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

Running title: Replication mechanism of TBEV in mouse primary neuron

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Summary

Neurological diseases caused by encephalitic flaviviruses are severe and associated with high level of mortality. However, detailed mechanisms of viral replication in the brain and features of viral pathogenesis remain poorly understood. We carried out the 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 flaviviruses multiplied well in the primary neuronal cultures from hippocampus, cerebral cortex, or cerebellum. Distribution of viral-specific antigen in the neuron varied; TBEV infection induced the accumulations of viral antigen in the neuronal dendrites to greater extent than did infection with other viruses. Viral structural, non-structural proteins, and double-stranded RNA were detected in the regions of which viral antigens accumulated in dendrites after TBEV replication. Replication of TBEV replicon after the infection of TBEV virus-like particles also induced the antigen accumulation, indicating that accumulated viral antigens were the results of the viral RNA replication. Further, electron microscopic observation confirmed that TBEV replication induced the characteristic ultrastructural membrane alterations in the neurites; newly formed laminal membrane structure containing virion-like structures. This is the first report describing viral replication in and ultrastructural alterations of the neuronal dendrites, possibly causing the neuronal dysfunction. These findings encourage further study to understand the molecular mechanisms of viral replication in brains and the pathogenicity of neurotropic flaviviruses.
Introduction

*Flavivirus* is a genus in the family *Flaviviridae*, and consists of positive-polarity single-strand RNA viruses with lipid envelopes (Gould & Solomon, 2008; Lindenbach, 2007; Schmaljohn & McClain, 1996). The flavivirus genome encodes one poly polyprotein, which is cleaved into three structural proteins, the core, premembrane, and envelope (E) proteins, and seven non-structural (NS) proteins within a single long open reading frame (Chambers *et al.*, 1990). *Flavivirus* contains over 70 members, many of which are arthropod-borne human pathogens (Lindenbach, 2007; Mackenzie *et al.*, 2004; Mackenzie & Williams, 2009; 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 *Aedes* and *Culex* families are the major vector 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 human causes various manifestations, hemorrhagic 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. All of WNV, JEV, and TBEV are principal neurotropic flaviviruses causing encephalitic diseases in humans. Common symptoms are headache, vomiting, ataxia, and paralysis. Differences in neurologic symptoms have been reported in the infection of
each virus. JEV infection triggers acute spasm and development of a dull pathognomonic Parkinsonian syndrome (Ooi et al., 2008; Solomon et al., 1998). Cognitive function is compromised upon 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 neurologic manifestations are rare (Anastasiadou et al., 2013; Sejvar et al., 2003). On histopathological examinations, all these viruses mentioned above induce typical nonsuppurative 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 viruses, including the brainstem, the cerebral cortex, the caudate putamen, and the cervical spinal cord (Hayasaka et al., 2009; Kimura et al., 2010). However, it remains unclear how viral replication and pathogenicity contributes to the neurologic manifestations.

Primary culture has been developed for maintaining brain cells (Banker & Cowan, 1977), and such cultures can be used to investigate 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 response affected by virus invasion, including lyssavirus, herpesvirus, and flaviviruses (Lewis & Lentz, 1998) (Perkins et al., 2002) (Chen et al., 2011). Primary cultured neurons could provide detailed information about flavivirus replication in neurons.

In the present study, we used primary neuronal cultures to explore replicative and neuropathogenic features of encephalitic flaviviruses. We revealed 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, 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, green), astroglial marker (Glial fibrillary acidic protein: GFAP, red), and DAPI (blue), via indirect immuno fluorescent assay (IFA). Fig. S1 shows that the primary cultures contained principally neurons (70–80%) and astroglial cells (20%), and lacked microglial cells (data not shown).

To compare growth kinetics of encephalitic flaviviruses, primary cultures from each region were infected with TBEV, WNV, or JEV at an multiplicity of infection (MOI) of 0.1, and viral titers in the culture supernatant were measured at various time points, the experiments were repeated four times. Fig. 1a and 1b show that viral titers peaked at 48 h.p.i., and the titers did not differ among studied primary cultures. The viral titer 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 was similar all studied primary cultures. Thus, cerebral cortex cells were used in all subsequent experiments. No obvious morphological change evident upon light microscopy, apart from slight dendritic degeneration in TBEV-infected neurons (Fig. 1c).

Distribution of viral antigen in primary neuronal cultures

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

Detailed images of accumulated viral antigens are shown in Fig. 2b. Antigen accumulations varied in diameter, being 5–10 μm on the major and 3–5 μm on the minor axis (Fig. 2b iii, iv, vi, and vii). Viral antigens were surrounded by MAP2 in structures that appeared to be swollen (Fig. 2b iv and 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 in the cell body principally, thus minimally the dendrites (Fig. 3a i and ii). From 48–72 h.p.i., viral antigen accumulated in the dendrites (Fig. 3a iii and iv, white arrows). However, viral antigen in WNV or JEV infected cells was located principally in the cell body (thus minimally in the dendrites) at all timepoints examined (Fig. 3a v-viii, and ix-xii). WNV antigen accumulated in dendrites of several neurons by 72 h.p.i. (Fig. 3a viii, white arrows), but such accumulations were fewer compared with in neurons infected with TBEV (Fig. 3b).

In a previous study, TBEV infection triggered microtubule re-arrangement in neuroblastoma cells (Ruzek 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 and e-j), anti-β3-tublin (Fig. 4b and k-p), anti-calreticulin (Fig. 4c and q-v), or anti-synaptophysin (Fig. 4d and w-ab) antibodies. However, no obvious change in microtubules distribution was evident in infected primary neuronal cultures.
Accumulated viral antigens in TBEV infected cells were localized with MAP2 (Fig. 4i–j), β3-tublin (Fig. 4n–p), or calreticulin, which is distributed in 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 it was not directly associated with rearrangement of microtubules and synaptic vesicles.

**Effect of perturbation of microtubule 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, and g–i). Thus, the viral antigen accumulations were affected by microtubule.

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

Viral constituents in 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, iii, iv, and vi, white arrows). In addition, double stranded RNA: dsRNA (reflecting viral genome replication) was also present (Fig. 6b vi and vii). These results suggest 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 the most of the cording region for viral structural proteins. The VLPs can enter cells, and replicate within, but cannot produce a progeny virus. Fig. 6c shows that viral antigen also accumulated in
dendrites after infection of the TBEV-VLPs (Fig. 6c i–iv, white arrows). Thus, viral protein accumulations did not require expression of viral structural proteins.

**The ultrastructure of flavivirus infected primary cultured neuron.**

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 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 of 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 and 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 infection 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 neuro-pathogenicity of TBEV, the detailed feature of the replication mechanism in the neural cells is still unknown. We used primary cultures of brain cells to
comparatively examine the replication of several flaviviruses. Viral antigen distribution in infected
primary neuronal cultures differed when such 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, LMS.

Each studied flavivirus multiplied effectively in primary neuronal cultures from several brain
regions, indicating that use of such cells is appropriate when investigating flavivirus infection. But,
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 less detected 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 granule cells (Gelpi et al., 2005; Hayasaka et
al., 2009). Two possible reasons may 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 viruses are subsequently to spread
efficiently through the brain. Second, 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 that 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 astroglias), 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. Interferon treatment of primary neuronal cultures may render viral replication patterns similar to those observed in vivo.

Infection with tick-borne flaviviruses was associated with accumulations of viral antigens in the dendrites of infected neurons, but this was not true of mosquito-borne flaviviruses. The accumulations contained structural proteins, non-structural proteins, and dsRNA. Accumulations were also evident upon the replication of replicon RNA after infection with VLPs of TBEV. Flaviviruses replicate at ER membranes, and buds 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 & Couve, 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 similarly accumulated viral antigens in the dendrites previously (Li et al., 2005; Matsuda et al., 2005). Such accumulation has been considered to reflect inhibition of viral protein transportation in dendrites in which cytoskeleton has been disrupted. However, we found that the mechanism of antigen accumulations during TBEV infection were quite different. This is the first report to show the tick-borne flaviviral replication in dendrites.

TBEV infection caused a characteristic ultrastructural change in neurite membranes of infected neuron. An LMS developed, lying parallel to microtubules, and virion-like structures were observed both inside and outside of this structure. The co-localization of the viral antigen and ER marker indicated the LMS were derived from ER-membrane. LMS-like membranes were previously observed in glioblastoma cells infected with TBEV (Ruzek et al., 2009). Flavivirus infection induces typical alterations in ER membranes. The membranes assume vesicle packets (VPs) and convoluted membranes (CM) (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 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 complex (formed by viral non-structural proteins) may be transported along dendritic membrane. Another important transport mechanism involves formation of RNA granules. Recently, mRNA transportation to the dendrites, and local translational control therein, have been described in neuron (Kiebler & DesGroseillers, 2000; Kohrmann et al., 1999; Muramatsu et al., 1998; Sinnamon & Czapinski, 2011). Specific mRNAs form RNA granules containing several different RNA binding proteins 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, Far Eastern and European subtype 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 characteristics in viral antigen accumulation in dendrites.

Alteration in membrane structure and accumulation of viral proteins in dendrites may cause the neuronal dysfunction and degeneration in vivo. LMS formation and viral protein accumulation induced ultrastructural changes in neurites, including compression of the microtubules, the obstruction of trafficking pathways, and reconstitution of membrane structure. Such changes may affect synaptic function and induce neurite degeneration leading to 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, being in recognition, memory, and behavioral 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). Especially, fragile-X syndrome is caused by disruption of local protein synthesis in dendrites, and triggers hyperactivity and greater response 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 by TBEV infection might cause disruption of local protein synthesis in dendrites, resulting in 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 phenochromocytoma of the rat adrenal
medulla. Such arrest was caused by interaction between 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 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 the neural dendrites. These findings encourage further study to understand the molecular mechanism of viral replication in brains and the pathogenicity of neurotropic flaviviruses, and also promote study to learn how to prevent and cure viral infections.

Methods

Cell culture

Baby hamster kidney-21 (BHK-21) cells were grown at 37°C in minimum essential medium (Life Technologies Co., Carlsbad, CA) supplemented with 8% (v/v) fetal bovine serum 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) fetal bovine serum and penicillin/streptomycin.

Pregnant Slc: ICR mice were purchased from Japan SLC Inc. (Shizuoka, Japan), 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 & Scheiffele, 2007; Viesselman 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, Co., Franklin Lakes, NJ) 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× B27 supplement (Life Technologies). Dissociated cells were seeded into eight-well glass chamber slide (Matsunami Glass Ind., Osaka, Japan) coated with cell matrix type IC (Nitta Gelatin Inc., Osaka, Japan). The cells 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 (AB062063.2) (Takashima et al., 1997). The Sofjin-HO strain of TBEV was isolated from the brain of a human patient in Khabarovsk (Russia) in 1937 (AB062064.1) (Zilber & Soloviev, 1946). The Guriev strain of OHFV was isolated from human blood (AB507800). The recombinant viruses of these strains were recovered from infectious cDNA clones as previously described (Hayasaka, 2004) (Takano et al., 2011) (Yoshii et al., 2011). The WNV 6-LP strain was isolated from a New York City isolate, NY99-6922 (AB185914.2). The WNV-6LP was propagated in a suckling mouse, and passed three times in BHK-21 cells and once in C6/36 cells. The JEV Sw/Mie40/2004 was isolated from a pig (AB241118.1). The JEV Sw/Mie40/2004 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 (KUNZ, 1981). The LGTV TP21 strain (unknown passage history) was
isolated from an *Ixodes* tick (AF253419.1). Working stocks of the all viruses were propagated once in BHK-21 cells, and stored at −80 °C. All viral infections were conducted in the 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 react with both structural and non-structural 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 monoclonal antibody was used to detect the dsRNA, product of viral genome replication (English and Scientific Consulting, Szirak, Hungary). Chicken anti-MAP2, rabbit anti-GFAP, anti-β3 tubulin, and anti-synaptophysin polyclonal antibodies were the products of Abcam plc. (Cambridge, UK). Rabbit anti-calreticulin polyclonal antibodies were products of Affinity BioReagents, inc. (Golden, Colorado). The secondary antibodies, anti-mouse IgG, anti-rabbit IgG, and anti-chicken IgG conjugated with AlexaFluor488 or AlexaFluor555, were purchased from Life Technologies.

**Construction of VLPs of TBEV**

The plasmids Oshima REP (Hayasaka *et al.*, 2004) and pTBECME (Yoshii *et al.*, 2005) were used to construct VLPs of TBEV. The TBEV replicon was transcribed from the Oshima REP plasmid using a mMMESSAGE 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, Co.,
Madison, WI). 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 48 h post-transfection and cleared 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 MOI of 0.1. After viral adsorption for 1 h, half of the culture medium was replaced. Medium was harvested at 12 h, 24 h, 48 h, 72 h post-infection (h.p.i.), and stored at −80°C. Cells were fixed and stained with toluidine blue, either subjected to IFA or viewed using TEM. Unless otherwise stated, TBEV Oshima 5-10 strain was used for TBEV infection.

**Viral titration**

Monolayers of BHK-21 cells, prepared in multi-well plates, were incubated with serial dilutions of viruses for 1 h, and next overlaid with minimum essential medium containing 2% (v/v) FBS and 1.5% (w/v) carboxymethyl cellulose. After 3–5 days of incubation, cells were fixed and stained with a solution of 0.25% (w/v) crystal violet in 10% (v/v) buffered formalin. Plaques were counted and viral titers expressed as plaque-forming unit (PFU)/ml.

**Toluidine blue staining**

After 48 h.p.i. of growth, infected primary neuronal cultures were fixed in 4% paraformaldehyde (w/v) for 20 min at 37°C, and next washed with 0.1 M glycine in PBS. Staining with toluidine blue followed, and images were viewed by using BZ-9000 fluorescence microscope (Keyence, Osaka,
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 next 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 next blocked with 2% (w/v) bovine serum albumin. 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 the Slowfade Gold antifade reagent with DAPI (Life Technologies), and observed via BZ-9000 (Keyence) or LSM 700 confocal laser scanning microscopy (Carl Zeiss Microscopy Co., Ltd., Jena, Germany). Images were processed using the BZ-2 Analyser (Keyence) or ZEN 2009 (Carl Zeiss Microscopy) software.

Cytoskeletal perturbation
After viral 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 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 solution of 2.5% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 M phosphate buffer, at 4°C. After washing with 0.1 M phosphate buffer, cells were post-fixed in 1% (w/v) osmium tetroxide and dehydrated in a graded series of alcohol. Cells were next embedded in a Quetol 812, DDSA, and MNA mixture (Nisshin EM, Tokyo, Japan). Ultrathin sections were stained with uranyl acetate and lead citrate and visualized via JEM-1400plus.
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References


Figure legends

Fig. 1. Virus production in primary neuronal cell cultures
(a) TBEV growth kinetics in primary cell cultures. Hippocamal (closed circles), cerebral cortex (crosses), and cerebellal (open triangles) cells were infected with TBEV at an MOI of 0.1 and, at the indicated time points, the media were harvested and virus titers determined by plaque forming assay. Error bars: standard deviations.
(b) Virus production levels in primary cultures of cells from various brain regions. Cells from the hippocampus (Hip), cerebral cortex (Cor), and cerebellum (Cer) were infected with TBEV, WNV, or JEV at an MOI of 0.1. Virus titers in supernatants at 48 h.p.i. were measured.
(c) Infected neurons were stained with toluidine blue. Cultured cerebral cortex cells were infected with TBEV or WNV at an MOI of 0.1. Infected cells and mock-infected cells were fixed at 48 h.p.i. and stained with toluidine blue. Scale bars: 50 μm.

Fig. 2. Distribution of viral antigens and MAP2 in primary neuronal cultures
(a) Viral antigen distribution at 48 h.p.i.. Cultured cerebral cortex cells were infected with TBEV (i−v), WNV (vi−x), or JEV (xi−xv), at an MOI of 0.1. The infected cells were fixed at 48 h.p.i., and stained with antibodies against MAP2 (green), antiseras against each virus (red), and DAPI (blue). Panels (iv, v, ix, x, ixv, and xv) show magnifications of dendrites from the indicated regions of the merged images. Scale bars: 50 μm.
(b) Viral protein accumulations in TBEV-infected dendrites. Infected cells were fixed at 48 h.p.i. and stained with antibodies against MAP2 (green) and an antiserum against a tick-borne flaviviruses (red). The images were collected with a confocal laser scanning microscopy. Panels (termed Region1: ii−iv, and Region2: v−vii) show magnifications of dendrites in the indicated region of panel (i).
Accumulations of viral antigens in dendrites are indicated by white arrows.

**Fig. 3. Time-course changes in viral antigen distribution**

(a) IFA images showing the changes over time in viral antigen distributions in primary neuronal cultures. Cultured cerebral cortex cells were infected with TBEV (i–iv), WNV (v–viii), or JEV (ix–xii) at an MOI of 0.1. The infected cells were fixed at the indicated time points (12, 24, 48, and 72 h.p.i.), and stained with antisera against each virus (red). Accumulations of viral antigens are indicated by white arrows. Scale bars: 50 μm.

(b) The extent of viral antigen accumulations in infected cells. Cultured cerebral cortex cells were infected with TBEV, WNV, or JEV at an MOI of 0.1, fixed the indicated timepoints (12, 24, 48, and 72 h.p.i.), and stained with antisera against each virus and DAPI. The numbers of infected cells and antigen accumulations in such cells were counted in four different microscopic fields. Error bars: standard deviation. **: P<0.01 by Tukey’s test.

**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 MOI 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 and e–j), β3-tublin (b and k–p), carleticulin (c and q–v), and synaptophysin (d and w-ab) were used as organelle markers. The accumulations of viral antigens are magnified in panels (h–j, n–p, t–v, and z–ab). Accumulations of viral antigens are indicated by white arrows. Scale bars: 50 μm.

**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 MOI 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). Scale bars: 50 μm.

Fig. 6. Viral constituents present in antigen accumulations in dendrites
(a) Cells were infected with TBEV at an MOI 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 E protein (i–iii) and NS3 (iv–vi) were used. Scale bars: 50 μm.
(b) Cells were infected with TBEV at an MOI 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). The accumulation of viral antigens is magnified in panels (ii–iv). The images were collected with a confocal laser scanning microscopy.
(c) Cells were infected with VLPs of TBEV and fixed at 48 h.p.i.. Neurons were stained with an antibody against MAP2 (green), an antiserum against a tick-borne flaviviruses (red), and DAPI (blue). Accumulations of viral antigens are magnified in panels (iv and v). Scale bars: 50 μm.

Accumulations of viral antigens are indicated by white arrows.

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, and f), cells infected with TBEV (d, e, and h–l), and cells infected with WNV (g). (a–c) Mock-infected neurons contained structurally intact organelles (Nu: Nucleus, Cyto: Cytosol, ER: endoplasmic reticulum, and Mit: Mitochondria). (d) TBEV infected neurons exhibited cytoplasmic condensation, and reactive lysosomes were also observed (Lys: Lysosome). (e) Virion-like structures coated with lipid bilayers were evident (white arrows). (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. (j-l) Representative images of the regions (surrounding those shown in h, and i). Virion-like structures coated with lipid bilayers were observed both inside and outside the observed structures (white arrows).

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.
The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see:

http://www.textcheck.com/certificate/vOO0vI
Fig. 1

(a) Viral titer (log10 PFU/ml) over time (h.p.i.):
- Hippocampus
- Cortex
- Cerebellum

(b) Comparison of viral titer (log10 PFU/ml) for different regions and viruses:
- TBEV
- WNV
- JEV

(c) Histological images:
- Mock infected
- TBEV
- WNV
Fig. 2

(a)

TBEV

MAP2

Virus Antigen

Merge

Mag

(i)

(ii)

(iii)

(iv)

(v)

(vi)

(vii)

(viii)

(ix)

(x)

(xi)

(xii)

(xiii)

(xiv)

(xv)

(b)

MAP2

Virus Antigen

Merge

Region 1

Region 2

(i)

(ii)

(iii)

(iv)

(v)

(vi)

(vii)
Fig. 3

(a) 12 h.p.i.  24 h.p.i.  48 h.p.i.  72 h.p.i

TBEV

WNV

JEV

(b) Number of the accumulation per infected cells

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Fig. 4

Mock infected

MAP2  β-Tublin3  Calreticulin  Synaptophysin

TBEV

MAP2  Virus Antigen  Merge  Mag
β-Tublin3  Virus Antigen  Merge  Mag
Calreticulin  Virus Antigen  Merge  Mag
Synaptophysin  Virus Antigen  Merge  Mag

TBEV

(a)  (b)  (c)  (d)  (e)  (f)  (g)  (h)  (i)  (j)  (k)  (l)  (m)  (n)  (o)  (p)  (q)  (r)  (s)  (t)  (u)  (v)  (w)  (x)  (y)  (z)  (aa)  (ab)
Fig. 5

Mock infected

TBEV

WNV

(a) MAP2

(b) Virus Antigen

(c) Merge

(d) (e) (f)

(g) (h) (i)
Fig. 6

(a) α-E protein  Infected serum  Merge

α-NS3 protein  Infected serum  Merge

(b) α-E protein  dsRNA  Merge

Magnification

(c) MAP2  Virus Antigen  Merge  Mag

(i)  (ii)  (iii)  (iv)  (v)
Fig. 7 (1/2)

(a) Mock

(b) ER

(c) Mit

(d) TBEV

(e) Virion

Nu
Cyto

1.0 μm

5.0 μm