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REVIEW

Flaviviruses, an expanding threat in public health: focus on dengue, West Nile, and Japanese encephalitis virus

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Abstract The flaviviruses dengue, West Nile, and Japanese encephalitis represent three major mosquito-borne viruses worldwide. These pathogens impact the lives of millions of individuals and potentially could affect non-endemic areas already colonized by mosquito vectors. Unintentional transport of infected vectors (*Aedes* and *Culex* spp.), traveling within endemic areas, rapid adaptation of the insects into new geographic locations, climate change, and lack of medical surveillance have greatly contributed to the increase in flaviviral infections worldwide. The mechanisms by which flaviviruses alter the immune and the central nervous system have only recently been examined despite the alarming number of infections, related deaths, and increasing global distribution. In this review, we will discuss the expansion of the geographic areas affected by flaviviruses, the potential threats to previously unaffected countries, the mechanisms of pathogenesis, and the potential therapeutic interventions to limit the devastating consequences of these viruses.

Keywords Vaccines · Mosquito · Fever · Bioterrorism · Brain

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Introduction

The genus *Flavivirus* is composed of approximately 73 arthropod-borne viruses, or arboviruses, that infect rodents, pigs, birds, non-human primates, humans, and other mammalian hosts. Several members of this virus family which include the dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), St. Louis encephalitis virus, and yellow fever virus are associated with important human diseases which are transmitted by arthropod vectors. Hepatitis C virus is a notable exception as while it is related to the other medially important *flaviviruses*, it is one of the few members of this viral family that is not vector-borne. Most of these viruses can cause a wide variety of clinical manifestations and complications such as undifferentiated fever, capillary leakage–hemorrhagic disease, and encephalitis which can potentially lead to death. Most flaviviruses are zoonotic and depend upon non-human animal vectors for their survival, replication, and dispersal with the exception of DENV which propagates mainly in humans. While the evolutionary event that led to the increased spread of the viruses is still unknown, population movements, rapid urbanization, and widespread deforestation have contributed to the expansion of the pathogens into previously non-endemic areas (Bhatt et al. 2013; Petersen and Marfin 2005). In addition, burgeoning travel to endemic areas and the slow increase in global temperatures due to climate changes have allowed the expansion of DENV, WNV and JEV into new territories (Beatty et al. 2005; Caminade et al. 2012; Hansen 2006; Rahmstorf et al. 2007). Despite the adaptation, wild reservoirs of DENV are still maintained in subtropical and tropical areas that support a mosquito–monkey–mosquito transmission cycle (Gubler 2002). While most flaviviruses are endemic in tropical areas, the distribution of several members of the viral family such as WNV has extended to temperate areas including the USA within the past decade (Caminade et al. 2012).

Flavivirus epidemiology and global dispersal

Flaviviruses are important human pathogens that have plagued mankind, accounting for millions of mortality worldwide (Table 1). The first recorded epidemic of dengue-like disease was reported between 1779 and 1780 when outbreaks occurred in Asia, Africa, and North America (Gubler 1998, 2002). Since its emergence, four DENV closely related but genetically distinct serotypes have been identified (Zanotto et al. 1996). Each serotype is believed to have emerged from a common ancestor and evolved separately (Kawaguchi et al. 2003) despite possessing different antigenicity and causing different degrees of illness severities including dengue hemorrhagic fever (DHF; currently being changed to severe dengue as indicated by WHO). Variations within each serotype have been genetically detected, though not limited to, within the envelope protein (E) and non-structural protein, NS5, within the viral genome. WNV is being transmitted within the US population since the first reported case in 1999 (see Table 1 for details). Since then, the total number of West Nile virus infection cases has been increasing annually within the USA (http://www.cdc.gov/westnile/resources/pdfs/cummulative/99_2013_cummulativeHumanCases.pdf). Currently, DENV is endemic in at least 100 countries throughout Asia, the Pacific, the Americas, Africa, and the Caribbean (Fig. 1). Approximately one million infected individuals suffer from either dengue fever or dengue hemorrhagic fever annually with dengue-related deaths estimated to occur between 1 and 5 % of those infected as reported by WHO (http://whqlibdoc.who.int/publications/2009/9789241547871_eng.pdf) (see Table 1). DENV infections are often self-limiting and range from asymptomatic to relatively mild, undifferentiated illness, with the patient eventually making full recovery. However, approximately 5 % of symptomatic cases progress to a more severe disease whose manifestations include fever, myalgia, vomiting, and acute abdominal pain. The symptoms then progress further to hypotension, tachycardia, decreased peripheral perfusion, perisoreositis, and myocarditis (Krishnamurti et al. 2001; Malavige et al. 2004). In addition to these symptoms, hemorrhage develops approximately 7 days post-infection with no observed circulatory collapse (Krishnamurti et al. 2001). Factors influencing disease severity are not well understood. A predisposition to severe illness in secondary infections due to antibody-dependent enhancement of infection is often cited (Kliks et al. 1989).

In the USA and its territories, DENV transmission primarily occurs in tropical and subtropical areas such as Puerto Rico, the US Virgin Islands, American Samoa, and the USA-affiliated Pacific Islands (Imrie et al. 2006; Mohammed et al. 2010a; Rigau-Perez et al. 2001; Tomashek et al. 2009), where dengue is endemic. Dengue has been found to be the most frequent cause of febrile illness among US

Table 1 Flavivirus epidemiology, infection, and complications

Virus	At risk individuals	Annually reported new infection cases	Symptoms	Neurologic disease	Mortality rate
Japanese encephalitis virus (JEV)	Children–young adult (0–15 years old)	35,000–50,000 new cases (in JEV-endemic countries)	Pyrexia, cephalalgia, vomiting, neurologic dysfunction, muscle weakness, seizures, loss of motor function	Parkinsonism Flaccid paralysis Coma Acute encephalitis	~25–30 %
Dengue (DENV)	No age distinction	One million cases (globally)	Pyrexia, myalgia, vomiting, cephalalgia, abdominal pain, bleeding, low blood pressure, tachycardia, seizures	Encephalitis	~1–5 %
West Nile virus (WNV)	Adults and immunocompromised persons (≥50 years old at greater risk of developing more severe disease and complications)	Ranging from 20 to 5,674 cases annually between 1999 and 2012 in the USA; reports of WNV infection and mortality rates reported in Canada and Mexico	Fever, cephalalgia, nausea, vomiting, myalgia, muscle weakness, lower back pain, loss of motor function	Encephalitis Meningitis	~3–15 %

Data collected from the Centers for Disease Control (www.cdc.gov) and review by Malavige et al., Post-Graduate Medical Journal 80(948):588–601, http://www.cdc.gov/westnile/resources/pdfs/cummulative/99_2012_CasesAndDeathsClinicalPresentationHumanCases.pdf, and http://www.cdc.gov/westnile/resources/pdfs/cummulative/99_2012_cummulativeHumanCases.pdf

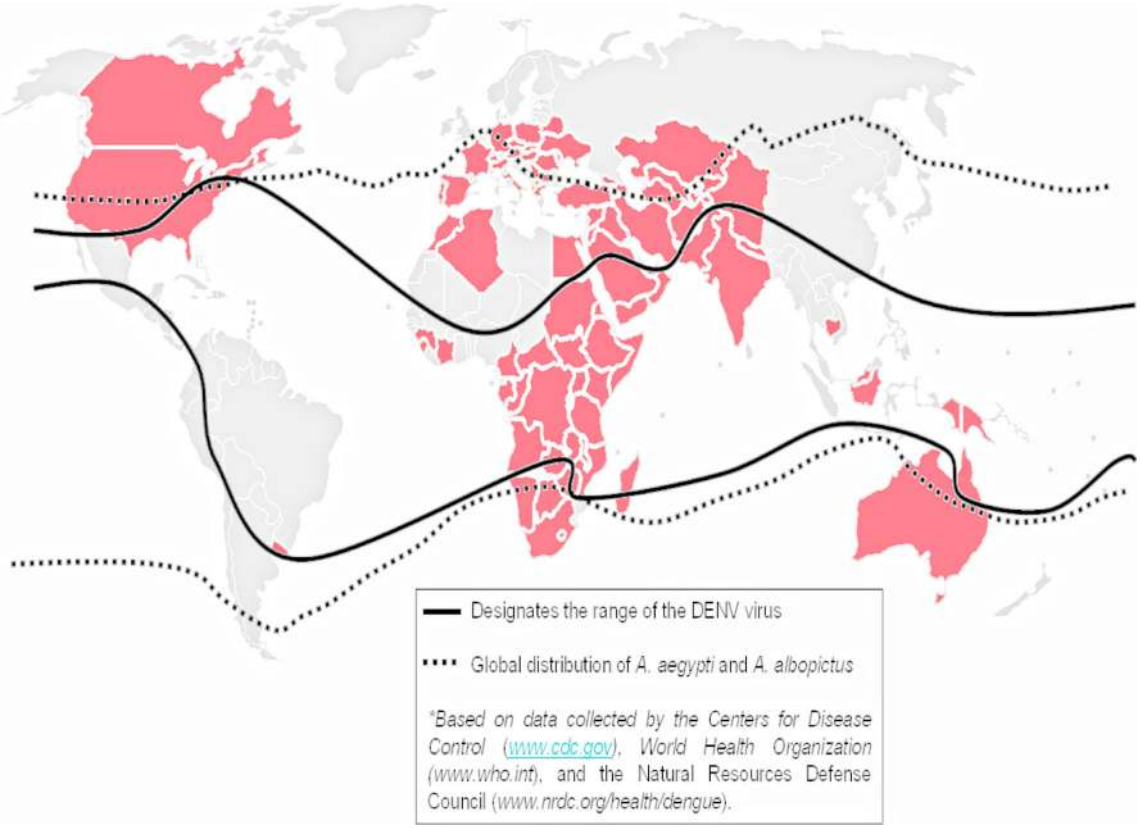

Fig. 1 Global distribution of DENV in relation to its arthropod vectors. DENV is endemic in parts of Asia, Australia, Africa, and Latin America (area between the *solid lines*) and slowly expanding to other parts of the globe. Currently, DENV is located within the distribution range of *A. aegypti* and *A. albopictus* (*dotted line*). The presence of these mosquito vectors in non-DENV endemic areas suggests the potential for the expansion of the virus into new regions if optimal conditions are present. Countries highlighted in red represent areas that are endemic to WNV or have reported cases of the virus. Similar to DENV, the expansion of WNV is due to numerous reasons which include increased travel to WNV endemic areas and the introduction of suitable arthropod within these regions

travelers returning from Asia, Latin America, and the Caribbean (Freedman et al. 2006; Mohammed et al. 2010b; Sharp et al. 2012). In addition, outbreaks of dengue occur sporadically in non-endemic areas where the mosquito vectors exist (Brunkard et al. 2007; Graham et al. 2011; Radke et al. 2012; Ramos et al. 2008), Hawaii (Effler et al. 2005), and Florida (Radke et al. 2012). Meanwhile, WNV infections have been reported throughout the continental USA. While the risk of WNV infection within various age groups remains the same, the potential for developing neurologic complications due to WNV infection increases with age. In the past 10 years, approximately 40,000 individuals have become infected with WNV in the USA (http://www.cdc.gov/westnile/resources/pdfs/cummulative/99_2013_cummulativeHumanCases.pdf), of which ~20 % developed neuroinvasive diseases (i.e., encephalitis and meningitis) with 12 % fatality rate (Lindsey et al. 2010). Interestingly, ~80 % of WNV-infected individuals are asymptomatic.

Within days following infection with JEV, the host begins to exhibit clinical manifestations of the disease beginning with cephalalgia, vomiting, and pyrexia which last approximately 1 week (Sarkari et al. 2012b), after which neurologic disorders ensue, hallmarked by Parkinsonism, flaccid paralysis, and coma. Up to one third of infected patients acquire acute encephalitis, which can often lead to complications and patient fatality (Lee et al. 2012; Sarkari et al. 2012a, b). Indeed up to 50 % of patients who contract the virus often die due to complications. Interestingly, patients who enter convalescence remain seropositive against JEV despite persistent viremia (Ravi et al. 1993). Currently, there are no reported JEV cases within the USA.

Distribution of viral vectors

DENV is transmitted to humans by the mosquito vectors *Aedes aegypti* and *Aedes albopictus*. Similarly, the global dissemination of WNV (Fig. 2) and JEV (Fig. 3) relies on the *Culex* species of mosquitoes for their dispersal, in particular *Culex quinquefasciatus*. Unlike WNV, JEV is currently localized in parts of Asia and surrounding islands and the northern region of Queensland, Australia. These mosquitoes, in particular *A. aegypti*, have become widely distributed across tropical and subtropical areas, including vast areas of the USA (Figs. 1 and 2). The spread of the DENV in the USA

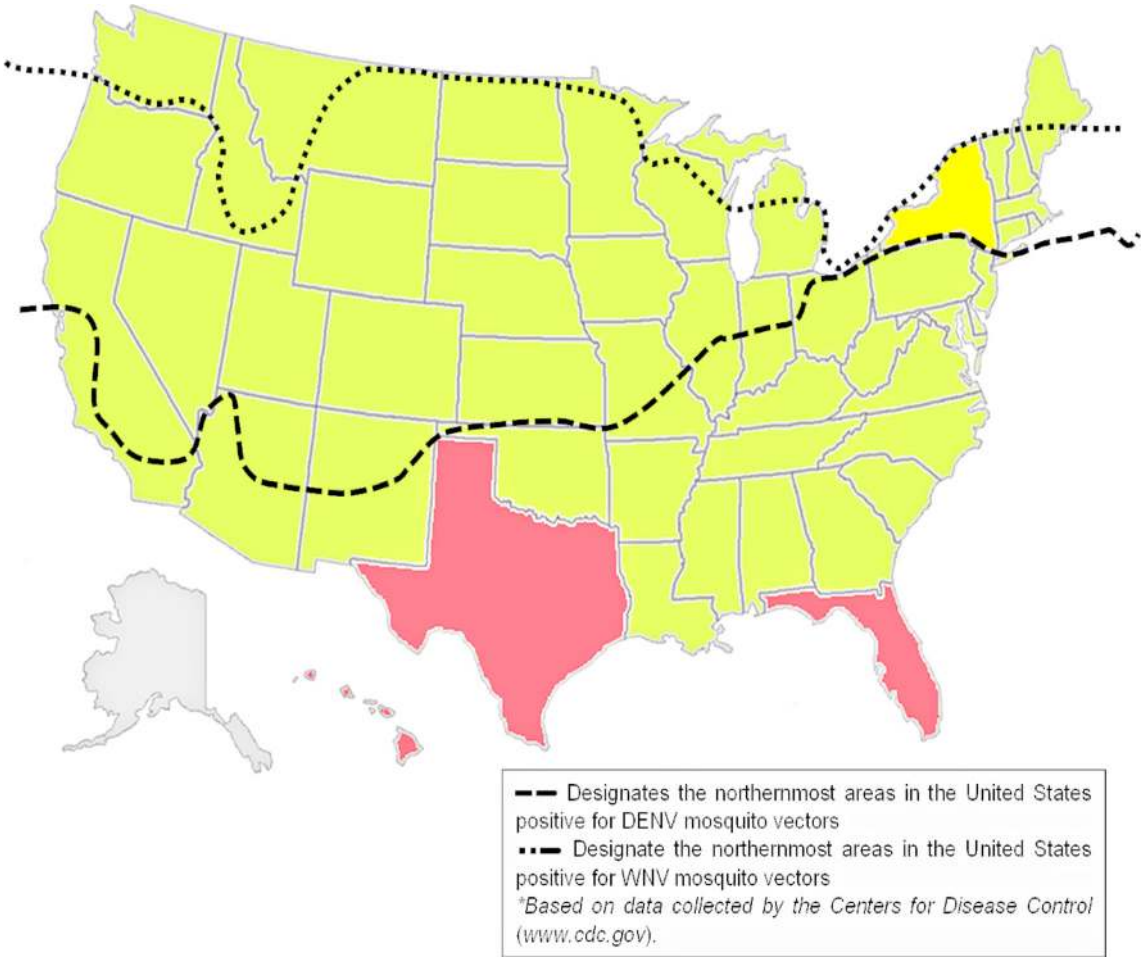

Fig. 2 Distribution of WNV and DENV infections have been reported in the USA. To date, WNV infections (*green*) have been documented throughout the USA with the exception of Alaska (*gray*). Texas, Florida, and New York are currently the only states in which both WNV and locally acquired DENV (*red*) have been reported. While some cases of DF have been reported in New York (*yellow*), DENV infections have been ruled out as travel-associated cases. The *dotted line* designates the northernmost distribution of the WNV mosquito vector, *Culex* sp., while the *dashed line* illustrates the northernmost territory of the DENV mosquito vector, *Aedes* sp., in the USA

is exacerbated by the expanded range of *A. albopictus* in recent years, reaching as far as New England (Fig. 2) (Mousson et al. 2005). In addition, mosquito species capable of transmitting WNV have also been discovered to be as far north as Canada (www.hc-sc.gc.ca). The spread of WNV as well as DENV is facilitated by the dispersal of suitable arthropod vectors which has been accelerated by rapid urbanization, increased travel into endemic countries, and adaptation to climate changes. Together these events are expected to contribute to increases in the number of individuals affected by these pathogens (Anders et al. 2011; Petersen and Marfin 2005).

Typically, flaviviral transmission into humans occurs within 2 weeks of viremia following the initial feeding from an infected host. After entering a naïve mosquito in the blood meal, the virus will require an additional 8–12 days of incubation before it can be re-transmitted to another human. Once infected with the virus, the mosquito remains infectious for the remainder of its life. Symptoms among infected human hosts typically develop within 7 days after the mosquito bite and lasts between 3 and 14 days. While some individuals do not develop any significant symptoms, they can still successfully transmit these viruses to others via mosquitoes. DENV is unique among other flaviviruses as they are the only virus within the family which utilizes humans as its amplifying host. Among WNV and JEV, viral amplification has been documented to occur within various mammalian hosts including horses, sheep, pigs, and goats; in the case of JEV, the virus has also been reported in bats as detected by qPCR (Liu et al. 2013; Olaleye et al. 1990; Pauvolid-Correa et al. 2011; Peiris et al. 1992). The expansion of WNV and JEV across different geographical regions is believed to be mediated by the seasonal migration of birds (Reed et al. 2003).

Dengue is endemic to many parts of the tropics and subtropics, with outbreaks closely correlated with the completion of the annual monsoon season as reported by the WHO. During this time, an increase in the *Aedes* sp. mosquito population is also typically observed. Furthermore, the risk of contracting DHF also increases as more humans become

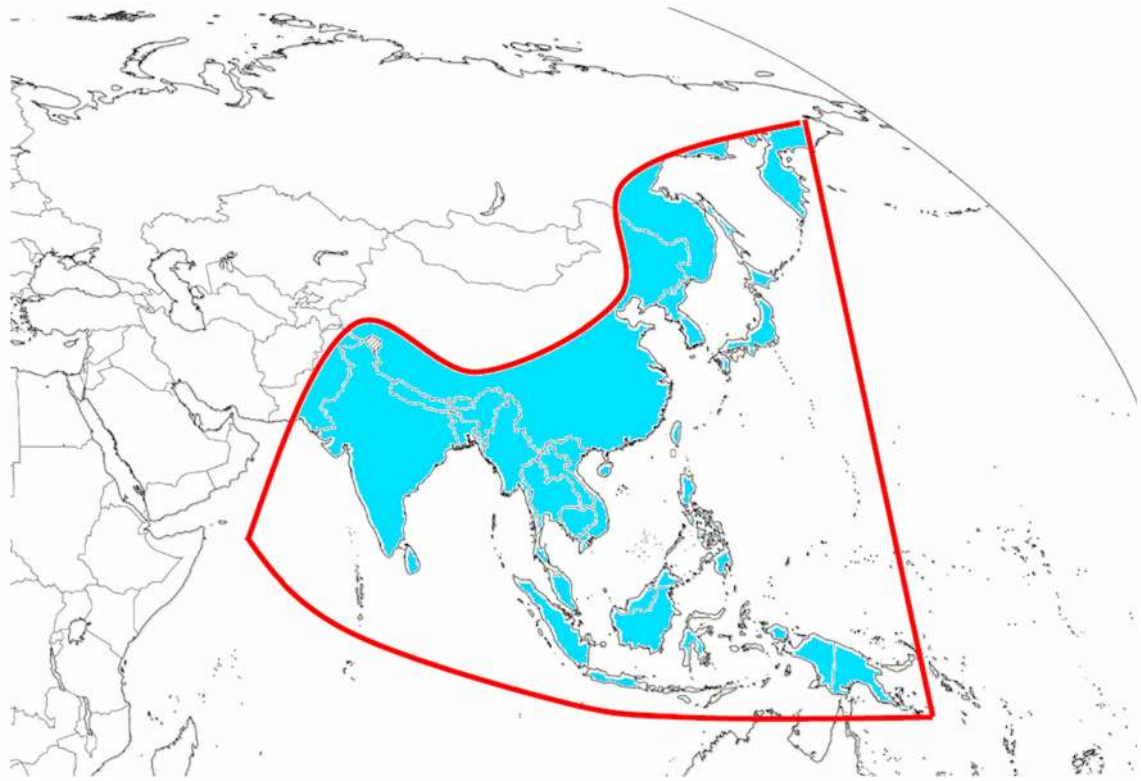

Fig. 3 Distribution of JEV. JEV is currently endemic within south, east, and southeast Asia (*highlighted in blue*) and its surrounding islands, indicated by the *red border*. To date, there are no reported cases of JEV outside of this region; however, the expansion of its mosquito vector, *Culex* sp., to other countries worldwide makes spreading of this dangerous pathogen a significant threat

infected with the virus during this short time period. A combination of optimal environmental conditions that facilitate an increase in infected arthropod vectors, greater presence of individuals with no immunity to one of the four virus types (DENV-1–4), and an opportunity for infected vector–host contact are required for the onset of DENV and other flavivirus epidemics. Interestingly, despite the induction of lifelong protective immunity against one DENV serotype, protection against the virus is partial and transient against other serotypes (Chen et al. 2004). Secondary infection by a different DENV serotype often leads to increased severity and carries a higher risk of susceptibility to DHF and patient mortality. DHF is a life-threatening illness characterized by internal bleeding, serious brain compromise, organ failure, and eventually death. In some cases, dengue shock syndrome is observed among DF and DHF patients. While it is generally accepted that tertiary and quartenary DENV infections are asymptomatic, the lack of clinical data that accurately determine multiple DENV infections among affected individuals and the importance of ADE make the surveillance of the virus and its treatment important. There are currently no approved vaccines or therapies for the disease. Treatment of infected patients is mostly palliative, with an administration of fluids and an occasional blood transfusion in the event of severe hemorrhage. The lack of treatment for DENV and related flaviviruses (i.e., WNV and JEV) makes these diseases debilitating and deadly.

In the past, DHF cases in the USA were only observed among travelers who have returned from visits to dengue-endemic areas. However, data from the Centers for Disease Control (CDC, http://www.cdc.gov/dengue/) and the National Resources Defense Council (NRDC, http://www.nrdc.org/health/dengue/) indicate that localized cases of DENV infection have already been detected in Hawaii, Texas, Florida, and New York along with the mosquitoes that are responsible for harboring the virus (Fig. 2). While the US population currently has no immunity to the virus due to infrequent interactions between infected individuals and viral vectors which are necessary for successful dissemination of DENV, the presence of DENV within the USA suggests the imminent risk of infection among millions of Americans. Furthermore, the rapid spread of the WNV throughout the USA suggests the possibility for DENV to become prevalent within the US given the proper existing conditions.

Flavivirus genetics and life cycle

Flaviviruses are composed of a single-stranded positive-sense RNA genome (~11 kilobases) packaged into a 40–60-nm virion comprising of a spherical nucleocapsid core coated in an icosahedral envelope (Harris et al. 2006; Rodenhuis-Zybert et al. 2010). The entire flaviviral genome consists of ten genes

encoding a large polyprotein that is post-translationally cleaved by host and viral proteases to produce three structural (protein capsid, C; prM/M protein, and envelope protein, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Fig. 4) (Leyssen et al. 2000). The NS region encodes for various important proteases, in particular NS3 and NS2B, which are important in the auto-processing of the virus, mediation of viral genome replication, and virus packaging (Clyde and Harris 2006). The transcription of these genes is controlled by the 5′- and 3′-untranslated regions (UTR) flanking the viral genome (Appaiahgari and Vrati 2012b; Leyssen et al. 2000; Lindenbach et al. 2007).

The mechanisms of flaviviral infection of the host cell and its life cycle are not fully understood. The current consensus is that endocytosis of the viral particle is important in the successful infection of the cell and the production of progeny viruses (Fig. 5). Attachment of the DENV and other flaviviruses to cells utilizes multiple potential receptors that facilitate the attachment and internalization of the virus (i.e., CD14, R80, heparin sulfate, C-type lectin receptors, DC-SIGN, and mannose receptors) (Rodenhuis-Zybert et al. 2010); however, the exact mechanism by which DENV and other flaviviruses use these molecules for cellular internalization is still under investigation. The attached virus is internalized into an endosomal compartment which acidifies to facilitate the fusion of the viral envelope with the endosomal compartment (Fig. 5). This fusion of the viral envelope is due to the rearrangement of the capsid proteins, resulting in the release of the virus into the host cell (van der Schaar et al. 2008). The viral RNA is released into the host cytoplasm and transported to the endoplasmic reticulum (ER) where it undergoes two different fates: first, the positive sense RNA is translated to produce a polyprotein that is post-translationally cleaved into structural and non-structural proteins (listed earlier) or, second, the genetic material is converted into a negative sense RNA by viral NS5 RNA-dependent RNA polymerases (RdRp) and used to produce positive-stranded RNA copies (Fig. 5). The viral genome is then packaged within the cytoplasm by the action of protein C to form the nucleocapsid, while the prM and E proteins heterodimerize within the lumen of the ER and initiate viral budding (Kuhn et al. 2002; Zhang et al. 2004). Nascent virion particles formed within the ER travel through the secretory pathway and into the Golgi apparatus. Changes in pH within the trans-Golgi network trigger the dissociation of the prM/E heterodimers activating the cellular endo-protease furin. Activation of this protease leads to cleavage of the prM protein to generate protein M (membrane associated) and the peptide pr (Rauscher et al. 1997; Yu et al. 2008; Zybert et al. 2008). The cleavage of this protein complex results in a mature, fully infectious virion.

Immunopathogenesis and central nervous system pathogenesis

Studies of DENV immunopathogenesis are highly limited due to the nature of the disease and lack of animal models that recapitulate the disease. However, results obtained from humanized mice show some similarities in the development and progression of the disease which is typically observed in humans. Thus, these models will play an important role in elucidating flaviviral pathogenesis now and in the future (Mota and Rico-Hesse 2011). Epidemiological studies have identified some of the risk factors important for disease development and severity following flaviviral infection. These factors include age (younger people are most susceptible to DENV and JEV infection, while older or immunocompromised individuals are affected by WNV), high body mass index, viral strain, gender, genetic variation of the major histocompatibility complex (MHC)-class I-related sequence B, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), tumor necrosis factor-α (TNF-α) and phospholipase C epsilon 1 genes, environmental conditions such as mosquito spread and temperature, and secondary infection with a different viral strain (in the case of dengue) (Anders et al. 2011; Khor et al. 2011; Nguyen et al. 2005; Simmons et al. 2012).

In DENV infection, the serotype-specific life immunity acquired following the initial infection by the virus does not provide complete protection to other infecting serotypes. In fact, subsequent DENV infections increase the development

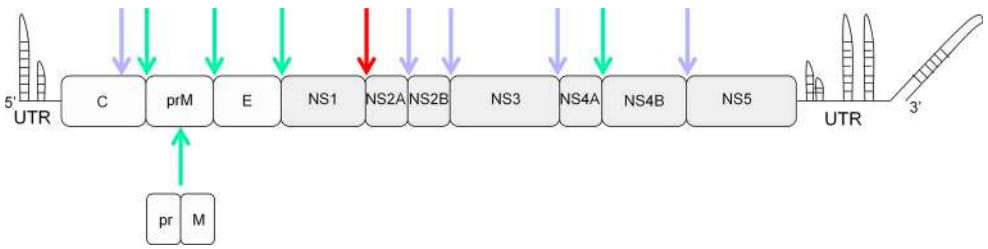

Fig. 4 Flavivirus genome. The *flavivirus* genome consists of a single-stranded positive-sense RNA encoding a polyprotein post-translationally cleaved by host proteases (sites designated by the *blue arrows*) and viral proteases (sites designated by *green arrows*). The site designated by the *red arrow* is cleaved by a yet to be identified protease. Processing of the polyprotein produces three structural (*white boxes*) and seven non-structural genes (*gray boxes*). The prM protein is then later cleaved within the Golgi to release the M protein important for the maturation of the virus. Translation of viral RNA is controlled by the untranslated regions (*UTR*) located at the 5′- and 3′-ends of the RNA

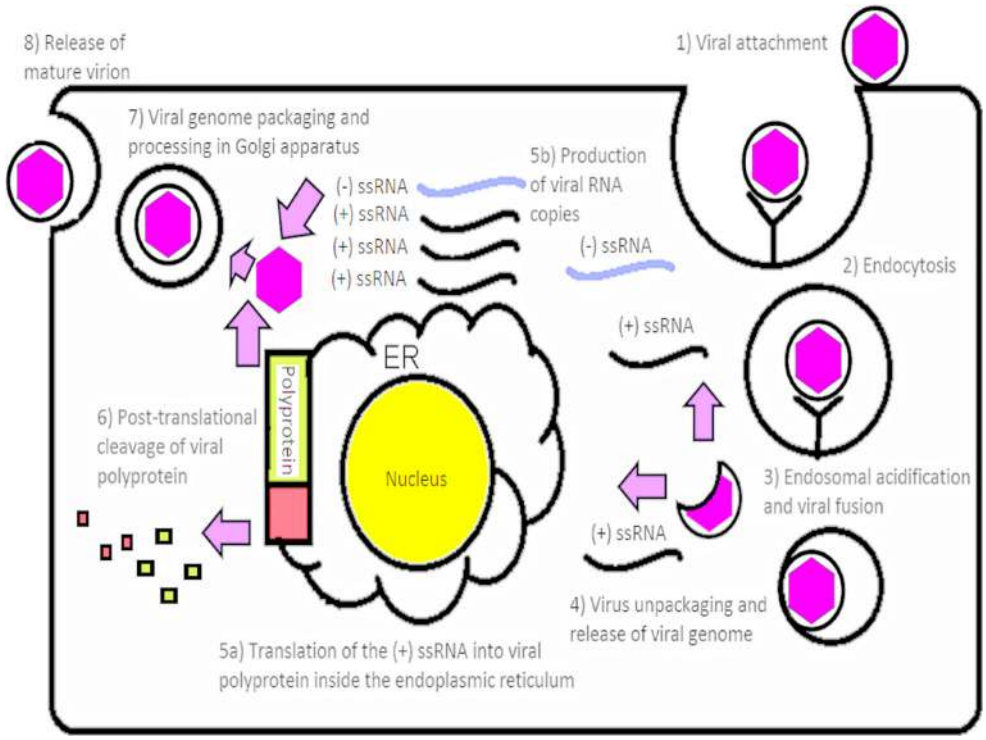

Fig. 5 Flavivirus life cycle. Following attachment to the host extracellular surface (*1*), the virus is endocytosed (*2*) and encapsulated inside an endosomal vacuole. Acidification of the endosomal compartment alters the E protein, causing the fusion of the virus with the endosome (*3*), facilitating virion release into the intracellular compartment where it is unpackaged (*4*). The released viral genome undergoes two different fates: the viral genome is either transported to the endoplasmic reticulum where it is translated into a polyprotein (*5a*) or converted into a negative-sense RNA to make positive-sense RNA copies (*5b*). The large polyprotein is post-translationally processed (*6*), producing structural and non-structural components important for virus assembly and maturation. The viral genome is packaged into a capsid and transported to the Golgi where it is coated by the E/M protein complex (*7*) to produce a mature virion (*8*)

of more severe dengue illnesses such DHF (Halstead et al. 2002; Kliks et al. 1988). The increased disease severity during secondary DENV infections is potentially due to the role of memory cells and antibodies. This is the concept for antibody-dependent enhancement (ADE) of secondary dengue infections (Halstead and O'Rourke 1977). During ADE, antibodies generated after the initial infection recognize different DENV serotypes during secondary infections and facilitate increased binding and internalization of the virus via the Fc receptors on leukocytes and blood–brain barrier (BBB) cells. The adherence and infection of the BBB by DENV leads to inflammatory cytokine and chemokine production (i.e., TNF-α, MCP-1/CCL2, IL-2, IL-6, IL-8, IL-10, and IL-12) as well as other inflammatory factors that lead to BBB compromise and CNS dysfunction (Anderson et al. 2011; Chaturvedi et al. 2000; Chen 2012; Gubler 1998; Malavige et al. 2004; Priyadarshini et al. 2010; Restrepo et al. 2008a, b). This provides potential targets for therapeutic intervention against succeeding DENV infections.

Among leukocyte populations, dendritic and natural killer (NK) cells are the first ones to become infected. The infection of these cells occurs through pathogen-recognizing receptors, such as toll-like receptors and DC-SIGN, and elicits inflammatory signals that lead to a Th_2 immune response and eventual vascular barrier dysfunction (Chaturvedi et al. 2000). Monocyte/macrophages and lymphoid cells (T and B cells) have also been described as major preferential targets for flaviviruses due to the presence of viral RNA or proteins in infected cells which have been collected from lymphoid tissues (Durbin et al. 2008; Jessie et al. 2004; King et al. 1999; Lin et al. 2002; Mentor and Kurane 1997; Srikiatkhachorn et al. 2012). The high viral titers within the germinal centers of these tissues also suggest that these sites can harbor viral particles that can disseminate and infect other sites within the host. Interestingly, flaviviruses have developed strategies to circumvent the host immune response limiting clearance either by inhibiting IFN secretion (Munoz-Jordan et al. 2003; Umareddy et al. 2008) or by reducing antigen presentation through the limitation of MHC and co-stimulatory molecule expression (Palmer et al. 2005). The loss of these molecules will lead to an impairment of $CD4^+$ T lymphocyte activation and $CD8^+$ T lymphocyte response and thus leading to the loss of an immune response against the viruses and disease development (Aleyas et al. 2009, 2010; Chase et al. 2011).

The in vivo cellular targets of DENV and other flaviviruses in the CNS remain to be fully characterized; however, our laboratory and others have observed that primary human astrocytes and brain microvascular endothelial cells can be infected by DENV (Daep, Munoz-Jordan and Eugenin, unpublished

data). Given the potential importance of astrocytes as HIV reservoirs within the central nervous system (CNS) (Eugenin and Berman 2007; Eugenin et al. 2011), these cells may also function to maintain DENV reservoirs within the brain. To date, the full mechanism of flavivirus infection of the CNS remains poorly understood due to early studies which did not detect pathological signs of viral invasion within the CNS (Burke 1968; Nathanson and Cole 1970). Deeper examination of CNS pathology following DENV infection, however, will provide groundbreaking information that will elucidate the role of the blood–brain barrier, leukocytes, and CNS inflammation following flaviviral infection in the development of dengue fever (DF) and DHF.

Older manuscripts indicate that DENV antigens can be found in several tissues including neurons in the cerebrum, Purkinje's and granular cells in the cerebellum, astrocyte, microglia, and cells of the choroid plexus (Bhoopat et al. 1996a). However, earlier reports describing DHF pathology indicates no brain compromise and clearly stated that minimal damage was observed (Burke 1968; Nathanson and Cole 1970) despite the devastation of the CNS following infection by DENV and other flaviviruses. In addition, positive detection of flaviviral antigens without viral RNA (Jessie et al. 2004) indicated viral uptake by endocytosis and/or phagocytosis and not by productive infection that result in progeny virions. Recent improvements in research techniques could provide detection assays with increased sensitivity and improved accuracy. In agreement, recent publications have demonstrated the presence of dengue antigens in the CNS due to the detection of viral proteins, immunoglobulin, and RNA (Araujo et al. 2011; Lima et al. 2011; Miagostovich et al. 1997a, b). Viral infiltration and subsequent infection of the CNS have been linked directly to CNS viral replication. Thus, the mechanism of invasion of the CNS requires reexamination and further studies. Later in this review, we will discuss all these mechanisms.

Role of leukocytes in CNS disease

Although there is a general consensus that flaviviruses can infect mononuclear cells in vivo, only few cell types have been identified to be targeted for infection in vitro. Indeed autopsy studies clearly indicated that presence of flaviviruses within the Langerhan's cells located in the skin (Clyde et al. 2006). However, the participation of these or other cell types in flaviviral pathogenesis has yet to be fully explored. Subsequently, we will discuss the evidence of leukocyte infections by flaviviruses.

Dendritic cells

Following a bite from an infected mosquito, flaviviruses including DENV, WNV, and JEV are inoculated into the human body and immediately interact with resident dendritic cells (DC) found in the epidermis and dermis layers of the skin. These cells originate from the bone marrow as $CD34^+$ progenitors and later differentiate into lymphoid/plasmacytoid (pDC; IL-3- and CD40 ligand-dependent maturation) or myeloid lineage (mDC; GM-CSF-dependent maturation). Following entry of DENV into DC, it has been observed that the total number of circulating DCs decreases and unresponsive thereby altering the host immune response. Furthermore, in vitro experiments utilizing mature PBMC-derived DCs showed an altered profile of inflammatory production (Hober et al. 1996a, b). However, infiltration of infected DCs into the lymph nodes activates $CD4^+$ and $CD8^+$ T lymphocytes and elicits an adaptive immune response against the virus. Thus, flaviviruses use DCs as gateway cells to infiltrate and successfully infect their human host.

Monocyte/macrophages

Monocytes are one of the natural hosts of DENV (Durbin et al. 2008) and are implicated in the pathogenesis of DF and DHF (Halstead 1988; Kliks et al. 1989). Interestingly, depletion of monocytes from murine models resulted in a tenfold increase in viral load, suggesting that monocytes are also important in controlling viral infection (Fink et al. 2009). DENV infection also accelerates the differentiation of monocytes into macrophage and facilitates cellular transmigration into the CNS where inflammatory cytokines, chemokines, viral proteins, and other inflammatory factors are produced. Inflammation within the CNS leads to compromise and loss of endothelial function and potentially BBB dysfunction. Indeed DENV-infected $CD16^+$ monocytes produce IL-1β, TNFα, CCL2, CCL3, and CCL4 (Azeredo et al. 2010). These cytokines and chemokines have been shown to be tightly involved in the loss of BBB integrity and the development of CNS disease in the context of HIV CNS infection (Roberts et al. 2012a). These observations are also consistent with other viral diseases that compromise the CNS; for instance, it has been reported that HIV-infected patients have an elevated $CD14^+CD16^+$ monocyte population which can transmigrate into the CNS in response to CCL2 and produce inflammatory factors that disrupt the BBB (Eugenin et al. 2006b; Williams et al. 2012). However, to date, there are very few studies describing the neuropathogenesis of flaviviruses in this cell linage.

Lymphocytes

The role of lymphocytes in disease development following flaviviral infection has not yet been fully characterized due to various observed conundrums within the disease pathogenesis. In vitro studies have shown that DENV can infect as well as activate both $CD4^+$ and $CD8^+$ T cells (Mentor and Kurane 1997;

Pang et al. 2007). Indeed DENV-specific $CD4^+$ and $CD8^+$ T cells have been identified among patients with DF (Gagnon et al. 1996), with the latter lymphocyte population important for controlling viral spread and replication within the host. However, studies have shown that $CD8^+$ cytotoxic lymphocytes also contribute to DF pathogenesis and thus negatively impacting the health of the infected individual. The arrival of $CD8^+$ T cells at the infection sites initiates the destruction of infected cells, facilitating further production of inflammatory factors that lead to CNS damage and vascular endothelial dysfunction, resulting in BBB permeability (Mongkolsapaya et al. 2003; Rothman 2009; Screaton and Mongkolsapaya 2006). By inducing the production of inflammatory cytokines, chemokines, and other immune factors (e.g., IFN-γ TNF-α, IL-1β, IL-2, IL-4, Il-6, IL-7, IL-8, IL-18, CCL2, interferons, and several soluble proteins such as CD4, sTNFR, and CD8) during primary and secondary infections, the severity of the disease escalates and leads to the vascular permeability observed during DHF (Azeredo et al. 2010; Davis et al. 2008a; Hober et al. 1993; Kurane et al. 1991a, b). This supports the idea that T cell-produced inflammatory molecules are key factors that facilitate disease development following flavivirus infection, with some promoting vascular permeability. This results in compromised BBB integrity and CNS dysfunction. Interestingly, despite JEV, WNV, and DENV belonging in the flaviviral family and causing similar outcomes, the viruses differ in their effects on the host immune system. Indeed it has previously been shown that IFN-γ activity is inhibited by DENV by targeting STAT2 degradation, whereas JEV and WNV do this by inhibiting STAT1 (Ashour et al. 2009; Laurent-Rolle et al. 2010; Lin et al. 2006; Mazzon et al. 2009).

B lymphocytes infected by DENV have been detected in both the spleen and bone marrow (Durbin et al. 2008; King et al. 1999; Lin et al. 2002), with active viral replication verified by PCR and Western blot analyses (Lin et al. 2002). Similar to T lymphocytes, the exposure of B cells to DENV in vitro induced the production of inflammatory cytokine and DENV-specific immunoglobulins, both of which have been shown to be important in DENV pathogenesis and DF development. Since antibody production is essential in amplifying infection and inflammation, this suggests that the dysregulation of B cells is critical because they normally produce immunity against the serotype-specific E protein antigen (Mathew et al. 2011). During secondary infection, B lymphocyte response is predominately cross-reactive to other serotypes, with the produced heterologous antibodies having greater avidity for the virus than homologous antibodies. This increased avidity for the pathogen is important during ADE of viral infection as observed in DENV pathogenesis (Lin et al. 2002).

During ADE of virus infection, DENV utilizes memory antibodies generated from previous DENV infection to bind to Fc receptors located on the extracellular surface, facilitating their entry into the host cell. Interestingly, vaccination against any DENV serotypes results in full cytotoxic activity, cell proliferation, and controlled secretion of regulatory cytokines (Dharakul et al. 1994; Kurane et al. 1989; Malavige et al. 2004). While vaccination with the right serotype can evoke an effective immune response, some B cells produce autoantibodies that undermine immunity and contribute to DENV pathogenesis (Malavige et al. 2004). Thus, B cells are targeted for DENV infection and contribute to disease development.

Similar to DENV, JEV infection elevates the levels of inflammatory molecules and chemokines (e.g., IFN-α, IFN-γ, IL-6, IL-8, IL-10, CXCL19, CXCL10, CXCL11, TNF-α, MIF, VEGF, sCD4, sCD8, TNFR, sIL-2, IL-1RA, and TGF-β) within the host. In addition, the secretion of matrix metalloproteinase-9 (MMP-9) at sites of infection (Tung et al. 2010), especially within the CNS, has been shown to be important in facilitating the breakdown of the endothelium, leading to plasma leakage and BBB destruction. Similarly, the activation of lymphocytes and increased number of $CD14^+$ cells following JEV infection has been correlated with the severity of the disease (Paessler and Walker 2013; Rothman 2009). Identifying mechanisms behind these changes is essential in targeting and designing therapeutic interventions that limit the devastating consequences of flavivirus infection.

Blood–brain barrier dysfunction and CNS compromise

The blood–brain barrier is a physical and highly specialized barrier separating the peripheral circulation from the CNS. This barrier is composed of multiple cell types, including brain microvascular endothelial cells (BMVEC), astrocytic end feet, pericytes, and perivascular macrophages, all of which are in close contact to neurons, glial cells, and microglia (Spindler and Hsu 2012b). Due to the high expression of tight junction proteins, the BBB is normally impermeable to most peripheral molecules and cells and restricts the diffusion of ions and small molecules.

Several viruses alter the BBB integrity and function, including HIV-1, human T lymphotropic viruse 1 (HTLV-1), lymphocytic choriomeningitis (LCMV), WNV, and simian immunodeficiency virus (SIV). The mechanism of BBB compromise involves transmigration of leukocytes and these viruses into the CNS parenchyma, leading to BBB disruption, inflammation, and leukocyte transmigration into the CNS (Buckner et al. 2006; Eugenin and Berman 2003; Eugenin et al. 2006b; Spindler and Hsu 2012a; Williams et al. 2012). HIV transmigration and BBB disruption are the best examined cases of CNS viral invasion. Our laboratory and others have demonstrated that HIV infection alters BBB function by three different mechanisms: (1) alteration of the migratory properties

of leukocytes, (2) changes in BBB permeability, and (3) secretion of factors that compromise leukocyte migration and BBB integrity. We and others have also shown that HIV infection of leukocytes alters the expression of several adhesion molecules involved in leukocyte transmigration and increases the levels of important chemokine receptors such as CCR2, which increases the sensitivity to CCL2, a key chemokine involved in the pathogenesis of HIV CNS disease (Eugenin et al. 2006b; Roberts et al. 2012b). In addition, we demonstrated that BBB permeability among neuroAIDS patients is compromised at two stages: first, during productive HIV infection of leukocytes and, second, following increased expression of CCL2 in the CNS. This combination results in BBB disruption potentially due to enhanced secretion of MMPs and other factors that highly compromise the expression and function of key barrier tight junction proteins such as occludin, claudin-1, and ZO-1 (Roberts et al. 2012a, b). Some factors involved in HIV neuropathogenesis include cleaved forms of adhesion molecules, such as PrP^{c} and PECAM-1, with the latter competing with the PECAM-1–PECAM-1 or PrP^{C}–PrP^{C} interactions between endothelial cells. This direct competition leads to destabilization of the endothelium, compromising BBB integrity, thereby facilitating leukocyte migration into the CNS (Eugenin et al. 2006a; Roberts et al. 2012a). However, our experiments using primary BMVECs indicate that CCL2 binding to its corresponding receptor alone has profound effects on tight junction protein expression and localization, resulting in BBB compromise by altering β-catenin distribution and interaction with adherence junctions and PECAM-1 (Roberts et al. 2012a, b). Thus, HIV CNS invasion is a highly regulated process and involves several host and viral components, but whether these mechanisms are similar in flaviviruses is totally unknown.

Among the best described cases of CNS infection involves WNV. Due to the close relation of WNV with DENV and JEV, we expected the mechanism of neuropathogenesis of these viruses to be similar (Fig. 6). WNV attacks the brain, causing neurotropic effects that include encephalitis and fever. In mouse models, WNV-associated neurologic disease is characterized by BBB disruption, increased leukocyte infiltration, inflammation, and neuronal loss (Glass et al. 2005; Samuel and Diamond 2006). Some of these effects have been associated with the loss of BBB function due to degradation of junctional proteins such as ZO-1, claudin-1, occludin, JAM-A, β-catenin, VE-cadherin by secreted metalloproteinases (MMP) including -1, -3, and -9 (Roe et al. 2012a, b). In addition, WNV infects human BMVECs, which could lead to a compromised BBB by inflammatory mechanisms described previously (Verma et al. 2009). Experiments using WNV-infected endothelial cells indicate an up-regulation of MHC–I and II, ICAM-1, VCAM-1, and E- and P-selectin (King et al. 2003), which can contribute to leukocyte adhesion to the BBB and leukocyte infiltration into the CNS. During WNV infection, $CD19^{+}B220\text{-}BST\text{-}2^{+}$ leukocytes have been described as a major leukocyte population that transmigrates into the CNS and contributes to the development of encephalitis (Brehin et al. 2008). However, the mechanisms by which these cells transmigrate into the CNS remain unknown.

Flavivirus infection of the CNS, especially with DENV, has been reported by a few studies, with contradictory results. Viral RNA has been detected in the cerebrospinal fluid obtained from encephalitic individuals (Kumar et al. 2008;

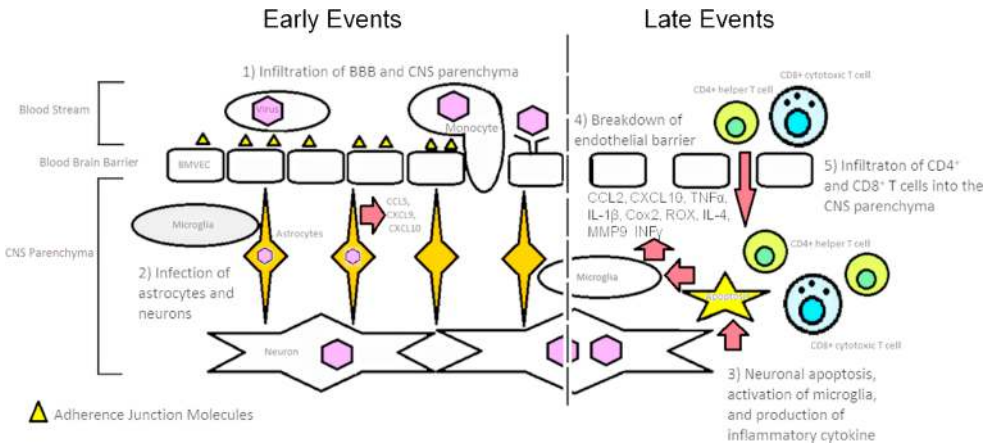

Fig. 6 Infection of CNS by flaviviruses. *1* Infection of the CNS occurs either through the adherence of the virus to molecules present on the surface of brain microvascular endothelial cells or infiltration of infected monocytes across the BBB. Viral infiltration then leads to infection of the BBB and CNS cell populations. *2* Infection of human astrocytes leads to chemokine production, facilitating further recruitment of monocytes and macrophages. *3* Neurons infected by flaviviruses undergo apoptosis and activates the resident microglia population which produces an inflammatory response. Production of inflammatory cytokines (e.g., TNF-α, IL1β, INF-γ, and IL-4), chemokines (e.g., CCL2, CCL5, CXCL9, CXCL10), inflammatory enzymes (COX2), and matrix-metalloproteinases (MMPs) leads to degradation of the endothelial barrier and release of inflammatory factors (*5*) recruiting $CD4^{+}$ and $CD8^{+}$ T lymphocytes into the CNS parenchyma. Infiltration of $CD4^{+}/CD8^{+}$ T lymphocytes leads to further inflammation and eventually CNS damage

Miagostovich et al. 1997b). In addition, DENV antigens have been detected in neurons, astrocytes, and microglia (Bhoopat et al. 1996b; Ramos et al. 1998). While in situ hybridization for viral particles in neurons, BMVECs, and glial cells were negative (Jessie et al. 2004), perivascular macrophages were positive for flaviviral NS3 protein. Further studies of these and other CNS cells are required to clarify their role in the neuropathogenesis of DENV and other flaviviruses. Thus, it is unclear whether proteins or RNA is a product of replication, viral uptake, or both.

Mechanisms of neuronal compromise

For a long time, the neurologic involvement of flavivirus infection was not considered and was only associated with peripheral infection, such as loss and extravasation of fluid, hyponatremia, and systemic failure. However, some reports indicating the unequivocal presence of viral proteins and genetic viral material in the brain and CSF of infected individuals suggest otherwise (Araujo et al. 2012; Soares et al. 2006; Solomon et al. 2000). Thus, the nature and mechanisms of CNS injury induced by these viruses must be examined. Several members of the flavivirus family also induced neuronal apoptosis in association with post-mitotic neuronal apoptosis that cannot be regenerated by neuronal stem cells (Ogata et al. 1991; Pekosz et al. 1996). Experiments in cell lines indicate that DENV can directly infect neurons (Despres et al. 1998), resulting in permanent damage.

Despite the problems with animal models for flavivirus infections, DENV has been easily adapted to invade the CNS of rodents. Normally, newborn mice are insensitive to non-neuroadapted mouse DENV strains. By passaging the virus within a murine host, the virus is neuroadapted to the murine host with highly neurovirulent strains of the virus selected and amplified (Despres et al. 1998; Sabin 1952). This selection process produces viruses that are capable of inducing neuronal apoptosis in infected mice, especially in the hippocampus and cerebral cortex where viral CNS replication has been observed (Despres et al. 1998). In addition, several DENV infection animal models display interesting effects on neuroinflammation characterized by up-regulation of important chemokines (e.g., CCL2, CCL5, CXCL1, and CXCL2) and inflammatory cytokines (e.g., TNF-α and IFN-γ) that facilitate leukocyte infiltration into the CNS (Amaral et al. 2011a, b).

Mice infected intracerebrally with DENV-3 show progressive meningo-encephalitis characterized by CNS infiltration of neutrophils and mononuclear cells by a mechanism that correlates with a chemokine-dependent mechanism that includes CCL2 and CXCL12 (Amaral et al. 2011a, b). These results show the importance of these cell types in the development of CNS disease during DENV infection. Interestingly, similar mechanisms of CNS damage have been observed in mouse models of hepatitis virus (JHMV), which resulted in animal mortality (Zhou et al. 2003). Neutrophils have a biphasic role during WNV infection: first, serving as a reservoir for viral replication and, second, aiding in viral clearance (Bai et al. 2007). However, these conflicting roles of neutrophils during WNV pathogenesis have not been examined further.

It has been observed that WNV causes limbic seizures by a mechanism involving *N*-methyl-D-aspartic acid receptor activation. Blocking this receptor activation could therefore abrogate limbic seizures and prolong animal survival (Getts et al. 2007). In addition to the neuronal damage induced by WNV infection, infiltration of leukocytes (e.g., neutrophils and mononuclear cells) was detected in the CNS parenchyma, similar to what was seen during DENV-3 infection of the CNS. WNV-mediated recruitment of microglial precursors (i.e., $Ly6c^{+}$ inflammatory monocytes) indicates that the virus can pass through the BBB by infected leukocyte transmigration into the CNS and induce local inflammation (Getts et al. 2008). Once in the CNS, the virus can infect and replicate within neurons and astrocytes, but not microglia (Cheeran et al. 2005; Diniz et al. 2006; Hussmann et al. 2013) despite data illustrating microglial activation and secretion of cytokines and chemokines that lead to CNS dysfunction.

Meanwhile, infection of CNS cells with JEV, especially neurons, results in massive CNS compromise, encephalitis, and death (Chen et al. 2004; German et al. 2006; Ghoshal et al. 2007). Apoptosis induced by JEV has been linked to three related mechanisms: (1) direct infection of neurons by the virus, (2) infection of other CNS cells such as microglia and astrocytes, and (3) general inflammation (Chen et al. 2004; Das and Basu 2008; Das et al. 2008; Raung et al. 2005, 2007; Swarup et al. 2008). It has been shown that inflammatory cells play an important role in the onset, progression, and severity of JEV-mediated encephalitis (Khanna et al. 1991; Mathur et al. 1988; Singh et al. 2000). Furthermore, reports indicate that the levels of inflammation can be a predictor of patient outcome following infection (Ravi et al. 1997). However, compared to other flaviviruses, the extent of glial infection during JEV is unclear. It is known that JEV triggers neuronal apoptosis by inducing endoplasmic reticulum (ER) stress through p38 mitogen-activated protein kinase (MAPK)-dependent and death-related transcription factor CHOP (C/EBP homologous protein)-mediated pathways (Su et al. 2002). This is in agreement with WNV which also uses CHOP and mitochondrial pathways to induce apoptosis (Chu and Ng 2003; Medigeshi et al. 2007). Additionally, phosphatidylinositol 3-kinase (PI3K), AKT, superoxide, arachidonic acid, caspases, and alterations in bcl-2/Bax also participate in apoptosis (Courageot et al. 2003; Jan et al. 2000; Lee et al. 2005; Lin et al. 1997; Parquet et al. 2001). Our work in HIV, a clear neurotropic virus, indicates that despite the low numbers of HIV-infected astrocytes which support minimal to undetected replication, the bystander mechanism of cell death amplification can be used by the virus to induce apoptosis and inflammation using electrical and chemical synapses (Eugenin and Berman 2007;

Eugenin et al. 2011). Therefore, the number of virus-infected cells or the levels of viral replication are not good indicators of cellular damage.

Cytochrome C is a mitochondrial protein that has been shown to play an important role in cellular respiration. In addition, cytochrome C participates in cellular apoptosis following cell damage or infection. DENV infection triggers neuronal apoptosis by activation of phospholipase A2 (PLA2), superoxide anion generation, cytochrome C release from the mitochondria, caspase-3 activation, and NF-kB translocation (Jan et al. 2000). This is similar to our results which show that HIV infection of glial cells dysregulates cytochrome C. Despite the high amounts of cytotoxic intracellular cytochrome C, HIV-infected cells are protected from apoptosis (Eugenin and Berman 2013). Thus, future studies are required to identify the role of these factors in apoptosis, the generation of viral reservoirs, and the detection of flaviviruses.

Therapeutic approaches to flaviviral infections

Current management of flavivirus infections

There are no effective antiviral treatments or vaccines against DENV and WNV with only one vaccine available against JEV. Thus, infected patients can only provide with palliative care following diagnosis. DHF patients exhibiting hemorrhage as determined by lowered hematocrit levels are provided blood transfusions to improve clinical outcome. Furthermore, there are no treatments available that prevent, abolish, or reverse the devastating CNS consequences of flavivirus infection. Thus, elucidating the mechanisms utilized by flaviviruses to compromise the CNS will lead to better design of successful therapeutics against these pathogens. To date, virus control has largely involved either the administration of prophylactic measures (e.g., vaccines) or the control of flaviviral arthropod vectors, namely, the *Culex* and *Aedes* mosquito species. Indeed given the rapid global spread of these arthropods, the latter is the primary approach indicated by the CDC and WHO to contain the spread of DENV, WNV, and JEV. Introduction and spread of DENV and WNV, as well as the presence of suitable JEV vectors in non-endemic areas, will greatly increase the number of individuals who are threatened by these pathogens.

Vaccine development

Due to the lack of effective therapy against most flaviviruses, the focus on developing potential vaccines has escalated. Considering the increasing prevalence of WNV in the USA, as well as the globalization of DENV, development of successful vaccines is imperative for effectively preventing flaviviral-associated diseases (Chao et al. 2012; Ehrenfeld et al. 2009; Sutter et al. 2000). However, any such success requires further knowledge of flaviviral disease pathogenesis. Identifying viral and host components important for disease development may lead to new targets for vaccines and therapeutic agents against the viruses (Heinz and Stiasny 2012).

The flavivirus genome encodes for a polyprotein that is post-translationally cleaved into ten structural and non-structural components (see details in Fig. 5). Since all are important in the life cycle and pathogenesis of the virus, they have the capacity to be utilized for vaccine development. Vaccines for the three structural proteins C, prM, and E are already being developed, with the most emphasis on multivalent vaccines, which contain multiple immunostimulatory antigenic epitopes that simultaneously generate protection against multiple viruses and serotypes (Brewoo et al. 2012; Durbin et al. 2006; Ishikawa et al. 2011; Johnson et al. 2002, 2004). Numerous studies have successfully produced chimeric vaccines containing the viral envelope including the prM/E portion of the structural protein as well as regions encompassing important non-structural components (e.g., NS1 and NS5). The ADE mechanism facilitating secondary and other subsequent DENV infections is still a challenge that must be addressed in these vaccines. Indeed the limited heterotypic protection against other DENV serotypes has slowed DENV vaccine development. Production of multivalent vaccines eliciting simultaneous immunogenic responses against all DENV serotypes may lead to successful prevention of DF and DHF. There has been modest success in developing live-attenuated, intertypic DENV chimeras containing replacements of the C, prM, E, and/or NS1 regions of DENV-4 with the corresponding regions within the DENV-1 or DENV-2 genome (Bray and Lai 1991). Monkeys singly or mix inoculated with these chimeras successfully showed simultaneous seroprotection against DENV-1 and -2 while at the same time producing DENV-4-specific neutralizing antibodies (Bray et al. 1996). To date, however, there are no vaccines in use that can simultaneously target all four DENV serotypes. Therefore, creating chimeric, tetravalent vaccines protecting against all four DENV serotypes could lead to promising prophylactic therapies against this emerging virus and prevent the more severe disease, DHF. Sanofi Pasteur has shown exciting and groundbreaking results in the development of a tetravalent vaccine against all four DENV serotypes. Currently, the vaccine is in phase III clinical study which is being conducted in DENV endemic and non-endemic countries (www.dengue.info). Successfully producing tetravalent vaccines will alleviate the drawback of ADE associated with DENV infection and protect individuals from heterotypic infection with other DENV serotypes.

IMOJEV, meanwhile, is a chimeric JEV vaccine that integrated the prM and E genes of SA14-14-2 (Liu et al. 2011) into the yellow fever vaccine vector, YFV17D (Poland et al. 1981).

Remarkably, the vaccine carries a 100 % seroconversion rate with greater than 85 % seroprotection 6 months post-vaccination, with continued protection against heterologous serotypes up to 5 years post-inoculation (Appaiahgari and Vrati 2012a, b) as well as the ability to provide continuous protection after only a single immunization dose. More interesting is the reported cross-protection of IMOJEV against the JEV-related flaviviruses such as Murray Valley encephalitis virus and WNV–Kunjin strain; however, the mechanism for this cross-protection has not yet been identified (Lobigs et al. 2009). Finally, an important feature of IMOJEV is the incorporated high-fidelity RNA polymerase within the YF17D genome which decreases the likelihood that the virus will undergo genetic mutations and revert back into its virulent state (Pugachev et al. 2004). Together these positive attributes make IMOJEV a highly promising prophylaxis against JEV and, with further development, against other related flaviviruses.

The promising results observed during the development of multivalent DENV vaccines have led to the design of other viral chimeras integrating genomic markers from JEV, WNV, or DENV within the previously successful yellow fever virus vaccine backbone (Arroyo et al. 2004; Chambers et al. 1999, 2003; Guirakhoo et al. 1999, 2000; Monath et al. 1999; Querec et al. 2006). Unlike WNV and DENV, there has been significant progress in JEV vaccine development since the initial observation of acquired protection against JEV among accidentally exposed laboratory workers (Hammon and Sather 1973; Pulmanausahakul et al. 2011). Since then, there is now one vaccine currently licensed for use in the USA (IXIARO) along with three vaccines in JEV endemic countries (e.g., IMOJEV, SA14-14-2 live-attenuated vaccine, and the now discontinued JE-VAX). Following the discontinuation of JE-VAX due to some reported adverse reactions to the vaccine, IXIARO, a cell culture-based inactivated virus derived from the SA-14-14-2 strain, is the only inactivated vaccine being produced and administered today. Still the relatively limited effectiveness of IXIARO compared with live-attenuated vaccines (~80-90 %) (Halstead and Thomas 2011), reported reactions to the inactivated vaccine (Nazareth et al. 1994; Ohtaki et al. 1995; Plesner 2003; Plesner and Ronne 1997), and the requirement of multiple doses for complete protection (Pugachev et al. 2003) have led to searches for more immunogenic vaccines with less adverse effects. Production of live-attenuated SA14-14-2 vaccine in 1988 provided a seroconversion of 80 % after a single dose with subsequent immunizations producing efficacy rates of 95-99 % (Hase et al. 1993; Hennessy et al. 1996; Wills et al. 1992; Xin et al. 1988). While not as effective as IMOJEV, further development of SA14-14-2 could lead to a more effective live-attenuated vaccine. SA14-14-2 is an attenuated strain of JEV-SA14 produced through serial passaging of the virus within hamster kidney cells. Due to the low cost of its production, as well as minimal side effects, live-attenuated SA14-14-2 is being administered more prevalently than inactivated vaccines (Schioler et al. 2007). Indeed since its approval for public use, over 200 million children living in JEV-endemic areas have been immunized with the live-attenuated vaccine and accounts for over 80 % of the total number of JEV vaccinations (Appaiahgari and Vrati 2012b; Liu et al. 2011). While there have been no reports regarding SA-14-14-2 mutational reversion to its virulent state, such a risk remains a potential drawback for this and other live-attenuated viruses.

The reversion of live-attenuated viruses to their original pathogenic state and the side effects, including hypersensitivity against components within inactivated vaccines, suggest that there is a need for new prophylactic treatments against JEV and other flaviviruses. Since the initial concept of using plasmids as immunogens two decades ago, the development of DNA vaccines has advanced dramatically, leading to the production of immunogenic agents which are in various phases of clinical trials (Ferraro et al. 2011). For instance, DNA vaccines have started to be developed as prophylactic therapies against a variety of human pathogens including HIV (Ramirez et al. 2013), *Plasmodium falciparum* (Chuang et al. 2013), and *Mycobacterium tuberculosis* (Okada et al. 2012) with promising results. Inoculations of the host with engineered viral protein expression plasmids are sufficient to elicit a robust cytotoxic $CD8^+$ and helper $CD4^+$ T lymphocyte response as well as the production of protective antibodies against the expressed immunogens (Alarcon et al. 1999). This exciting technology has some important implications especially in the development of DENV vaccines as it could provide concurrent protection against all DENV serotypes, thereby limiting the adverse effects of ADE, of which current vaccines have been unable to circumvent. This novel approach is being applied to develop new flavivirus vaccines that target the surface-exposed E and prM and NS1 proteins which were previously shown to be highly immunogenic and elicit a favorable immune response (Ahsan and Gore 2011; Azevedo et al. 2013; Costa et al. 2006a, b, 2007; Davis et al. 2008b; Kulkarni et al. 2012; Lu et al. 2013; Schneeweiss et al. 2011). A DNA vaccine targeting DENV (designated D1ME100) and WNV previously entered in phase 1 of clinical trials produced favorable results (Beckett et al. 2011). Both D1ME100 and WNV vaccines elicited strong, long-lasting immune response in all test subjects as quantified by virus-specific IgG and IgM production. Even more interesting, despite only being immunized against DENV1, was that the D1ME100-vaccinated subjects showed significant IFNγ response following inoculation with E proteins derived from all four DENV serotypes (Beckett et al. 2011). Together these data show great promise for DNA vaccines in the prevention of flavivirus infections as well as infections by any pathogen lacking available treatments or vaccines.

Viral inhibitors

In order to successfully infect a suitable host, a virus must attach to the host's extracellular surface and penetrate into the cytosol. There, the viral genome is unpackaged, transcribed, and translated. Progeny viruses are then processed and packaged by host and viral machineries to produce mature, infectious virions. While vaccines have been highly successful in preventing important human infections such as polio and smallpox, research for antiviral therapeutic agents (i.e., small molecule inhibitors) is still being pursued for one major reason: identifying homologous targets across related pathogens can lead to the design of pharmaceutical drugs that will help eradicate numerous related human diseases. Close dependence of the flaviviral life cycle for host cellular processes allowed researchers to utilize these host functions in their design of antiviral agents. Indeed numerous cellular components have been targeted, including viral adherence to the host extracellular surface (De Burghgraeve et al. 2012; Schmidt et al. 2012), nucleic acid and protein synthesis (Noisakran et al. 2008; Oh et al. 2006; Paranjape and Harris 2007; Qing et al. 2009), intracellular trafficking and signaling (Hirsch et al. 2005; Hong et al. 2013), and the host immune response (Dikeakos et al. 2010; Mishra et al. 2009; Proudfoot et al. 1999). The ADE of DENV infection, which has hindered the production of successful DENV vaccines, has also been the primary reason for the design of more effective viral inhibitors.

Only a few inhibitors are currently available that prevent flaviviral attachment and entry. Due to the importance of E protein in flaviviral entry and its numerous binding targets on host cell membranes, it has been the focus for viral entry inhibitors (Liao et al. 2010; Schmidt et al. 2012). Schmidt et al. (2012) has shown that a DENV E protein inhibitor, designated 1662G07, and its analogs could successfully prevent DENV-2 fusion with host endosomes at micromolar to sub-micromolar concentrations (IC_{90}=0.75–2 μM) when tested in vitro (Schmidt et al. 2012). In addition, the inhibitor has been shown to be effective against DENV-1 and -2 serotypes.

LCTA-949, a replication inhibitor of hepatitis C (Obeid et al. 2011), is another compound being developed against flaviviral infections, in particular DENV (De Burghgraeve et al. 2012). Treatment with LCTA-949 (12.5 μM) was observed to prevent DENV entry into host cells by limiting viral attachment to the cell membrane; however, this inhibitory activity occurs only when added concurrently with the virus in vitro. This suggests that the binding affinity of the virus for the host cell membrane may be greater than viral adherence to the inhibitor. Even more interesting is the ability of LCTA-949 to competitively inhibit the attachment of antibody-opsonized DENV on the host cell surface. This suggests the compound's potential inhibitory activity against ADE of DENV infection. Due to its incapacity to reverse pre-existing DENV infections, LCTA-949 must be developed further in order for it to become clinically relevant. Inhibition of other flaviviral pathogens (i.e., hepatitis C and yellow fever viruses) and interference with ADE-DENV infections may make this novel compound a viable therapeutic agent against flaviviral infections in the future.

Similar to virus attachment and entry, post-translational processing of the translated viral genome is important for a successful viral life cycle and production of mature, infectious virions. Indeed host signalases and viral proteases (NS2b and NS3) have been shown to be required for cleavage of the flaviviral polyprotein into biologically active components (Appaiahgari and Vrati 2012b; Leyssen et al. 2000). Inhibiting these proteases has been proposed to abate virus formation and maturation (Filocamo et al. 1999). The importance of viral proteases during flavivirus pathogenesis, as well as in other viral infections, makes them attractive targets for therapeutic intervention. For instance, one anti-HIV therapy involves the use of a protease inhibitor, Duranvir (Prezista; Janssen Therapeutics), which targets the HIV-1 PR protease important for the processing of the Gag/Gag-polyprotein (Phung and Yeni 2011). This same approach is being applied towards flaviviruses by targeting the NS3 protease (Chappell et al. 2008; Lescar et al. 2008; Leyssen et al. 2000). In fact, a protease inhibitor (Boceprevir; Merck) targeting the NS3 protease of HCV has yielded impressive results in clinical trials (Manns et al. 2011). During the study, HCV loads among treated patients were drastically reduced as compared with naïve subjects. The activity of Boceprevir has not yet been tested against other related flaviviruses. Current results, however, suggest that Boceprevir and other NS3-targeting protease inhibitors are excellent candidates for treatment against DENV, WNV, and JEV infections.

Another potential enzyme targeted for flavivirus therapy is guanylyltransferase (GTase) encoded within the NS5 regions of the flavivirus genome. It has previously been shown that GTases, in concert with viral RNA triphosphatase, methytransferase, and nucleoside 2′-*O*-methyltransferase, regulate flavivirus genome translation and preserve viral RNA integrity by methylating the 5′-end UTR of the flavivirus genome. (Issur et al. 2009). This, together with its conservation among related flaviviruses, suggests that GTases could be targeted for therapeutic intervention against members of this virus family (Bollati et al. 2010; Egloff et al. 2002; Geiss et al. 2009). Indeed inhibition of GTase activity using the compound (*E*)-[3-[5-[4-*tert*-butylbenzylidene-4-oxo-2-thioxo-1,3-thiazolidin-3-yl] propanoic acid (BG-323) prevented DENV and WNV replication (Stahla-Beek et al. 2012). Furthermore, this same study showed that BG-323 may also have anti-viral activity against the yellow fever virus. It is possible to speculate that given its broad activity against flaviviruses, BG-323 could be further developed into a successful therapy against flavivirus infections, that is, against the severe diseases DF and DHF.

Vector control: a potential effective way to protect the population

The spread of mosquito vectors is a critical event in the transmission cycle of DENV, WNV, and JEV. Thus, preventing or reducing viral transmission depends upon either the control of these insect vectors or interruption of the human–vector interaction. Some of these measures are included in the following list which provides a comprehensive approach in designing better policies and programs to detect, eliminate, and attack the critical stages of vector development and viral transmission:

- Elimination of potential habitats by controlling local environments facilitating the successful reproduction of the mosquitoes—These recommendations involve simple steps targeting the early stages of vector development such as better management of natural and man-made stagnant water sources as well as chemical and biological control methods of mosquito populations (e.g., insecticides, repellants, mosquito fish, and *Bacillus thuringiensis*). These simple steps will help reduce the presence of larvae and adult mosquito populations, thereby limiting the potential contact between infected arthropod vectors and humans.
- Politics and social advocacies are essential factors which allow the implementation of policies educating individuals about the global threat of flaviviral dispersion. Designing better policies will provide the general population and health care workers important medical training and preparation to face these important viral threats. This is even more important in areas previously not endemic to DENV, WNV, or JEV as health workers are not typically trained to identify these infectious diseases and thus potentially confuse the infections with other flu-like maladies. Given the increasing global spread of these viruses and its mosquito vectors, dispersal of medical information as well as policies aimed at preventing the further spread of the pathogens is necessary.
- A better communication between health organizations and the population is essential in preventing disease spread. The spread of WNV, DENV, and JEV may be influenced by the lack of information about viral transmission, disease identification, disease treatment, and infection statistics. These problems are exacerbated among resource-poor countries where flaviviruses are typically found. Lack of resources and health services could impede the public diffusion of educational information important in controlling either the viruses or its insect vectors. Proper education would help alleviate this problem as it will lead to productive discussions regarding the control of these pathogens, subsequently preventing viral transmission and the diseases associated with the infection.

Concluding remarks and future directions

The emergence of WNV in the USA, the worldwide expansion of DENV, the continuous threats of JEV, and the rapid dispersal of suitable mosquito vectors make research involving these pathogens imperative. It is clear that drastic global changes are affecting the spread of flaviviruses and their vectors. Further, with the rapidly expanding human population and the encroachment of these viruses in previously non-endemic areas, a global health problem involving these viruses is imminent. Thus, understanding how these viruses cause human diseases such as DHF is imperative. In addition, the ever increasing threats of new infections make the development of novel, effective vaccines and therapeutic agents vital. The sparse, conflicting information available regarding the pathogenesis of these viruses and the cell types, tissues, and organs they infect, however, make it difficult to produce successful drugs against any of these pathogens. Thus, until successful treatments and more effective vaccines are available, the best method of eliminating new infections in public and medical institutions is to stop viral vector spread and prevent becoming bitten by mosquitoes through the copious use of repellents.

The mechanisms of CNS dysfunction induced by flavivirus infection are poorly explored or ignored. The cell targets of flaviviruses and the replication pattern used by these viruses are still a matter of debate and require further investigation. Identifying how flaviviruses alter host cell physiology will provide the basis for understanding their pathogenesis and will subsequently lead to the design of more effective therapeutic agents against the viruses within the CNS. Recent advancements in research techniques and animal models and our data using primary human astrocytes and BMVECs provided critical evidence, suggesting that flaviviruses are indeed neurotropic. Further, identifying how these viruses regulate leukocyte activation and CNS transmigration as well as dysregulation of BBB function leading to CNS compromise and human disease is critically important.

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