV genotypes I and III resulted in the development of viremia and encephalitic lesions without any outward signs of clinical disease [16].

Subsequent studies using the hamster model have demonstrated the role of axonal infection and transport of WNV for direct access to neural tissues [111, 143]. The use of the larger hamster as a model host has allowed direct experimental inoculation of the sciatic nerve [143, 184]. In addition to the role that Syrian golden hamsters have played in establishing the role of retrograde axonal transport in WNV neuroinvasion, hamsters have also proven useful for identifying alternative CNS infection routes for closely related members of the JEV complex. For instance, hamsters were used to demonstrate that SLEV can enter the CNS by an olfactory route of infection [106].

Additional studies using electromyography in WNV-inoculated hamsters have also provided correlations between autonomic dysfunction related to respiratory distress [112] and gastrointestinal [185] symptoms following WNV infection related to peripheral neuronal infection. This model has thus provided much insight not only into neuroinvasive

mechanisms of flaviviruses but also into subsequent pathological manifestations that can be attributed to motor neuron infection, death and viral persistence.

## Future directions

Small-animal models have proven to be valuable resources in understanding flavivirus encephalitis. In particular, the advent of KO mouse models have permitted detailed characterization of a number of host factors that influence disease or protection. New developments in mouse biology, such as the Collaborative Cross inbred mouse resource, may provide a rational approach for identifying suitable mouse strains to accurately model infection for flaviviruses that fail to elicit classical disease in existing laboratory rodent lines. Additionally, this resource may enable the discovery of novel combinations of host genes that conspire to elicit disease [109]. Continuing developments in our understanding of mouse biology will serve to foster further discoveries on the genesis of flavivirus pathology and virus-host interactions.

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