Tick-borne flaviviruses alter membrane structure and replicate in dendrites of primary mouse neuronal cultures

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Neurological diseases caused by encephalitic flaviviruses are severe and associated with high levels of mortality. However, detailed mechanisms of viral replication in the brain and features of viral pathogenesis remain poorly understood. We carried out a comparative analysis of replication of neurotropic flaviviruses: West Nile virus, Japanese encephalitis virus and tick-borne encephalitis virus (TBEV), in primary cultures of mouse brain neurons. All the flaviviruses multiplied well in primary neuronal cultures from the hippocampus, cerebral cortex and cerebellum. The distribution of viral-specific antigen in the neurons varied: TBEV infection induced accumulation of viral antigen in the neuronal dendrites to a greater extent than infection with other viruses. Viral structural proteins, non-structural proteins and dsRNA were detected in regions in which viral antigens accumulated in dendrites after TBEV replication. Replication of a TBEV replicon after infection with virus-like particles of TBEV also induced antigen accumulation, indicating that accumulated viral antigen was the result of viral RNA replication. Furthermore, electron microscopy confirmed that TBEV replication induced characteristic ultrastructural membrane alterations in the neurites: newly formed laminal membrane structures containing virion-like structures. This is the first report describing viral replication in and ultrastructural alterations of neuronal dendrites, which may cause neuronal dysfunction. These findings encourage further work aimed at understanding the molecular mechanisms of viral replication in the brain and the pathogenicity of neurotropic flaviviruses.

INTRODUCTION

Flavivirus is a genus in the family Flaviviridae, and consists of positive-polarity ssRNA viruses with lipid envelopes (Gould & Solomon, 2008; Lindenbach, 2007; Schmaljohn & McClain, 1996). The flavivirus genome encodes one polyprotein, which is cleaved into three structural proteins – the core, pre-membrane and envelope (E) proteins – and seven non-structural (NS) proteins within a single long ORF (Chambers et al., 1990). The genus Flavivirus contains over 70 members, many of which are arthropod-borne human pathogens (Lindenbach, 2007; Mackenzie & Williams, 2009; Mackenzie et al., 2004; Schmaljohn & McClain, 1996). Recently, many outbreaks have been reported, and flaviviruses are attracting global attention as emerging or re-emerging infectious diseases (Balogh et al., 2010; Chung et al., 2013; Danis et al., 2011; McMichael et al., 2006; Morse, 1995; Vong et al., 2010). Flaviviruses are divided into four distinct evolutionary lineages: mosquito-borne, tick-borne, no known vector and insect-only flaviviruses (Billoir et al., 2000; Cook et al., 2012; Crabtree et al., 2003; Kuno et al., 1998). Mosquitoes of the Aedes and Culex families are the major vectors of mosquito-borne flaviviruses, including yellow fever virus, dengue virus, West Nile virus (WNV) and Japanese encephalitis virus (JEV) (Gould & Solomon, 2008). Ixodidae ticks carry tick-borne flaviviruses, including tick-borne encephalitis virus (TBEV) and Langat virus (LGTV) (Lindquist & Vapalahti, 2008).

Flavivirus infection of humans causes various manifestations such as haemorrhagic disease, encephalitis, biphasic fever, flaccid paralysis and jaundice (Gould & Solomon,
2008). Encephalitis, a neurological manifestation of disease, is particularly problematic. This condition is associated with high-level mortality and severe sequelae. WNV, JEV and TBEV are all principally neurotropic flaviviruses causing encephalitic diseases in humans. Common symptoms are headaches, vomiting, ataxia and paralysis. Differences in neurological symptoms have been reported in the infections caused by each virus. JEV infection triggers acute spasms and development of a dull pathognomonic Parkinsonian syndrome (Ooi et al., 2008; Solomon et al., 1998). Cognitive function is compromised in several cases of TBEV infection: patients develop photophobia, irritability and sleeping disorders (Czupryna et al., 2011; Kaiser, 1999; Mickiene et al., 2002). WNV infection triggers development of systemic symptoms generally, but neurological manifestations are rare (Anastasiadou et al., 2013; Sejvar et al., 2003). On histopathological examination, these viruses all induce typical non-suppurative encephalitis, including necrosis of neurons (associated with shrunken perikarya), perivascular and vascular infiltration of mononuclear cells, and neuronophagia. The distribution of viral antigens in the cerebellum differs, but viral antigens are seen in several brain regions among all these viruses, including the brainstem, cerebral cortex, caudate putamen and cervical spinal cord (Hayasaka et al., 2009; Kimura et al., 2010). However, it remains unclear how viral replication and pathogenicity contribute to the neurological manifestations.

Primary culture techniques have been developed for maintaining brain cells (Banker & Cowan, 1977), and such cultures can be used to investigate the detailed intracellular activities of neurons (Ishihara et al., 2009; Okabe, 2013; Wang et al., 2009). This approach has been used to explore not only physiological functions but also neuronal responses affected by viral invasion, including in lyssavirus, herpesvirus and flavivirus infections (Chen et al., 2011; Lewis & Lentz, 1998; Perkins et al., 2002). Primary cultured neurons could provide detailed information about flavivirus replication in this cell type. In the present study, we used primary neuronal cultures to explore the replicative and neuropathogenic features of encephalitic flaviviruses. We found that the replicative properties of mosquito and tick-borne flaviviruses differed significantly.

**RESULTS**

Replication of neurotropic flaviviruses in primary neuronal cultures

Prior to experiments using infectious viruses, the cell components of primary cultured brain cells were examined. Primary neuronal cultures were prepared from hippocampi, cerebral cortices and cerebella, and stained for a neuronal marker (microtubule-associated protein 2: MAP2), astroglial marker (glial fibrillary acidic protein: GFAP) and DAPI, using an indirect immunoﬂuorescent assay (IFA). The primary cultures contained principally neurons (70–80%) and astroglial cells (20%) (Fig. S1, available in the online Supplementary Material) and lacked microglial cells (data not shown).

To compare the growth kinetics of encephalitic flaviviruses, primary cultures from each region were infected with TBEV, WNV or JEV at an m.o.i. of 0.1, and viral titres in the culture supernatant were measured at various time points. The experiments were repeated four times. Fig. 1(a) and (b) shows that viral titres peaked at 48 h post-infection (p.i.), and the titres did not differ among studied primary cultures. The viral titre of TBEV at 48 h p.i. was slightly higher than that attained by the other viruses, but the difference was not statistically significant. Viral growth kinetics were similar in all studied primary cultures. Thus, cerebral cortex cells were used in all subsequent experiments. No obvious morphological changes were evident upon light microscopy, apart from slight dendritic degeneration in TBEV-infected neurons (Fig. 1c).

**Distribution of viral antigens in primary neuronal cultures**

The distribution of viral antigens in primary neuronal cultures was examined by IFA. At 48 h p.i., infected cells were stained with MAP2 (a marker of neuronal cell body and dendrites), virus-specific antibodies and DAPI. Fig. 2(a) shows that the cell body distribution of viral antigens was similar in neurons infected with each virus studied. However, dendritic distributions were different in the infected neurons. Viral antigens were sparsely distributed in dendrites of cells infected with WNV or JEV [Fig. 2a(vii, viii, xii, xiii)]. On the other hand, elliptical antigen accumulations were evident in dendrites infected with TBEV [Fig. 2a(ii, iii), white arrows]. This form of antigen accumulation was also evident in neurons infected with the tick-borne flaviviruses, TBEV, Omsk haemorrhagic fever virus (OHFV) and LGTV (Fig. S2).

Detailed images of accumulated viral antigens are shown in Fig. 2(b). Antigen accumulations varied in diameter and were distributed in dendrites infected with WNV or JEV [Fig. 2b(iii, iv, vi, vii)]. Viral antigens were surrounded by MAP2 in structures that appeared to be swollen [Fig. 2b(iv, vii)]. In some large swellings, an unstained (hollow) region was evident within the accumulation of viral antigen [Fig. 2b(ii–iv)]. Changes over time in viral antigen distribution are shown in Fig. 3. In the early stages of TBEV infection, viral antigens were detected principally in the cell body, and thus minimally in the dendrites [Fig. 3a(i, ii)]. From 48 to 72 h p.i., viral antigen accumulated in the dendrites [Fig. 3a(iii, iv), white arrows]. However, viral antigen in WNV- or JEV-infected cells was located principally in the cell body and (thus minimally in the dendrites) at all time points examined [Fig. 3a(v–viii, ix–xii)]. WNV antigen accumulated in dendrites of several neurons by 72 h p.i.
In a previous study, TBEV infection triggered microtubule rearrangement in neuroblastoma cells (Růzek et al., 2009), possibly associated with the viral antigen accumulations in dendrites. As shown in Fig. 4, neurons mock infected (Fig. 4a–d) or infected with TBEV (Fig. 4e–ab) were co-stained with the anti-TBEV and anti-MAP2 (Fig. 4a, e–j), anti-β3-tubulin (Fig. 4b, k–p), anti-calreticulin (Fig. 4c, q–v) or anti-synaptophysin (Fig. 4d, w–ab) antibodies. However, no obvious changes in microtubule distribution were evident in infected primary neuronal cultures (Fig. 4k–p). Accumulated viral antigens in TBEV-infected cells were localized with MAP2 (Fig. 4h–j), β3-tubulin (Fig. 4n–p) or calreticulin, which is distributed in the endoplasmic reticulum (ER) membrane (Fig. 4t–v) but not with synaptophysin, a marker of synaptic vesicles (Fig. 4z–ab). Thus, the viral antigens accumulated in the ER of the dendrites, but were not directly associated with rearrangement of microtubules and synaptic vesicles.

**Effect of perturbation of microtubules on viral antigen distribution**

Addition of nocodazole (which disrupts microtubules) induced dendrite loss (Fig. 5a–c), and TBEV or WNV antigens were present in the neural cell body only (Fig. 5d–f, g–i). Thus, the viral antigen accumulations were affected by microtubules.

**Viral constituents of the protein accumulations in dendrites**

The viral constituents in the accumulations were investigated. Staining of TBEV-infected neurons with specific antibodies detecting structural (E) and non-structural (NS3) proteins showed that both proteins were present in the antigen accumulations [Fig. 6a(i, iii, iv, vi), white arrows], but such accumulations were fewer compared with neurons infected with TBEV (Fig. 3b).
arrows). In addition, dsRNA (reflecting viral genome replication) was also present [Fig. 6b(vi, vii)]. These results suggested that viral genome replication occurred in the regions of viral protein accumulations in dendrites.

To investigate the viral components required for the formation of the accumulated viral antigens, we next infected primary cultures with virus-like particles (VLPs) of TBEV (‘single-round’ infectious particles containing replicon RNA as a genome; Gehrke et al., 2003; Khromykh et al., 1998; Molenkamp et al., 2003; Reynard et al., 2011; Yoshii et al., 2008). The replicon RNA lacks most of the coding region for viral structural proteins. The VLPs can enter cells, and replicate within, but cannot produce progeny virus. Fig. 6(c) shows that viral antigen also accumulated in dendrites after infection with the TBEV VLPs [Fig. 6c(i–iv), white arrows]. Thus, viral protein accumulations did not require expression of viral structural proteins.

Ultrastructure of flavivirus infected primary cultured neurons

To observe the membrane structure of infected neurites, infected primary neuronal cultures were examined by transmission electron microscopy (TEM; Fig. 7). Mock-infected neurons had large nuclei, and the ER, mitochondria and Golgi apparatus were readily observed (Fig. 7a–c). However, virus-infected neurons exhibited cytoplasmic condensation with granular structures, and reactive lysosomes were evident (Fig. 7d). Apoptotic cells (identified by nuclear distortion or the presence of apoptotic bodies) were rare. The spherical virion-like structures coated with
lipid bilayer were observed in infected neurons (Fig. 7e). The cell bodies of neurons infected with TBEV or WNV were similar in appearance. Organized microtubules were observed in neurites of mock-infected neurons (Fig. 7f). After infection with WNV, degenerated membrane and granular structures appeared in neurites (Fig. 7g). In contrast, TBEV infection caused neurite swelling and appearance of elliptical structures (Fig. 7h, i). These structures were surrounded by laminal membranes and adjacent to microtubules (Fig. 7i). Virion-like structures coated with lipid bilayers were observed both inside and outside of these structures (Fig. 7j–l). Infection with either WNV or TBEV triggered neuronal cytoplasmic condensation. TBEV infection caused a characteristic ultrastructural change of membrane in the neurites: a laminal membrane structure (LMS) besides the microtubules. WNV infection was not associated with LMS formation.

**DISCUSSION**

Despite the importance of the neuropathogenicity of TBEV, detailed features of the replication mechanism of this virus in neural cells are still unknown. We used primary cultures of brain cells to comparatively examine the replication of several flaviviruses. Viral antigen distribution in infected neurons...
primary neuronal cultures differed when cells were infected with the mosquito and tick-borne neurotropic flaviviruses. IFA and TEM studies revealed that dendritic replication of tick-borne flaviviruses caused abnormal swelling of neurites and development of a specific structure, an LMS.

Each flavivirus studied multiplied effectively in primary neuronal cultures from several brain regions, indicating that use of such cells is appropriate when investigating flavivirus infection. However, the flaviviruses showed similar growth kinetics in primary cultures from several brain regions. Neurotropic flaviviruses have been reported to exhibit differences in distribution and multiplication among the various parts of the brain. WNV antigens were detected less frequently in granule cell neurons of the cerebellum compared with other neuronal populations (Omalu et al., 2003; Xiao et al., 2001). In contrast, JEV replicated well in granule cell neurons (Desai et al., 1995; German et al., 2006), and TBEV replicated throughout the cerebellum, including in granule cells (Gelpi et al., 2005; Hayasaka et al., 2009). Two possible reasons can be suggested to explain the difference in the in vivo and in vitro results. First, lack of glial cell maturation may influence viral replication. Mammalian neurons interact with glial cells soon after birth; the neurons mature and become myelinated (Baumann & Pham-Dinh, 2001). Some reports have emphasized that the presence of glial cells is important for effective flavivirus replication in the brain (Chen et al., 2010; Hussmann et al., 2013). It is possible that primary viral replication in glial cells is essential if the

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**Fig. 4.** Co-staining of TBEV antigen and cellular organelles. Neuronal cells were mock-infected (a–d) or infected with TBEV (e–ab) at an m.o.i. of 0.1 and fixed at 48 h p.i. The cells were stained with antibodies against organelle markers (green), an antiserum against tick-borne flaviviruses (red) and DAPI (blue). Antibodies against MAP2 (a, e–j), β3-tubulin (b, k–p), calreticulin (c, q–v) and synaptophysin (d, w–ab) were used as organelle markers. The accumulations of viral antigens are magnified (Mag) in panels (h–j, n–p, t–v and z–ab). Accumulations of viral antigens are indicated by white arrows. Bars, 50 μm.
Viruses are subsequently to spread efficiently through the brain. Secondly, incomplete maturation of the innate immune response of neurons may affect the susceptibility of such cells to flavivirus infection. The granule cells of the cerebellum have been reported to mount an effective innate immune response against viral infection (Cho et al., 2013). The primary cell cultures used in the present study were devoid of glial cells (except astroglia), and embryonic neurons may lack a well-developed innate immune system. Thus, the susceptibility of primary embryonic neuronal cultures to viral infection may differ from that of adult brains in vivo. IFN treatment of primary neuronal cultures may lead to viral replication patterns similar to those observed in vivo.

Infection with tick-borne flaviviruses was associated with accumulation of viral antigens in the dendrites of infected neurons, but this was not true of mosquito-borne flaviviruses. The accumulations contained structural proteins, NS proteins and dsRNA. Accumulations were also evident upon the replication of replicon RNA after infection with VLPs of TBEV. Flaviviruses replicate at ER membrane, and bud into the ER lumen (Lindenbach, 2007). Dendrites are known to contain free ribosomes and express satellite secretory pathways to secure synaptic plasticity (Martone et al., 1993; McCarthy & Milner, 2003; Ori-McKenney et al., 2012; Pierce et al., 2001; Ramirez & Couce, 2011). Together, the data suggest that tick-borne flaviviral replication in dendrites induced viral protein accumulation. Infection with rabies virus, influenza virus and other viruses was previously shown to cause similar accumulations of viral antigens in the dendrites (Li et al., 2005; Matsuda et al., 2005). Such accumulation has been considered to reflect inhibition of viral protein transportation in dendrites in which the cytoskeleton has been disrupted. However, we found that the mechanism of antigen accumulations during TBEV infection was quite different. This is the first report to show tick-borne flaviviral replication in dendrites.

TBEV infection caused a characteristic ultrastructural change in neurite membranes of infected neurons. An LMS developed, lying parallel to microtubules, and virion-like

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**Fig. 5.** Effect of microtubule perturbation on viral antigen distribution. Cells were mock-infected (a–c) or infected with TBEV (d–f) or WNV (g–i) at an m.o.i. of 0.1 and treated with nocodazole. The infected cells were fixed at 48 h p.i. and stained with antibodies against MAP2 (green), antisera against each virus (red) and DAPI (blue). Bars, 50 μm.

**Fig. 6.** Viral constituents present in antigen accumulations in dendrites. (a) Cells were infected with TBEV at an m.o.i. of 0.1 and fixed at 72 h p.i. The fixed cells were stained with antibodies against viral proteins (green), an antiserum against a tick-borne flaviviruses (red) and DAPI (blue). Two antibodies targeting the E protein (i–iii) and NS3 (iv–vi) were used. Bars, 50 μm. (b) Cells were infected with TBEV at an m.o.i. of 0.1 and fixed at 48 h p.i. The fixed cells were stained with antibodies against the TBEV E protein (green), an antibody against dsRNA (red) and DAPI (blue). Bar, 10 μm. The accumulation of viral antigens is magnified in panels (ii–iv). The images were collected with a confocal laser-scanning microscope. (c) Cells were infected with virus-like particles of TBEV and fixed at 48 h p.i. Neurons were stained with an antibody against MAP2 (green), an antiserum against tick-borne flaviviruses (red), and DAPI (blue). Accumulations of viral antigens are magnified in panels (iv) and (v). Bars, 50 μm. Accumulations of viral antigens are indicated by white arrows.
structures were observed both inside and outside of this structure. The co-localization of the viral antigen and ER markers indicated that the LMS was derived from ER membranes. LMS-like membranes were previously observed in glioblastoma cells infected with TBEV (Růzek et al., 2009). Flavivirus infection induces typical alterations in ER membranes. The membranes assume vesicle packets and convoluted membranes (Mackenzie, 2005), forming a platform on which viral genome replication and virion assembly proceed (Uchil & Satchidanandam, 2003; Welsch et al., 2009). It is possible that the LMS is formed via ER-derived membrane reconstitution triggered by the viral replication, and serves as the scaffold for dendritic viral replication and virion assembly. The unstained hollow regions evident when the accumulations of viral proteins were examined by IFA may be attributable to the fact that degenerated membranes are poorly permeable to antibodies.

A proposed model of LMS formation is shown in Fig. 8. Viral proteins are synthesized in dendrites (Fig. 8a). Membrane structures are reconstituted to form the LMS after such synthesis (Fig. 8b), and the LMS becomes multilayered and grows to compress the microtubules (Fig. 8c).

Time-course experiments revealed that viral proteins were synthesized principally in the neuronal cell bodies during the early stages of infection, becoming distributed in dendrites only later. Thus, TBEV genomic RNA (with or without viral proteins) was transported principally from cell bodies to dendrites. Viral genomic RNA bound to membrane-associated replication complexes (formed by viral NS proteins) may be transported along dendritic membranes. Another important transport mechanism involves formation of RNA granules. Recently, mRNA transportation to the dendrites, and local translational control therein, has been described in neurons (Kiebler & DesGroseillers, 2000; Köhrmann et al., 1999; Muramatsu

**Fig. 7.** Ultrastructural changes in primary neuronal cultures upon flaviviral infection. Primary neuronal cultures were examined by TEM; these included mock-infected cells (a–c, f), cells infected with TBEV (d, e, h–l) and cells infected with WNV (g). (a–c) Mock-infected neurons contained structurally intact organelles (Nu, nucleus; Cyto, cytosol; Mit, mitochondria). Bars: 1 μm (a); 500 μm (b, c). (d) TBEV-infected neurons exhibited cytoplasmic condensation, and reactive lysosomes were also observed (Lys, lysosome). Bar, 5 μm. (e) Virion-like structures coated with lipid bilayers were evident (white arrows). Bar, 200 μm. (f) Normal microtubule structure was observed in mock-infected neurites. (g) WNV triggered degeneration of membrane structure (white arrowhead) and the appearance of granular aggregates in the neurites. (h) TBEV infection triggered swelling of and development of elliptical membrane-encased structures in neurites. (i) A laminal membrane structure was observed adjacent to microtubules. Bars, 500 μm (f–i). (j–l) Representative images of the regions highlighted in (h) and (i). Virion-like structures coated with lipid bilayers were observed both inside and outside the observed structures (white arrows). Bars, 200 μm.
et al., 1998; Sinnamon & Czaplinski, 2011). Specific mRNAs form RNA granules containing several different RNA-binding proteins, which are transported along microtubules to dendrites in a kinesin-dependent manner (Bramham & Wells, 2007; Kanai et al., 2004). It is possible that viral genomic RNAs may hijack or mimic this transport mechanism. Microtubule-dependent formation of viral antigen accumulations in dendrites may support this hypothesis.

All tick-borne flaviviruses used in the present study, the far Eastern and European subtypes of TBEV, OHFV and LGTV accumulated viral antigens in dendrites. However, WNV and JEV formed smaller accumulations. Some previous studies used the primary brain cultures to investigate replication of mosquito-borne flaviviruses, but no mention was made of the viral antigen accumulation in dendrites (Chen et al., 2011; Diniz et al., 2006). Tick-borne flaviviruses share the characteristic of viral antigen accumulation in dendrites.

Alterations in membrane structures and accumulation of viral proteins in dendrites may cause neuronal dysfunction and degeneration in vivo. LMS formation and viral protein accumulation induced ultrastructural changes in neurites, including compression of the microtubules, obstruction of trafficking pathways and reconstitution of membrane structures. Such changes may affect synaptic function and induce neurite degeneration leading to the development of neurological disease. Synaptic connections are dynamically regulated via intracellular trafficking pathways, protein modifications and local protein synthesis in the dendrites (Bagni & Greenough, 2005; Kiebler & DesGroseillers, 2000; McCarthy & Milner, 2003; Steward & Schuman, 2003). Such connections play important roles in the brain, in recognition, memory and behavioural regulation. Dendritic degeneration occurs in some diseases associated with loss of cognitive function, including Alzheimer’s disease, fragile-X syndrome and Rett syndrome (Calon et al., 2004; Comery et al., 1997; Maezawa & Jin, 2010). In particular, fragile-X syndrome is caused by disruption of local protein synthesis in dendrites, and triggers hyperactivity and greater responses to low-intensity auditory stimuli in mouse model (Bagni & Greenough, 2005; Consortium, 1994). As observed in fragile-X syndrome, the abnormal membrane alterations in dendrites caused by TBEV infection might cause disruption of local protein synthesis in dendrites, resulting in the cognitive compromise observed in TBEV patients. In addition, viral protein accumulations in dendrites may affect neural function via the interaction of such proteins with host factors. In a previous study, TBEV replication arrested the neurite outgrowth in a cell line derived from a pheochromocytoma of the rat adrenal medulla. Such arrest was caused by interactions between the TBEV NS5 protein and host proteins Rac1 and Scribble (Wigerius et al., 2010). The latter proteins are involved in maintaining cell polarity, regulation of synaptic plasticity and synaptic vesicle dynamics (Roche et al., 2002). It is possible that the accumulated viral proteins affect the distribution and

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**Fig. 8.** Schematic diagram of LMS formation and the contribution thereof to neuro-pathogenicity. (a) Viral proteins are synthesized by free ribosomes in the dendrites. (b) Synthesized proteins form the LMS by modulating the structure of host membranes. (c) The LMS becomes enlarged and compromises microtubule linearity, thus obstructing trafficking pathways.
functionality of host proteins with which the viral proteins interact, in turn causing neural dysfunction and cell degeneration.

In conclusion, we have shown that mosquito and tick-borne flaviviruses replicated differently in primary neuronal cultures. Tick-borne flaviviruses induced ultrastructural membrane alterations and replication thereof was associated with accumulation of viral proteins in dendrites; this was not true of WNV or JEV. We have also shown, for the first time, that tick-borne flaviviruses replicate in neural dendrites. These findings encourage further investigations to understand the molecular mechanism of viral replication in brains and the pathogenicity of neurotropic flaviviruses, and also to learn how to prevent and cure viral infections by these viruses.

METHODS

Cell culture. Baby hamster kidney-21 (BHK-21) cells were grown at 37 °C in minimum essential medium (Life Technologies) supplemented with 8 % (v/v) FBS and penicillin/streptomycin. Human embryonic kidney 293T cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (Life Technologies), containing 10 % (v/v) FBS and penicillin/streptomycin.

Pregnant Skl:ICR mice were purchased from Japan SLC, and hippocampal, cerebral cortical and cerebellar neuronal cultures were established from brain cells of these animals. Neurons for primary culture were prepared from embryonic day 17–18 mouse embryos as described previously (Biederer & Scheifele, 2007; Viesselmann et al., 2011). Briefly, the hippocampus, cerebral cortex and cerebellum were dissected from embryonic brains into dissection medium: HBSS (Life Technologies) supplemented with 10 mM HEPES (Life Technologies) and 1 mM sodium pyruvate (Life Technologies). Tissues were treated with 0.0125 % (w/v) trypsin (Becton Dickinson) for 5 min at 37 °C, and gently dissociated via trituration in neuronal medium: neurobasal medium (Life Technologies) supplemented with 6 mM Glutamax (Life Technologies) and 1 x B27 supplement (Life Technologies). Dissociated cells were seeded into eight-well glass chamber slides (Matsumani Glass) coated with cell matrix type IC (Nitta Gelatin). The cells were propagated at 37 °C and were used after 6–7 days of culture. All animal experiments were approved by the President of Hokkaido University after review by the Animal Care and Use Committee of Hokkaido University.

Viruses. The TBEV Oshima 5-10 strain was isolated from a dog in Hokuto City (Japan) in 1995 (GenBank accession no. AB062063.2) (Takahashi et al., 1997). The Sofjin-HO strain of TBEV was isolated from the brain of a human patient in Khabarovsk (Russia) in 1937 (GenBank accession no. AB062064.1) (Silver & Soloviev, 1946). The Guriev strain of OHFV was isolated from a pig GenBank accession no. AB507800). The recombinant viruses of these strains were recovered from infectious cDNA clones as described previously (Hayasaka et al., 2004a; Takano et al., 2011; Yoshii et al., 2011). The WNV 6-LP strain was isolated from a New York City isolate, NY99-6922 (GenBank accession no. AB185914.2). The WNV-6LP was propagated in a suckling mouse, and passaged three times in BHK-21 cells and once in C6/36 cells. The JEV Sw/Mie4/2004 was isolated from a pig GenBank accession no. AB241118.1). The JEV Sw/Mie4/2004 was propagated in BHK-21 cells. The Hochosterwitz strain of TBEV (unknown passage history) was isolated from an Ixodes tick in Carinthia (Austria) in 1971 (Heinz & Kunz, 1981). The LGTV TP21 strain (unknown passage history) was isolated from an Ixodes tick (GenBank accession no. AF253419.1). Working stocks of all the viruses were propagated once in BHK-21 cells and stored at −80 °C. All viral infections were conducted under BioSafety Level 3 conditions, in a dedicated laboratory located in the Graduate School of Veterinary Medicine of Hokkaido University.

Antibodies. The following primary antibodies were used to perform IFA. Polyclonal mouse anti-LGTV (cross-reactive among the tick-borne flaviviruses), anti-WNV and anti-JEV antibodies were prepared from ascites of mice repeatedly immunized with LGTV TP21, WNV 6-LP and JEV Ja-GaR01, respectively. These antibodies reacted with both structural and NS viral proteins, respectively (data not shown). Rabbit polyclonal antibodies, prepared by immunization with recombinant E and NS3 proteins derived from Escherichia coli as described previously (Yoshii et al., 2004), were used to detect the TBEV E and NS3 proteins. The J2 mouse mAb (English and Scientific Consulting) was used to detect the dsRNA, the product of viral genome replication. Chicken anti-MAP2, rabbit anti-GEAP, anti-β3-tubulin and anti-synaptophysin polyclonal antibodies were from Abcam. Rabbit anti-calreticulin polyclonal antibodies were from Affinity Bioreagents. The secondary antibodies, anti-mouse IgG, anti-rabbit IgG and anti-chicken IgG conjugated with Alexa Fluor 488 or Alexa Fluor 555 were purchased from Life Technologies.

Construction of VLPs of TBEV. The plasmids Oshima REP (Hayasaka et al., 2004b) and pTBECME (Yoshii et al., 2005) were used to construct VLPs of TBEV. The TBEV replicon was transcribed from the Oshima REP plasmid using an mMESSAGE mMACHINE SP6 kit (Life Technologies), and transfected into human embryonic kidney 293T cells with the aid of a Trans IT mRNA transfection kit (Mirus Biology). After 5–6 h of culture, the cells were transfected with the pTBECME plasmid, which expresses the structural proteins of TBEV, with the aid of a TransIT-LT1 reagent (Mirus). The supernatant was harvested at 48 h post-transfection and clarified by centrifugation at 17,000 g for 5 min. VLPs in the supernatant were precipitated by 10 % (w/v) PEG 8000 and 1.9 % (w/v) NaCl followed by incubation for 2 h at 4 °C, and centrifugation at 16,000 g for 30 min. Pellets were resuspended in neurobasal medium and stored at −80 °C.

Infection of primary neuronal cultures. Primary neuronal cultures were infected at an m.o.i. of 0.1. After viral adsorption for 1 h, half of the culture medium was replaced. Medium was harvested at 12, 24, 48, 72 h p.i. and stored at −80 °C. Cells were fixed and stained with toluidine blue followed, and images were viewed by using BZ-9000 fluorescence microscope (Keyence).

Viral titration. Monolayers of BHK-21 cells, prepared in multi-well plates, were incubated with serial dilutions of viruses for 1 h, and then overlaid with minimum essential medium containing 2 % (v/v) FBS and 1.5 % (v/v) carboxymethyl cellulose. After 3–5 days of incubation, cells were fixed and stained with toluidine blue and either subjected to IFA or viewed using TEM. Unless otherwise stated, TBEV Oshima 5-10 strain was used for TBEV infection.

Toluidine blue staining. At 48 h p.i., infected primary neuronal cultures were fixed in 4 % paraformaldehyde (w/v) for 20 min at 37 °C and washed with 0.1 M glycine in PBS. Staining with toluidine blue followed, and images were viewed by using BZ-9000 fluorescence microscope (Keyence).

IFA. At 12–72 h p.i., infected primary neuronal cultures were fixed in 4 % (w/v) paraformaldehyde for 20 min at 37 °C and washed with 0.1 M glycine in PBS. Fixed cells were permeabilized by incubation in 0.1 % (v/v) Triton X-100 for 5 min at room temperature and blocked with 2 % (w/v) BSA. The cells were incubated at room temperature.
for 1 h with primary antibodies. After extensive washing, cells were incubated with secondary antibodies bearing fluorescent tags. The cells were enclosed with a solution of SlowFade Gold antifade reagent with DAPI (Life Technologies), and observed via BZ-9000 (Keyence) or LSM 700 confocal laser-scanning microscope (Carl Zeiss Microscopy). Images were processed using BZ-2 Analyser (Keyence) or ZEN 2009 (Carl Zeiss Microscopy) software.

Cytoskeletal perturbation. After virus adsorption for 1 h, half of the culture medium was exchanged, and nocodazole (final concentration 5 μM) was added to the medium. The cells were fixed 48 h later and the effect of cytoskeletal perturbation assessed using IFA.

TEM. Infected and mock-infected primary neuronal cultures growing in eight-chambered slides were directly pre-fixed overnight with a 1 % (w/v) osmium tetr-oxide and dehydrated in a graded series of alcohol. Cells were then post-fixed in 1 % (w/v) osmium tetr-oxide and dehydrated in a graded series of alcohol. Cells were then embedded in a Quetol 812, dodecyl succinic anhydride and methyl nadic anhydride mixture (Nishin EM). Ultrathin sections were stained with uranyl acetate and lead citrate and visualized using a JEM-1400plus (JEOL) microscope.

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