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

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Authors: Minato Hirano 1), Kentaro Yoshii 1), Mizuki Sakai 1), Rie Hasebe 2), Osamu Ichii 3), Hiroaki Kariwa 1)

1) Laboratory of Public Health, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan

2) Laboratory of Veterinary Hygiene, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan

3) Laboratory of Anatomy, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan

Corresponding author: Kentaro Yoshii,

Postal address: Laboratory of Public Health, Graduate School of Veterinary Medicine, Hokkaido University, kita-18 nishi-9, kita-ku, Sapporo, Hokkaido 060-0818, Japan

Tel/fax: +81-11-706-5213

E-mail: kyoshii@vetmed.hokudai.ac.jp

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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.

22 **Introduction**

23

24 *Flavivirus* is a genus in the family *Flaviviridae*, and consists of positive-polarity single-strand RNA
25 viruses with lipid envelopes (Gould & Solomon, 2008; Lindenbach, 2007; Schmaljohn & McClain,
26 1996). The flavivirus genome encodes one poly polyprotein, which is cleaved into three structural
27 proteins, the core, premembrane, and envelope (E) proteins, and seven non-structural (NS) proteins
28 within a single long open reading frame (Chambers *et al.*, 1990). *Flavivirus* contains over 70
29 members, many of which are arthropod-borne human pathogens (Lindenbach, 2007; Mackenzie *et*
30 *al.*, 2004; Mackenzie & Williams, 2009; Schmaljohn & McClain, 1996). Recently, many outbreaks
31 have been reported, and flaviviruses are attracting global attention as emerging or re-emerging
32 infectious diseases (Balogh *et al.*, 2010; Chung *et al.*, 2013; Danis *et al.*, 2011; McMichael *et al.*,
33 2006; Morse, 1995; Vong *et al.*, 2010). Flaviviruses are divided into four distinct evolutionary
34 lineages: mosquito-borne, tick-borne, no-known vector, and insect-only flaviviruses (Billoir *et al.*,
35 2000; Cook *et al.*, 2012; Crabtree *et al.*, 2003; Kuno *et al.*, 1998). Mosquitoes of *Aedes* and *Culex*
36 families are the major vector of mosquito-borne flaviviruses, including yellow fever virus, dengue
37 virus, West Nile virus (WNV), and Japanese encephalitis virus (JEV) (Gould & Solomon, 2008).
38 *Ixodidae* ticks carry tick-borne flaviviruses, including tick-borne encephalitis virus (TBEV) and
39 Langat virus (LGTV) (Lindquist & Vapalahti, 2008).

40

41 Flavivirus infection of human causes various manifestations, hemorrhagic disease, encephalitis,
42 biphasic fever, flaccid paralysis, and jaundice (Gould & Solomon, 2008). Encephalitis, a
43 neurological manifestation of disease, is particularly problematic. This condition is associated with
44 high-level mortality and severe sequelae. All of WNV, JEV, and TBEV are principal neurotropic
45 flaviviruses causing encephalitic diseases in humans. Common symptoms are headache, vomiting,
46 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

72

73 **Replication of neurotropic flaviviruses in primary neuronal cultures**

74 Prior to experiments using infectious viruses, cell components of primary cultured brain cells were
75 examined. Primary neuronal cultures were prepared from hippocampi, cerebral cortexes, and
76 cerebella, and stained for a neuronal marker (microtubule-associated protein 2: MAP2, green),
77 astroglial marker (Glial fibrillary acidic protein: GFAP, red), and DAPI (blue), via indirect
78 immuofluorescent assay (IFA). Fig. S1 shows that the primary cultures contained principally
79 neurons (70–80%) and astroglial cells (20%), and lacked microglial cells (data not shown).

80

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

90

91 **Distribution of viral antigen in primary neuronal cultures**

92 The distribution of viral antigens in primary neuronal cultures was examined via IFA. At 48 h.p.i.,
93 infected cells stained with MAP2 (a marker of neuronal cell body and dendrites, green),
94 virus-specific antibodies (red), and DAPI (blue). Fig. 2a shows that the cell body distribution of
95 viral antigens was similar in neurons infected with each virus studied. However, dendritic
96 distributions were different in infected neurons. Viral antigens were sparsely distributed in dendrites

97 of cells infected with WNV or JEV (Fig. 2a vii, viii, xii, and xiii). On the other hand, elliptical
98 antigen accumulations were evident in dendrites infected with TBEV (Fig. 2a ii and iii, white
99 arrows). This form of antigen-accumulation was also evident in neurons infected with the tick-borne
100 flaviviruses, TBEV, Omsk hemorrhagic fever virus (OHFV), and LGTV (Fig. S2).

101

102 Detailed images of accumulated viral antigens are shown in Fig. 2b. Antigen accumulations varied
103 in diameter, being 5–10 μm on the major and 3–5 μm on the minor axis (Fig. 2b iii, iv, vi, and vii).
104 Viral antigens were surrounded by MAP2 in structures that appeared to be swollen (Fig. 2b iv and
105 vii). In some large swellings, an unstained (hollow) region was evident within the accumulation of
106 viral antigen (Fig. 2b ii-iv).

107

108 Changes over time in viral antigen distribution are shown in Fig. 3. In the early stages of TBEV
109 infection, viral antigens were detected in the cell body principally, thus minimally the dendrites (Fig.
110 3a i and ii). From 48–72 h.p.i., viral antigen accumulated in the dendrites (Fig. 3a iii and iv, white
111 arrows). However, viral antigen in WNV or JEV infected cells was located principally in the cell
112 body (thus minimally in the dendrites) at all timepoints examined (Fig. 3a v-viii, and ix-xii). WNV
113 antigen accumulated in dendrites of several neurons by 72 h.p.i. (Fig. 3a viii, white arrows), but
114 such accumulations were fewer compared with in neurons infected with TBEV (Fig. 3b).

115

116 In a previous study, TBEV infection triggered microtubule re-arrangement in neuroblastoma cells
117 (Ruzek *et al.*, 2009), possibly associated with the viral antigen accumulations in dendrites. As
118 shown in Fig. 4, neurons mock-infected (Fig. 4a–d) or infected with TBEV (Fig. 4e–ab) were
119 co-stained with the anti-TBEV and anti-MAP2 (Fig. 4a and e-j), anti- β 3-tubulin (Fig. 4b and k-p),
120 anti-calreticulin (Fig. 4c and q-v), or anti-synaptophysin (Fig. 4d and w-ab) antibodies. However,
121 no obvious change in microtubules distribution was evident in infected primary neuronal cultures

122 (Fig. 4k–p). Accumulated viral antigens in TBEV infected cells were localized with MAP2 (Fig.
123 4h–j), β 3-tubulin (Fig. 4n–p), or calreticulin, which is distributed in endoplasmic reticulum (ER)
124 membrane (Fig. 4t–v), but not with synaptophysin, a marker of synaptic vesicles (Fig. 4z–ab). Thus,
125 the viral antigens accumulated in the ER of the dendrites, but it was not directly associated with
126 rearrangement of microtubules and synaptic vesicles.

127

128 **Effect of perturbation of microtubule on viral antigen distribution**

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

132

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

134 Viral constituents in accumulations were investigated. Staining of TBEV-infected neurons with
135 specific antibodies detecting structural (E) and non-structural (NS3) proteins showed that both
136 proteins were present in the antigen accumulations (Fig. 6a i, iii, iv, and vi, white arrows). In
137 addition, double stranded RNA: dsRNA (reflecting viral genome replication) was also present (Fig.
138 6b vi and vii). These results suggest that viral genome replication occurred in the regions of viral
139 protein accumulations in dendrites.

140

141 To investigate the viral components required for the formation of the accumulated viral antigens,
142 we next infected primary cultures with virus-like particles (VLPs) of TBEV (“single-round”
143 infectious particles containing replicon RNA as a genome) (Gehrke *et al.*, 2003; Khromykh *et al.*,
144 1998; Molenkamp *et al.*, 2003; Reynard *et al.*, 2011; Yoshii *et al.*, 2008). The replicon RNA lacks
145 the most of the coding region for viral structural proteins. The VLPs can enter cells, and replicate
146 within, but cannot produce a progeny virus. Fig. 6c shows that viral antigen also accumulated in

147 dendrites after infection of the TBEV-VLPs (Fig. 6c i–iv, white arrows). Thus, viral protein
148 accumulations did not require expression of viral structural proteins.

149

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

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

167

168 **Discussion**

169

170 Despite the importance of neuro-pathogenicity of TBEV, the detailed feature of the replication
171 mechanism in the neural cells is still unknown. We used primary cultures of brain cells to

172 comparatively examine the replication of several flaviviruses. Viral antigen distribution in infected
173 primary neuronal cultures differed when such cells were infected with the mosquito and tick-borne
174 neurotropic flaviviruses. IFA and TEM studies revealed that dendritic replication of tick-borne
175 flaviviruses caused abnormal swelling of neurites and development of a specific structure, LMS.
176
177 Each studied flavivirus multiplied effectively in primary neuronal cultures from several brain
178 regions, indicating that use of such cells is appropriate when investigating flavivirus infection. But,
179 the flaviviruses showed similar growth kinetics in primary cultures from several brain regions.
180 Neurotropic flaviviruses have been reported to exhibit differences in distribution and multiplication
181 among the various parts of the brain. WNV antigens were less detected in granule cell neurons of
182 the cerebellum compared with other neuronal populations (Omalu *et al.*, 2003; Xiao *et al.*, 2001). In
183 contrast, JEV replicated well in granule cell neurons (Desai *et al.*, 1995; German *et al.*, 2006), and
184 TBEV replicated throughout the cerebellum, including granule cells (Gelpi *et al.*, 2005; Hayasaka *et*
185 *al.*, 2009). Two possible reasons may be suggested to explain the difference in the *in vivo* and *in*
186 *vitro* results. First, lack of glial cell maturation may influence viral replication. Mammalian neurons
187 interact with glial cells soon after birth; the neurons mature and become myelinated (Baumann &
188 Pham-Dinh, 2001). Some reports have emphasized that the presence of glial cells is important for
189 effective flavivirus replication in the brain (Chen *et al.*, 2010; Hussmann *et al.*, 2013). It is possible
190 that primary viral replication in glial cells is essential if the viruses are subsequently to spread
191 efficiently through the brain. Second, incomplete maturation of the innate immune response of
192 neurons may affect the susceptibility of such cells to flavivirus infection. The granule cells of the
193 cerebellum have been reported that to mount an effective innate immune response against viral
194 infection (Cho *et al.*, 2013). The primary cell cultures used in the present study were devoid of glial
195 cells (except astroglia), and embryonic neurons may lack a well-developed innate immune system.
196 Thus, the susceptibility of primary embryonic neuronal cultures to viral infection may differ from

197 that of adult brains *in vivo*. Interferon treatment of primary neuronal cultures may render viral
198 replication patterns similar to those observed *in vivo*.

199

200 Infection with tick-borne flaviviruses was associated with accumulations of viral antigens in the
201 dendrites of infected neurons, but this was not true of mosquito-borne flaviviruses. The
202 accumulations contained structural proteins, non-structural proteins, and dsRNA. Accumulations
203 were also evident upon the replication of replicon RNA after infection with VLPs of TBEV.
204 Flaviviruses replicate at ER membranes, and buds into the ER lumen (Lindenbach, 2007). Dendrites
205 are known to contain free ribosomes and express satellite secretory pathways to secure synaptic
206 plasticity (Martone *et al.*, 1993; McCarthy & Milner, 2003; Ori-McKenney *et al.*, 2012; Pierce *et al.*,
207 2001; Ramirez & Couve, 2011). Together, the data suggest that tick-borne flaviviral replication in
208 dendrites induced viral protein accumulation. Infection with rabies virus, influenza virus, and other
209 viruses similarly accumulated viral antigens in the dendrites previously (Li *et al.*, 2005; Matsuda *et*
210 *al.*, 2005). Such accumulation has been considered to reflect inhibition of viral protein
211 transportation in dendrites in which cytoskeleton has been disrupted. However, we found that the
212 mechanism of antigen accumulations during TBEV infection were quite different. This is the first
213 report to show the tick-borne flaviviral replication in dendrites.

214

215 TBEV infection caused a characteristic ultrastructural change in neurite membranes of infected
216 neuron. An LMS developed, lying parallel to microtubules, and virion-like structures were observed
217 both inside and outside of this structure. The co-localization of the viral antigen and ER marker
218 indicated the LMS were derived from ER-membrane. LMS-like membranes were previously
219 observed in glioblastoma cells infected with TBEV (Ruzek *et al.*, 2009). Flavivirus infection
220 induces typical alterations in ER membranes. The membranes assume vesicle packets (VPs) and
221 convoluted membranes (CM) (Mackenzie, 2005), forming a platform on which viral genome

222 replication and virion assembly proceed (Uchil & Satchidanandam, 2003; Welsch *et al.*, 2009). It is
223 possible that LMS is formed via ER-derived membrane reconstitution triggered by the viral
224 replication, and serves as the scaffold for dendritic viral replication and virion assembly. The
225 unstained hollow regions evident when the accumulations of viral proteins were examined by IFA
226 may be attributable to the fact that degenerated membranes are poorly permeable to antibodies.

227

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

231

232 Time-course experiments revealed that viral proteins were synthesized principally in the neuronal
233 cell bodies during the early stages of infection, becoming distributed in dendrites only later. Thus,
234 TBEV genomic RNA (with or without viral proteins) was transported principally from cell bodies to
235 dendrites. Viral genomic RNA bound to membrane-associated replication complex (formed by viral
236 non-structural proteins) may be transported along dendritic membrane. Another important transport
237 mechanism involves formation of RNA granules. Recently, mRNA transportation to the dendrites,
238 and local translational control therein, have been described in neuron (Kiebler & DesGroseillers,
239 2000; Kohrmann *et al.*, 1999; Muramatsu *et al.*, 1998; Sinnamon & Czapinski, 2011). Specific
240 mRNAs form RNA granules containing several different RNA binding proteins are transported
241 along microtubules to dendrites in a kinesin-dependent manner (Bramham & Wells, 2007; Kanai *et*
242 *al.*, 2004). It is possible that viral genomic RNAs may hijack or mimic this transport mechanism.
243 Microtubule-dependent formation of viral antigen accumulations in dendrites may support this
244 hypothesis.

245

246 All tick-borne flaviviruses used in the present study, Far Eastern and European subtype of TBEV,

247 OHFV, and LGTV, accumulated viral antigens in dendrites. However, WNV and JEV formed
248 smaller accumulations. Some previous studies used the primary brain cultures to investigate
249 replication of mosquito-borne flaviviruses, but no mention was made of the viral antigen
250 accumulation in dendrites (Chen *et al.*, 2011; Diniz *et al.*, 2006). Tick-borne flaviviruses share the
251 characteristics in viral antigen accumulation in dendrites.

252

253 Alteration in membrane structure and accumulation of viral proteins in dendrites may cause the
254 neuronal dysfunction and degeneration *in vivo*. LMS formation and viral protein accumulation
255 induced ultrastructural changes in neurites, including compression of the microtubules, the
256 obstruction of trafficking pathways, and reconstitution of membrane structure. Such changes may
257 affect synaptic function and induce neurite degeneration leading to development of neurological
258 disease. Synaptic connections are dynamically regulated via intracellular trafficking pathways,
259 protein modifications, and local protein synthesis in the dendrites (Bagni & Greenough, 2005;
260 Kiebler & DesGroseillers, 2000; McCarthy & Milner, 2003; Steward & Schuman, 2003). Such
261 connections play important roles in the brain, being in recognition, memory, and behavioral
262 regulation. Dendritic degeneration occurs in some diseases associated with loss of cognitive
263 function, including Alzheimer's disease, fragile-X syndrome, and Rett syndrome (Calon *et al.*,
264 2004; Comery *et al.*, 1997; Maezawa & Jin, 2010). Especially, fragile-X syndrome is caused by
265 disruption of local protein synthesis in dendrites, and triggers hyperactivity and greater response to
266 low-intensity auditory stimuli in mouse model (Bagni & Greenough, 2005; Consortium, 1994). As
267 observed in fragile-X syndrome, the abnormal membrane alterations in dendrites by TBEV infection
268 might cause disruption of local protein synthesis in dendrites, resulting in cognitive compromise
269 observed in TBEV patients. In addition, viral protein accumulations in dendrites may affect neural
270 function via the interaction of such proteins with host factors. In a previous study, TBEV replication
271 arrested the neurite outgrowth in a cell line derived from a pheochromocytoma of the rat adrenal

272 medulla. Such arrest was caused by interaction between TBEV NS5 protein and host proteins-Rac1
273 and Scribble (Wigerius *et al.*, 2010). The latter proteins are involved in maintaining cell polarity,
274 regulation of synaptic plasticity, and synaptic vesicle dynamics (Roche *et al.*, 2002). It is possible
275 that the accumulated viral proteins affect the distribution and functionality of host proteins with
276 which the viral proteins interact, in turn causing neural dysfunction and cell-degeneration.

277

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

285

286 **Methods**

287

288 **Cell culture**

289 Baby hamster kidney-21 (BHK-21) cells were grown at 37°C in minimum essential medium (Life
290 Technologies Co., Carlsbad, CA) supplemented with 8% (v/v) fetal bovine serum and
291 penicillin/streptomycin. Human embryonic kidney 293T cells were cultured at 37°C in Dulbecco's
292 modified Eagle's medium (Life Technologies), containing 10% (v/v) fetal bovine serum and
293 penicillin/streptomycin.

294

295 Pregnant Slc: ICR mice were purchased from Japan SLC Inc. (Shizuoka, Japan), and hippocampal,
296 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; 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, 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 passaged 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 Hochsterwitz 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- $\beta 3$ 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.,

347 Madison, WI). After 5-6 h of culture, the cells were transfected with the pTBECME plasmid, which
348 expresses the structural proteins of TBEV, with the aid of a TransIT-LT1 reagent (Mirus). The
349 supernatant was harvested 48 h post-transfection and cleared by centrifugation at 17,000 g for 5 min.
350 VLPs in the supernatant were precipitated by 10% (w/v) PEG 8000 and 1.9% (w/v) NaCl followed
351 by incubation for 2 h at 4°C, and centrifugation at 16,000 g for 30 min. Pellets were resuspended in
352 neurobasal medium and stored at -80°C.

353

354 **Infection of primary neuronal cultures**

355 Primary neuronal cultures were infected at an MOI of 0.1. After viral adsorption for 1 h, half of the
356 culture medium was replaced. Medium was harvested at 12 h, 24 h, 48 h, 72 h post-infection (h.p.i.),
357 and stored at -80°C. Cells were fixed and stained with toluidine blue, either subjected to IFA or
358 viewed using TEM. Unless otherwise stated, TBEV Oshima 5-10 strain was used for TBEV
359 infection.

360

361 **Viral titration**

362 Monolayers of BHK-21 cells, prepared in multi-well plates, were incubated with serial dilutions of
363 viruses for 1 h, and next overlaid with minimum essential medium containing 2% (v/v) FBS and
364 1.5% (w/v) carboxymethyl cellulose. After 3-5 days of incubation, cells were fixed and stained with
365 a solution of 0.25% (w/v) crystal violet in 10% (v/v) buffered formalin. Plaques were counted and
366 viral titers expressed as plaque-forming unit (PFU)/ml.

367

368 **Toluidine blue staining**

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

372 Japan).

373

374 **IFA**

375 At 12–72 h.p.i., infected primary neuronal cultures were fixed in 4% (w/v) paraformaldehyde for 20
376 min at 37°C, and next washed with 0.1 M glycine in PBS. Fixed cells were permeabilized by
377 incubation in 0.1% (v/v) Triton X-100 for 5 min at room temperature, and next blocked with 2%
378 (w/v) bovine serum albumin. The cells were incubated at room temperature for 1 h with primary
379 antibodies. After extensive washing, cells were incubated with secondary antibodies bearing
380 fluorescent tags. The cells were enclosed with a solution of the Slowfade Gold antifade reagent with
381 DAPI (Life Technologies), and observed via BZ-9000 (Keyence) or LSM 700 confocal laser
382 scanning microscopy (Carl Zeiss Microscopy Co., Ltd., Jena, Germany). Images were processed
383 using the BZ-2 Analyser (Keyence) or ZEN 2009 (Carl Zeiss Microscopy) software.

384

385 **Cytoskeletal perturbation**

386 After viral adsorption for 1 h, half of the culture medium was exchanged, and nocodazole (final
387 concentration 5 μ M) was added to the medium. The cells were fixed 48 h later, and effect of
388 cytoskeletal perturbation assessed using IFA.

389

390 **TEM**

391 Infected and mock-infected primary neuronal cultures growing in eight-chambered slides were
392 directly pre-fixed overnight with a solution of 2.5% (w/v) glutaraldehyde and 2% (w/v)
393 paraformaldehyde in 0.1 M phosphate buffer, at 4°C. After washing with 0.1 M phosphate buffer,
394 cells were post-fixed in 1% (w/v) osmium tetroxide and dehydrated in a graded series of alcohol.
395 Cells were next embedded in a Quetol 812, DDSA, and MNA mixture (Nisshin EM, Tokyo, Japan).
396 Ultrathin sections were stained with uranyl acetate and lead citrate and visualized via JEM-1400plus

397 (JEOL Ltd., Tokyo, Japan).

398

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400

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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 μ m.

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), antisera 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 μ m.

(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 μ m.

(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), β 3-tubulin (b and k–p), carlenticulin (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 μ m.

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 μ m.

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 μ m.

(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 μ m.

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.

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Fig. 1

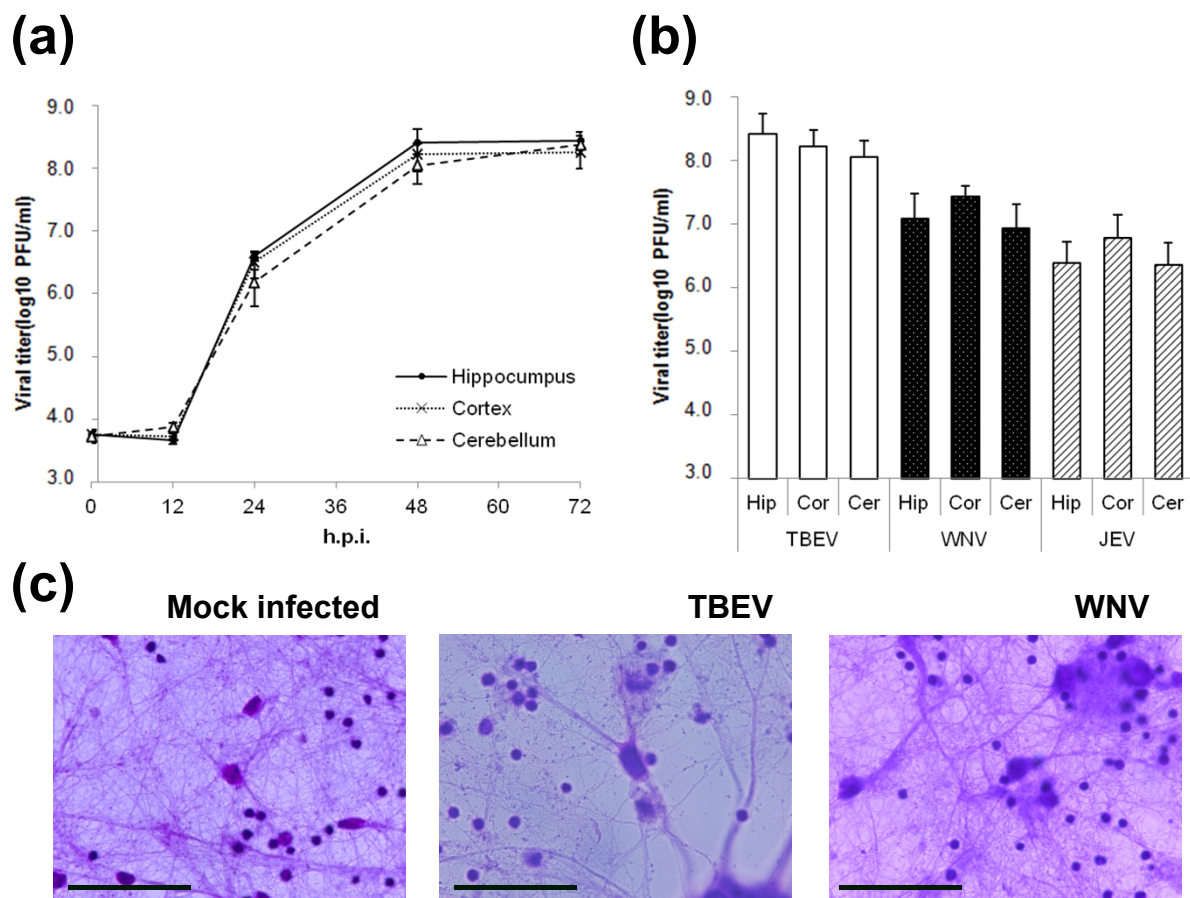


Fig. 2

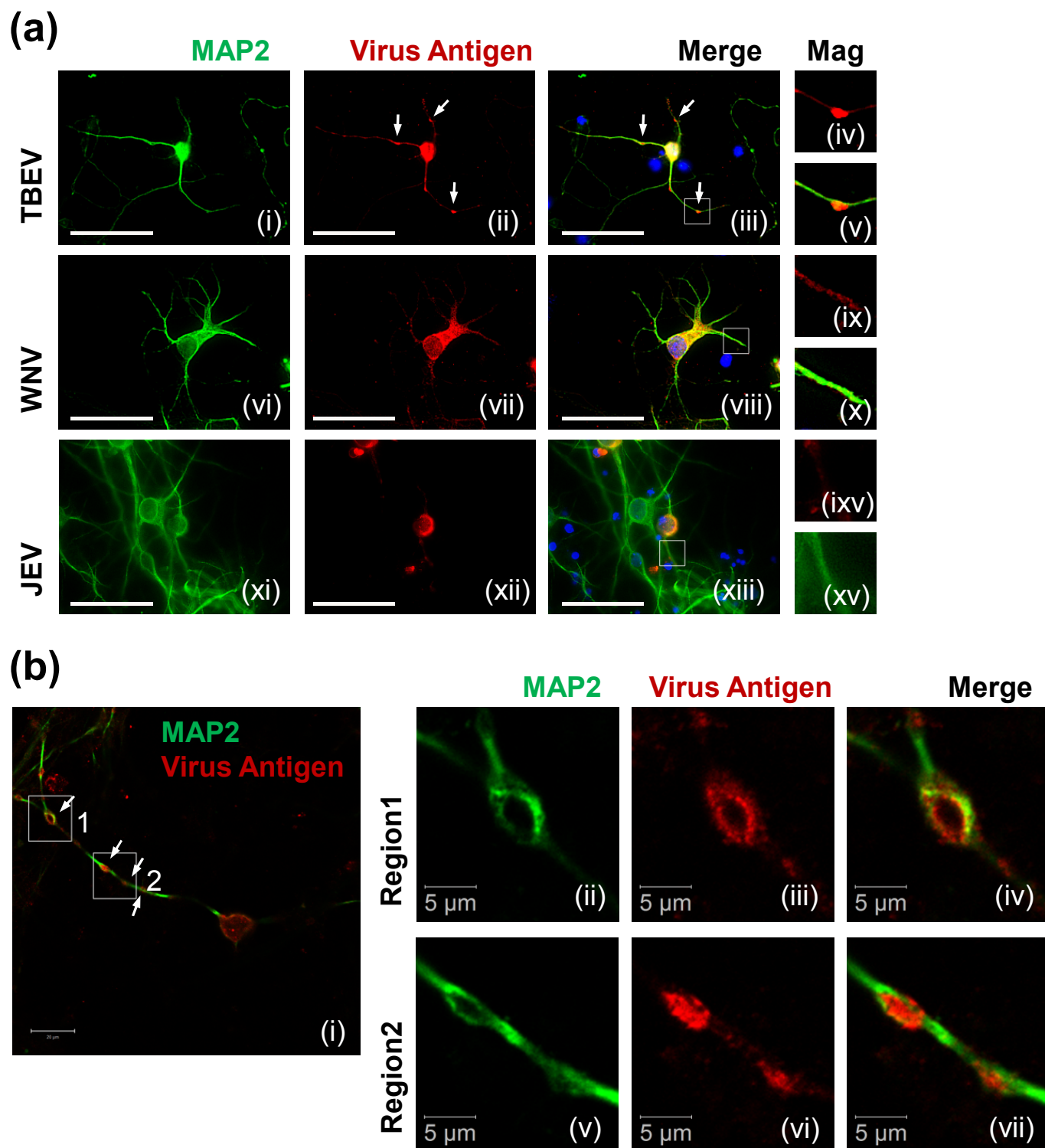


Fig. 3

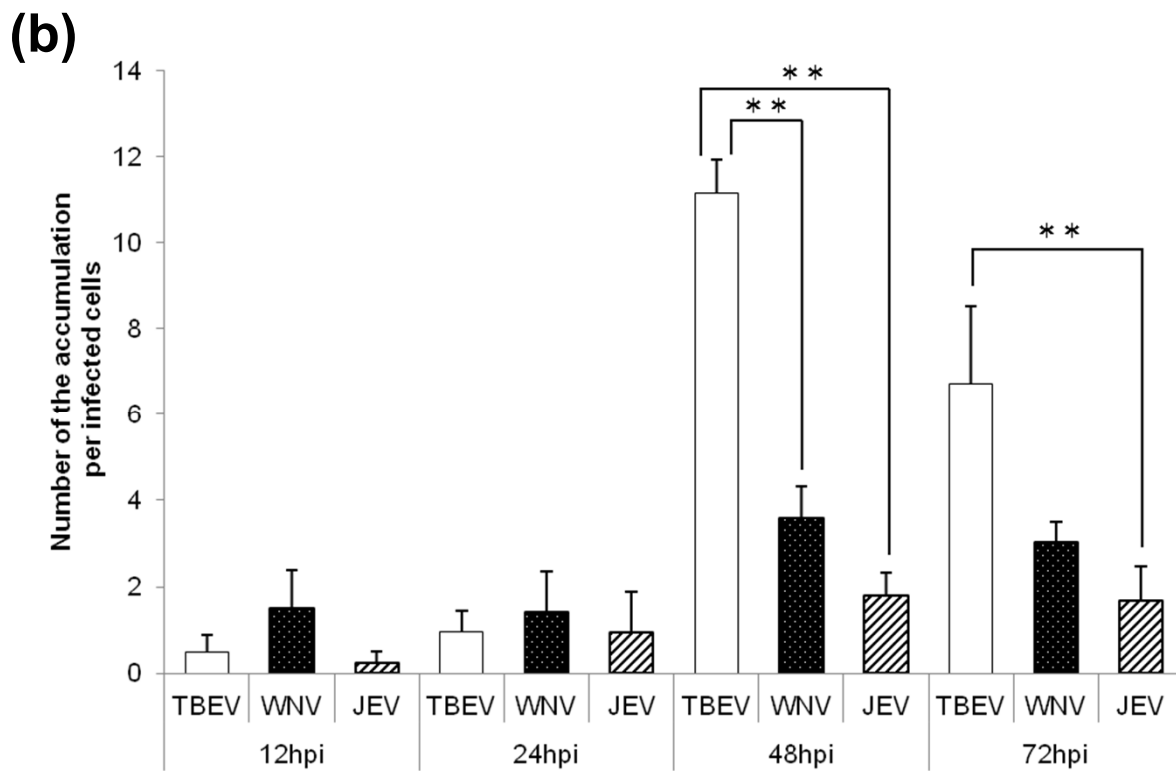
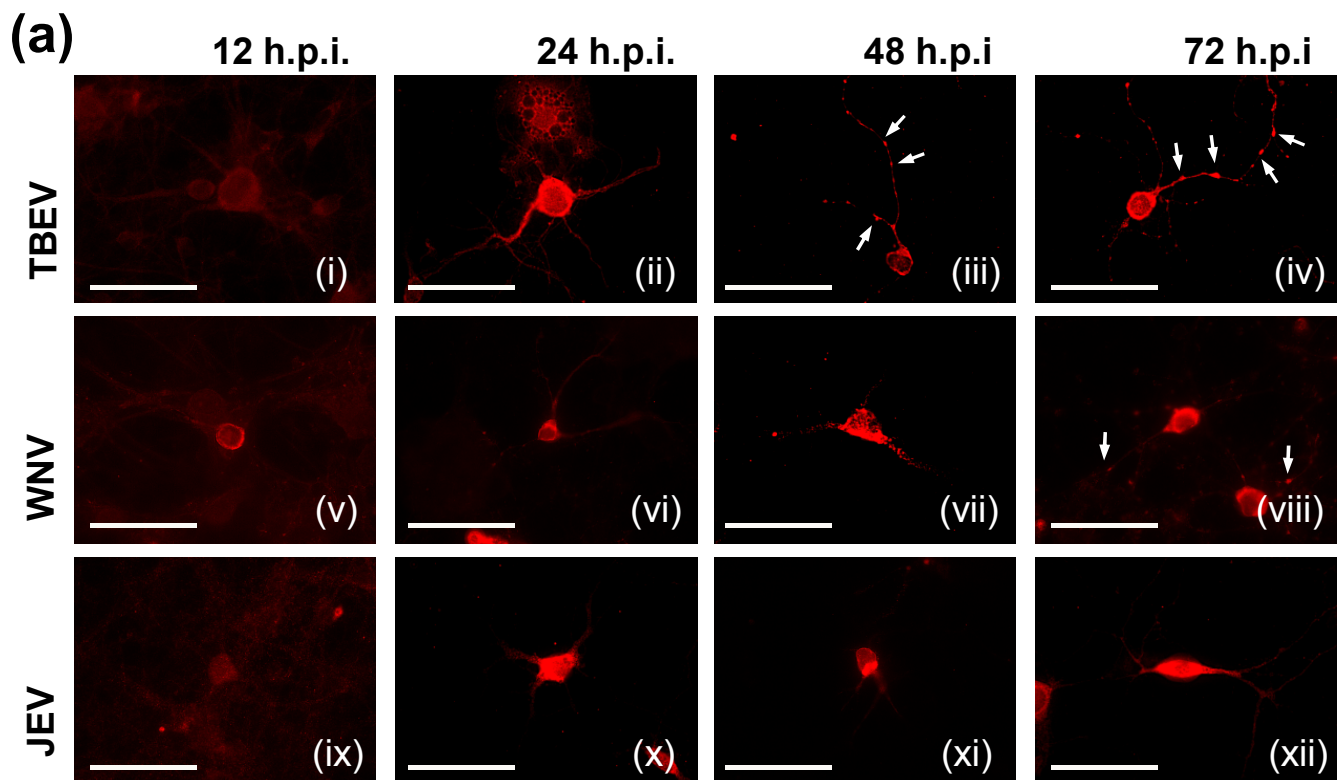


Fig. 4

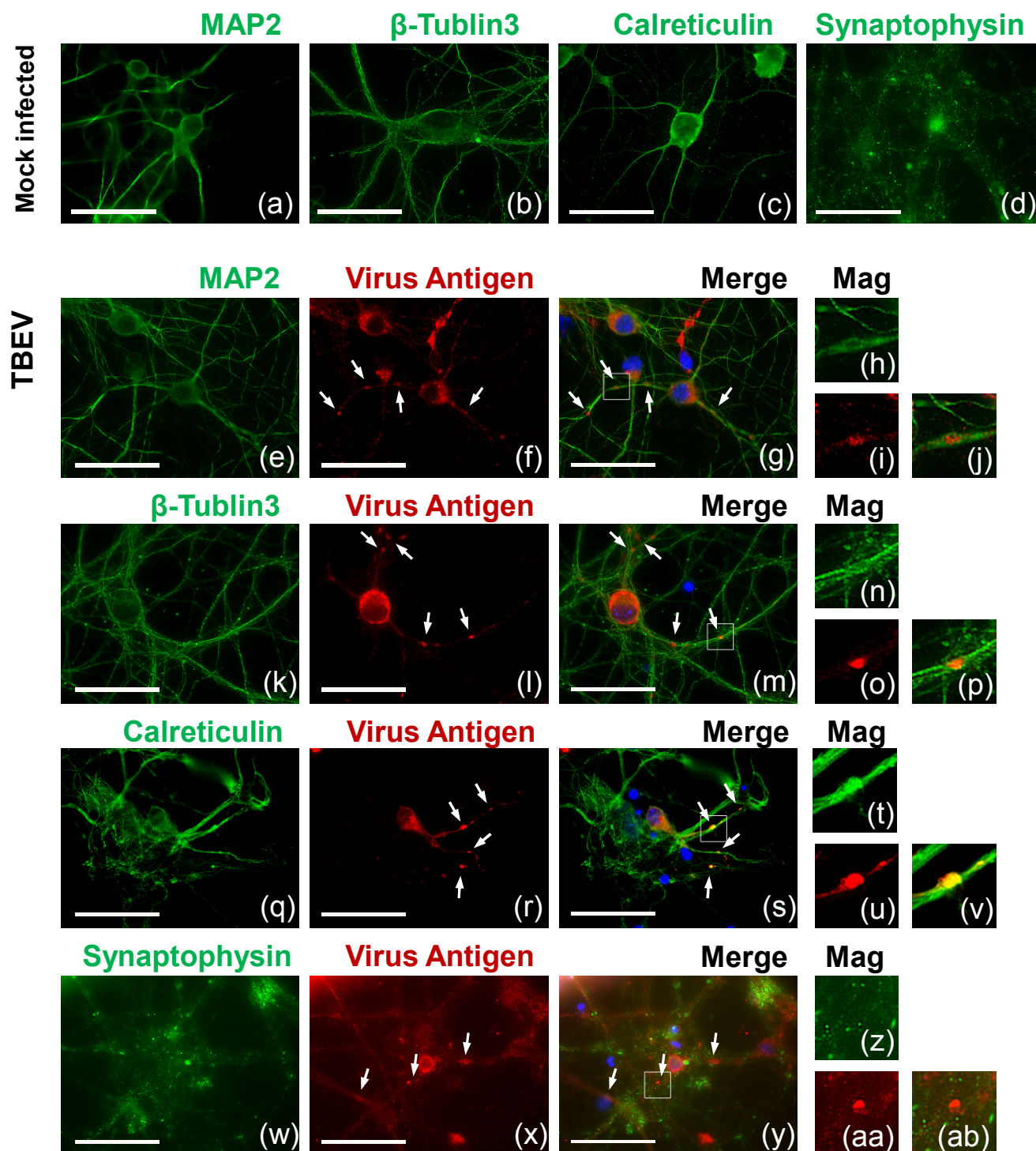


Fig. 5

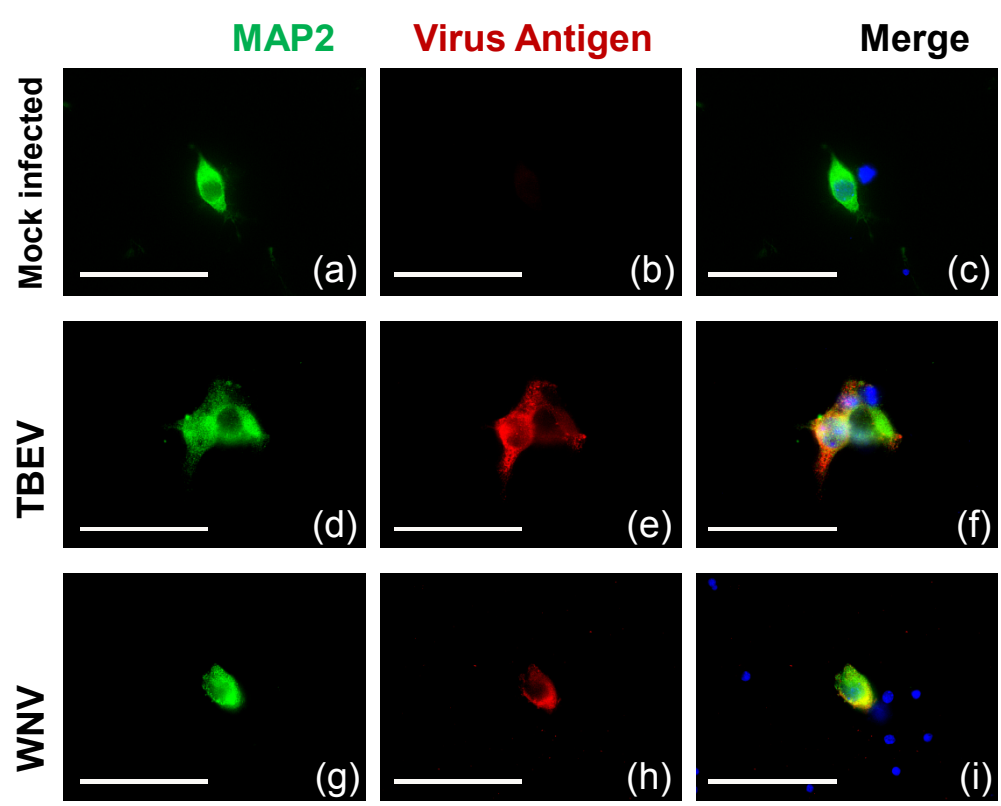


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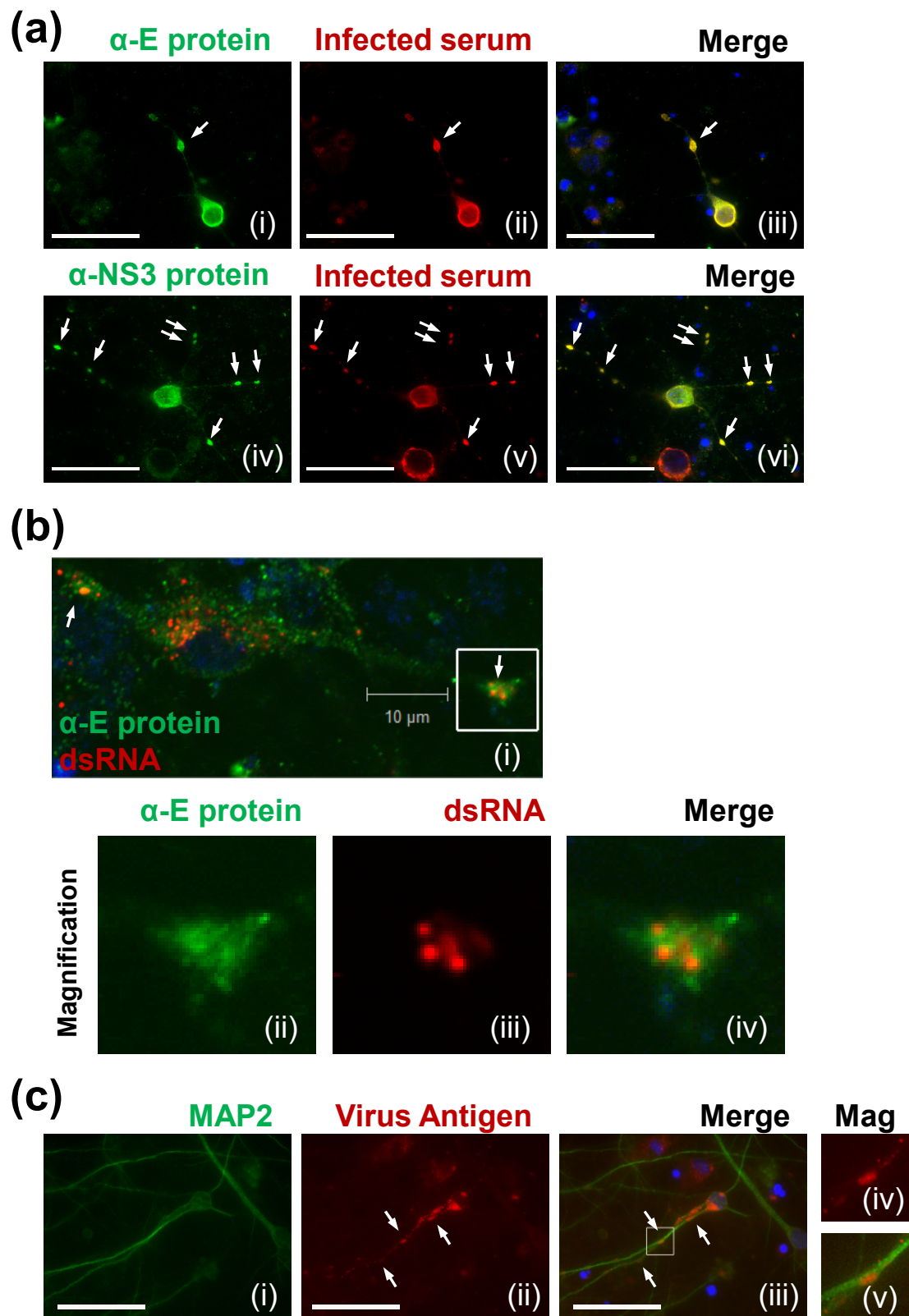


Fig. 7 (1/2)

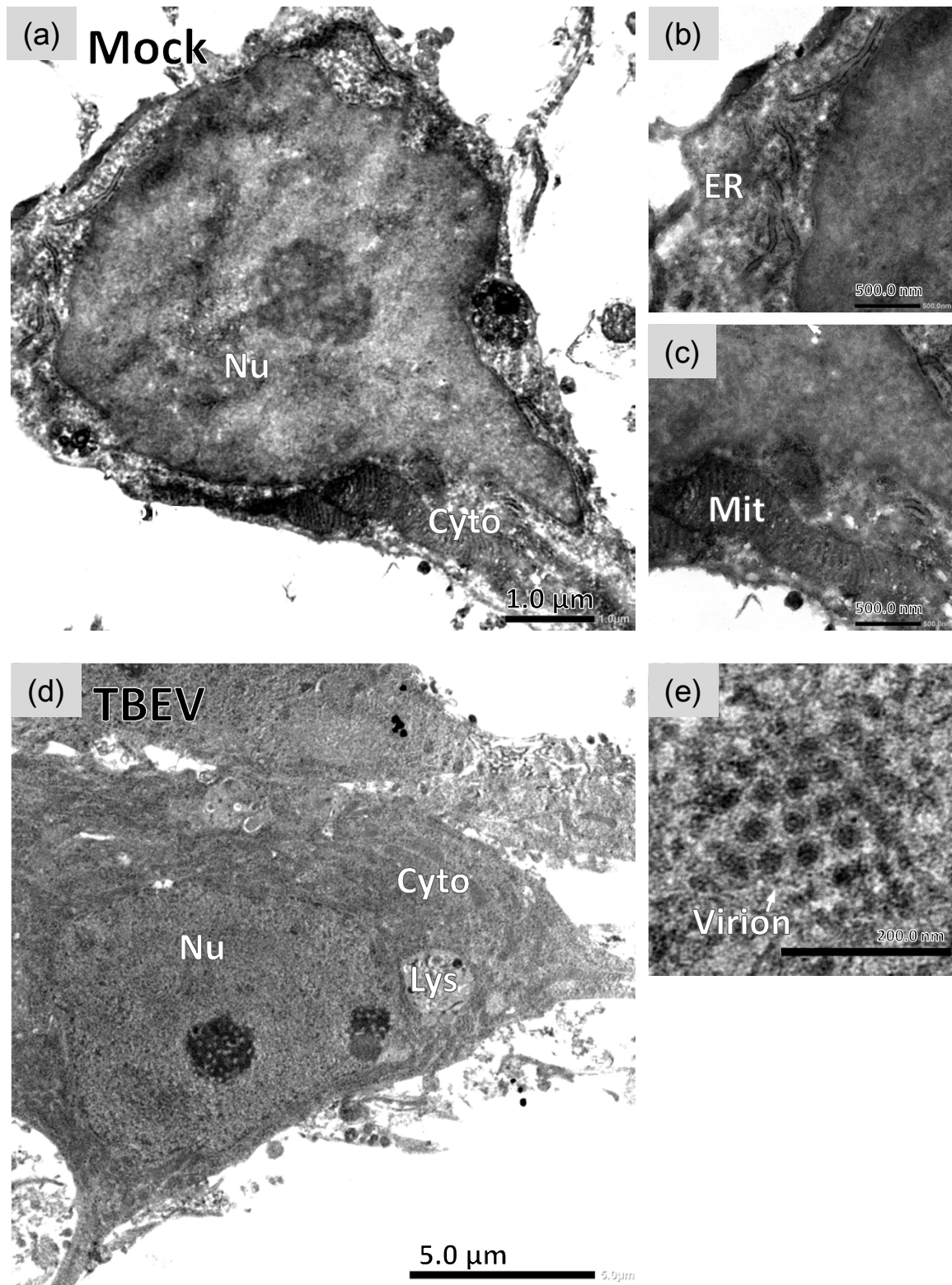


Fig. 7 (2/2)

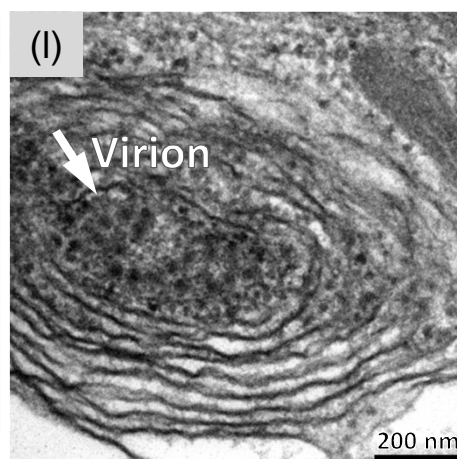
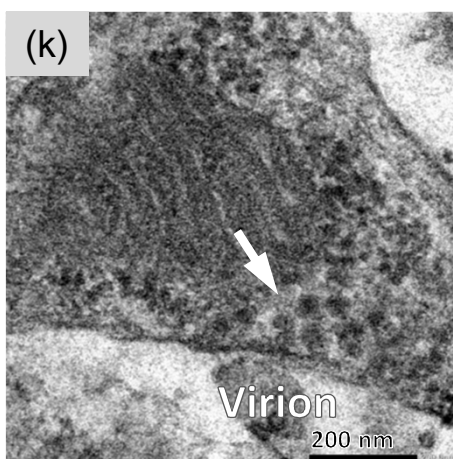
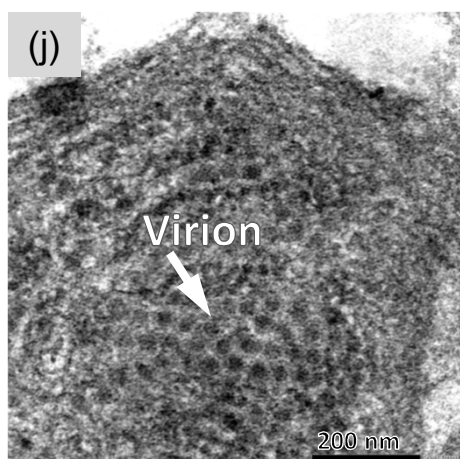
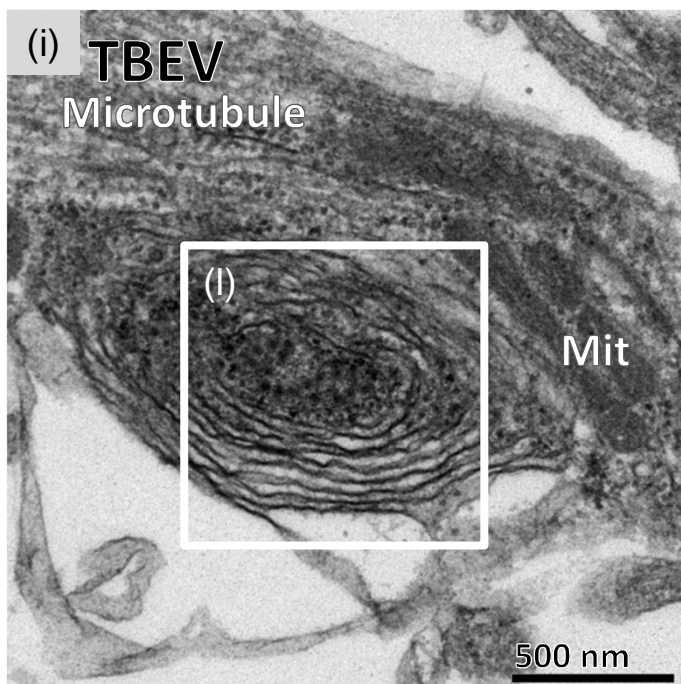
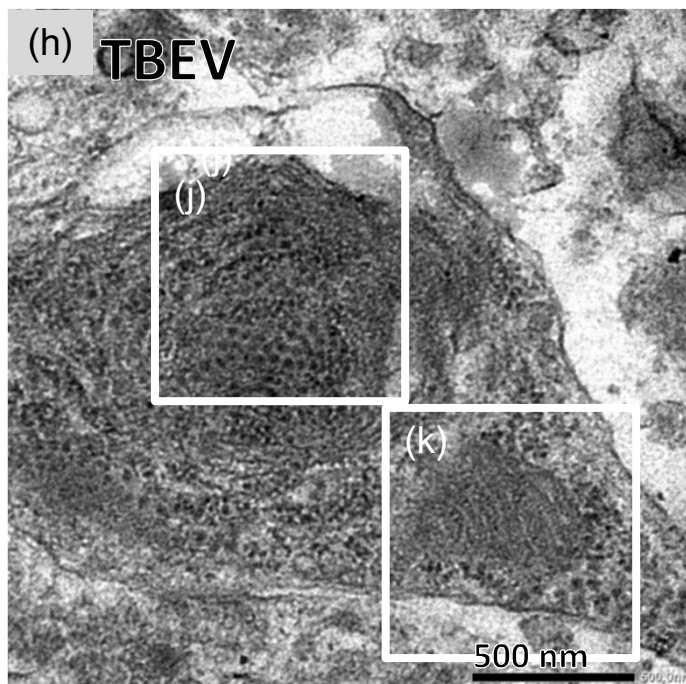
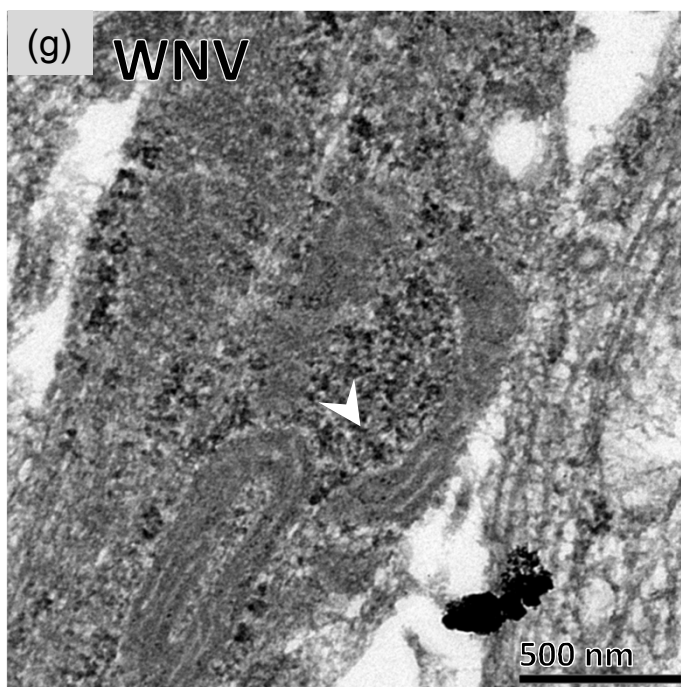
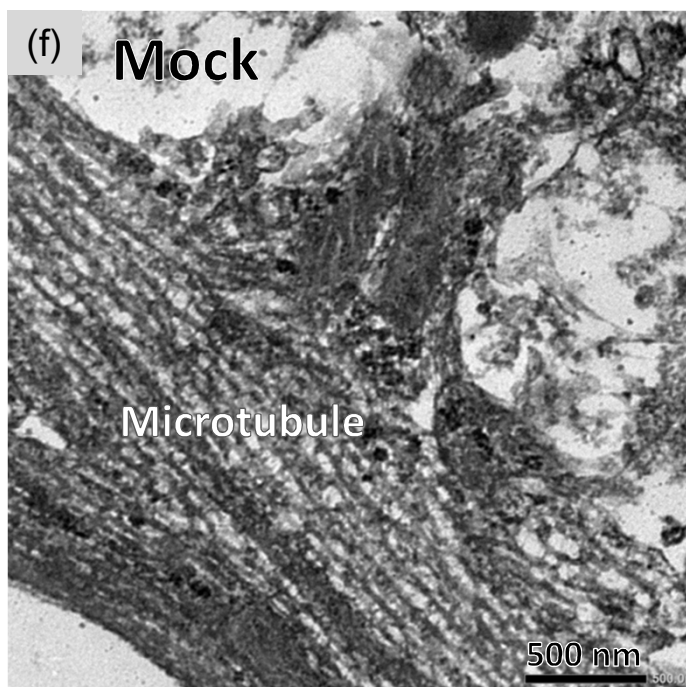


Fig. 8

