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A Critical Determinant of Neurological Disease Associated with Highly Pathogenic Tick-borne Flavivirus in Mice

running title: viral factors for neurological disease of TBEV

Kentaro Yoshii^{1)‡}, Yuji Sunden²⁾, Kana Yokozawa¹⁾, Manabu Igarashi³⁾, Hiroaki Kariwa¹⁾, Michael R Holbrook⁴⁾, Ikuo Takashima¹⁾

¹⁾Laboratory of Public Health, and ²⁾Laboratory of Comparative Pathology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan

³⁾ Division of Bioinformatics, Research Center for Zoonosis Control, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan

⁴⁾ NIAID Integrated Research Facility, 8200 Research Plaza, Ft. Detrick, Frederick, MD 21702, United States

#Corresponding author: Kentaro Yoshii, PhD., DVM.

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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Abstract

Tick-borne encephalitis virus (TBEV) and Omsk hemorrhagic fever virus (OHFV) are highly pathogenic tick-borne flaviviruses; TBEV causes neurological disease in humans while OHFV causes a disease typically identified with hemorrhagic fever. Although TBEV and OHFV are closely related genetically, the viral determinants responsible for these distinct disease phenotypes have not been identified. In this study, chimeric viruses incorporating components of TBEV and OHFV were generated using infectious clone technology and their pathological characteristics were analyzed in a mouse model to identify virus-specific determinants of disease. We found that only four amino acids near the C-terminus of the NS5 protein were primarily responsible for the development of neurological disease. Mutation of these four amino acids had no effect on viral replication or histopathological features, including inflammatory responses, in mice. These findings suggest a critical role of NS5 in stimulating neuronal dysfunction and degeneration following TBEV infection and provide new insights into the molecular mechanisms underlying the pathogenesis of tick-borne flaviviruses.

Importance

Tick-borne encephalitis virus (TBEV) and Omsk hemorrhagic fever virus (OHFV) belong to the tick-borne encephalitis serocomplex, genus *Flavivirus*, family *Flaviviridae*. Although TBEV causes neurological disease in humans while OHFV causes a disease typically identified with hemorrhagic fever. In this study, we investigated the viral determinants responsible for the different disease phenotypes using reverse genetics technology. We identified a cluster of only four amino acids in non-structural protein 5 primarily involved in the development of neurological disease in a mouse model. Moreover, the effect of these four amino acids was independent of viral replication property, and did not affect the formation of virus-induced lesions in the brain directly. These data suggest that these amino acids may be involved in the induction of neuronal dysfunction and degeneration in virus-infected neurons, ultimately leading to the neurological disease phenotype. These findings provide new insight into the molecular mechanisms of tick-borne flavivirus pathogenesis.

Introduction

Tick-borne encephalitis virus (TBEV) and Omsk hemorrhagic fever virus (OHFV) belong to the tick-borne encephalitis (TBE) serocomplex, genus *Flavivirus*, family *Flaviviridae*, which includes TBEV, Powassan virus, Langat virus, Louping ill virus, OHFV, Alkhurma virus (ALKV), and Kyasanur Forest disease virus (KFDV) (3). While the majority of TBE complex viruses cause encephalitis, OHFV, ALKV, and KFDV are known to cause hemorrhagic disease.

TBE is endemic to Europe, Russia, and Far-East Asia where about 10,000 cases are reported annually. TBEV can be divided into three subtypes: the Far-Eastern subtype, known as Russian spring summer encephalitis virus; the European subtype, known as Central European encephalitis subtype; and the Siberian subtype (12). In human patients, TBEV produces febrile illness, characterized by flu-like symptoms, followed by neurological symptoms including febrile headache, visual changes, paralysis, seizures, and coma (24). Among TBEV subtypes, the Far-Eastern subtype is particularly virulent, with mortality rates ranging from 20% to 60%. TBE therefore represents a significant threat to public health in endemic regions.

OHFV is endemic to a localized region near the Omsk Oblast of southwestern Siberia (37). Human OHFV infection results in clinical symptoms quite different from those caused by TBEV, characterized by high continuous fever, headache, muscle pain, dehydration, and often a distinct hemorrhagic syndrome including visceral hemorrhages of the nose, gums, uterus, and lungs. Unlike ALKV and KFDV, OHFV infections are rarely associated with neurological symptoms or sequelae (3).

Mice have been used as a reliable model with which to study disease progression following TBEV or OHFV infection. In our previous studies, we showed that virus-infected mice develop clinical signs and pathology similar to those seen in humans (15, 41). TBEV-infected mice

experience severe encephalitis resulting in paralysis, ranging from hind limb paresis to complete paralysis while OHFV-infected mice exhibit viscerotropic disease with limited signs of neurological disease despite multiplication of the virus in the brain.

The flavivirus genome consists of a positive-polarity, single-stranded RNA of approximately 11 kb, which encodes three structural proteins; i.e., the core (C), premembrane (prM), and envelope (E) proteins, and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), within a single open reading frame (4). The 5'- and 3'-UTRs predict secondary structures that are implicated in viral replication, translation, and packaging of the genome (13, 31). Although TBEV and OHFV exhibit >90% amino acid sequence identity (Table 1), the viral determinants responsible for disease-specific phenotypes have not been identified.

Recently, we constructed infectious cDNA clones of both TBEV and OHFV (14, 39, 50). In this study, we used these cDNA clones to generate TBEV-OHFV chimeric viruses with the specific objective of identifying viral determinants critical for the development of neurological disease in mice. Using this technology we were able to identify a four amino acid region near the C-terminus of the viral RNA-dependent RNA polymerase that is critical for neuropathogenesis. This discovery will allow us to further characterize specific virus-host cell interactions responsible for the development of severe disease.

Materials and Methods

Cells

BHK-21 cells were grown at 37°C in Eagle's minimum essential medium (E-MEM) supplemented with 8% fetal calf serum (FCS) and L-glutamine. Mouse neuroblastoma NA cells (kindly provided by Dr. Sugiyama and Dr. Ito of Gifu University) were maintained in E-MEM supplemented with

78 10% FCS. PC12 cells were maintained at 37°C in RPMI 1640 supplemented with 10% horse serum
79 and 5% FCS.

80

81 ***Viruses***

82 Recombinant TBEV (Oshima 5-10 strain) and OHFV (Guriev strain) were recovered from
83 infectious cDNA clones of the respective viruses (TBEV-pt and OHF-pt, respectively) as reported
84 previously (14, 50). To prepare the infectious cDNA clones of chimeric viruses (Fig 1), cDNA
85 fragments were synthesized by standard fusion-PCR and subcloned into TBEV-pt and OHF-pt in a
86 stepwise manner.

87 Infectious RNA was transcribed from the infectious cDNA clones using mMESSAGE
88 mMACHINE SP6 or T7 kits (Ambion, Austin, TX.) and transfected into BHK-21 cells using
89 TransIT-mRNA (Mirus Bio LLC, Madison, WI.), as described previously (49). At 3 dpi, viral
90 particles were collected from culture supernatants. Stocks of all viruses were propagated in BHK-21
91 cells. Successful recombination of recovered viruses was confirmed by sequencing of RT-PCR
92 fragments.

93 All experiments using recombinant viruses were performed according to the Law Concerning
94 the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of
95 Living Modified Organisms of Japan, a Japanese law ensuring compliance with the Cartagena
96 Protocol on Biosafety. All experiments using live viruses were performed in a BSL-3 facility.

97

98 ***Virus titration***

99 For viral titrations, monolayers of BHK-21 cells prepared in multi-well plates were incubated with
100 serial dilutions of virus for 1 h, overlaid with E-MEM containing 2% FCS and 1.5% carboxymethyl
101 cellulose (Sigma-Aldrich, St. Louis, MO.), and incubated for 5 days. Following incubation, the cells

102 were fixed and stained with 0.25% crystal violet in 10% buffered formalin. Plaques were counted
103 and expressed as plaque forming units (PFU)/mL.

104

105 ***Growth curves of the recombinant viruses***

106 BHK-21 cells were infected with each recombinant virus at a multiplicity of infection (MOI) of
107 0.01. After virus adsorption for 1 h, the inocula were removed. Cells were then washed with PBS
108 and incubated in E-MEM containing 2% FCS. Media were harvested at 24, 48, and 72 h
109 post-infection and stored at -80 °C until titration.

110

111 ***Pathogenicity of the recombinant viruses in mice***

112 Five- to six-week old BALB/c mice (Japan SLC Inc., Shizuoka, Japan) were challenged with
113 10,000 PFU of each virus subcutaneously. The physical conditions of the mice were observed and
114 the body weights were measured daily. Simple neurological assessments, including the following:
115 landing tests, balance tests and grasping tests, were performed as described previously (15).
116 Mice were scored based on the severity of neurological signs. Signs of paralysis and loss of balance
117 were typically associated with viral infection and were scored as 0 (absent), 1 (present), or 2
118 (severe). Mice were also weighed at each scoring session, with mice exhibiting >10% loss of body
119 weight defined as sick.

120 **Paralysis:** 0 = normal; 1 = dragging limbs or inversion of dorsum pedis; 2 = complete paralysis
121 and no spontaneous movement.

122 **Loss of balance:** 0 = normal; 1 = leaning of head or trunk posture to one side; 2 = inability to
123 retain posture and fall to one side, or circling movement to one side.

124 Total scores were quantified and expressed as means $\pm$ SEM. Neurological disease was defined as a
125 total score > 1.0.

126 For analysis of viral distribution in tissues, three to four mice were sacrificed on 1, 3, 5, 7, 9 and
127 11 dpi, and sera, brains, and spleens were collected following perfusion with cold PBS. Organs
128 were individually weighed, homogenized, and prepared as 10% suspensions (w/v) in PBS
129 supplemented with 10% FCS. Suspensions were then clarified by centrifugation (4,000 rpm for 5
130 min, at 4°C), and the supernatants were titrated.

131 The animal experiments were performed in accordance with the recommendations in the
132 Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in
133 Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports,
134 Science and Technology. The experimental protocols were approved by the Animal Care and Use
135 Committee of the Hokkaido University (approval number: 09-0071/11-0065).

136

137 ***Histopathology and immunohistochemistry***

138 Brains from infected mice were collected and fixed in 10% neutral phosphate-buffered formalin at 7
139 dpi or at terminal stages of infection for each virus. Paraffin embedded brains were cut into
140 4-μm-thick sections, stained with hematoxylin and eosin (HE), and examined by light microscopy.
141 For detection of viral antigens, the sections were incubated with rabbit polyclonal antibody against
142 flavivirus E protein (51), and stained using the streptavidin-biotin-immunoperoxidase complex
143 method (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). Sections were counterstained with Mayer's
144 hematoxylin. For detection of activated caspase-3, rabbit polyclonal anti-cleaved caspase 3 (1:500;
145 Cell Signaling Technology, Beverly, MA) was used.

146

147 ***Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay***

148 For the detection of DNA breaks in neuronal cells, sections were incubated with 0.02 mg/mL
149 proteinase K (Sigma) and treated with a methanol solution containing 3% H₂O₂ to block

150 endogenous peroxidase reactivity. Sections were then incubated with 0.5 μ M terminal
151 deoxynucleotidyl transferase (TdT) (Invitrogen, Carlsbad, CA) and 10 μ M biotin-16-dUTP (Roche,
152 Penzberg, Germany) in TdT buffer for 90 min at 37°C, followed by
153 peroxidase-conjugated-streptavidin. Sections were then counterstained with methyl green.

154

155 ***Quantitative RT-PCR of inflammatory cytokines***

156 Total RNA was extracted from the brains of infected mice using Isogen (Nippon Gene, Tokyo,
157 Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from
158 0.4- μ g of total RNA using M-MLV reverse transcriptase (Life Technologies, Carlsbad, CA,) and
159 oligo-dT primers. To quantify cytokine mRNA levels, real-time PCR was performed using a KAPA
160 SYBR FAST qPCR kit (Kapa Biosystems, Woburn, MA). Glyceraldehyde-3-phosphate
161 dehydrogenase (GAPDH) was used as an endogenous control. The primer sets used in this study are
162 listed in table 2.

163 Relative quantification of gene expression was normalized against GAPDH. Expression levels
164 are represented as the level of gene expression relative to uninfected controls.

165

166 ***Measurement of neurite length of PC12 cells***

167 PC12 cells were seeded onto collagen-coated eight-well chamber slides (Matsunami Glass Ind., Ltd.,
168 Kishiwada, Japan). Cells were infected with virus at a MOI of 10, and at 24 h post-infection, were
169 treated with 150 ng/mL rat 2.5S nerve growth factor (NGF) (BD, Franklin Lakes, NJ) in RPMI
170 1640 supplemented with 1% horse serum. After 72 h of treatment, cells were fixed in 4% (w/v)
171 paraformaldehyde for 20 min at 37°C, and observed by BZ-9000 (Keyence, Osaka, Japan). Cells
172 bearing neurites equivalent in length to the cell body diameter were scored using BZ-2 Analyser
173 software (Keyence). A total of 80 cells acquired from three independent experiments were

174 quantified.

175

176 ***Statistical analysis***

177 Data are expressed as means $\pm$ standard errors. The Tukey–Kramer test was used to determine
178 statistical significance of differences in the mean values of neurological scores (Table 3), viral titers
179 at each time point (Figs. 2, 3, 4, and 7), inflammatory cytokines (Fig. 6), and neurite outgrowth (Fig.
180 7). The Kaplan–Meier survival curves and the log-rank test were used to evaluate the survival of
181 infected mice (Table 3).

182

183 **Results**

184

185 **Effects of viral structural protein replacement on pathogenicity**

186 The viral structural proteins, prM and E, have been shown to play important roles in the tissue
187 tropism and neuropathogenesis of flaviviruses (9, 26, 35). To assess whether these proteins are
188 responsible for the differences in pathogenicity between TBEV and OHFV, the chimeric infectious
189 clones TBEV/OHF-ME and OHFV/TBE-ME were generated by replacing the prM and E genes of
190 each virus with those from the other species (Fig. 1A). Viable chimeric viruses were recovered from
191 cells transfected with synthetic mRNA derived from the plasmid template and sequencing of each
192 progeny virus confirmed their chimeric composition. Basic replication characteristics were
193 investigated in BHK-21 cells. Although TBEV grew more rapidly than OHFV ($P < 0.05$), similar
194 growth curves were obtained between parental viruses and the virus with replacement of the prM
195 and E genes (no significant differences), indicating that prM and E did not affect viral replication
196 properties in cultured cells.

197 The pathogenicities of the chimeric viruses were evaluated in BALB/c mice using non-chimeric

198 wild-type viruses as controls (i.e., TBEV-pt and OHFV-pt). Mortality rates greater than 70% were
199 seen in each group, with no significant differences in onset of disease and survival time between
200 groups (Table 3); however, animals could be readily divided into two groups based on clear
201 differences in disease phenotype. Mice infected with chimeric viruses containing the TBEV NS
202 protein genes (i.e., TBEV-pt or TBEV/OHF-ME) began to show general signs, such as hunched
203 posture, ruffled fur, and general malaise at 7-9 days post-infection (dpi). The majority of mice that
204 succumbed to infection with either TBEV-pt or TBEV/OHF-ME (81.0% and 93.3%, respectively,
205 Table 3) showed typical indications of neurological illness such as loss of balance, paresis,
206 hind-limb paralysis, or tremor in the final stage of disease, similar to the observations with other
207 neurotropic flaviviruses. In contrast, OHFV-pt- or OHFV/TBE-ME-infected mice exhibited general
208 signs of illness (hunched posture, ruffled fur, and general malaise), but the majority of animals
209 exhibited mild or no signs of neurological illness (OHFV-pt, 6.4%; OHFV/TBE-ME, 14.2%). In
210 semi-quantitative neurological assessments, the animals showed little or no indication of a physical
211 inability to perform the assessment tests. In the final stages of infection, OHFV-pt- and
212 OHFV/TBE-ME-infected mice exhibited obvious signs of weakness and were unable to complete
213 the assessments, but they attempted to perform the required tasks. The neurological scores for
214 severity in mice infected with either TBEV-pt or TBEV/OHF-ME were significantly higher than
215 those in mice infected with OHFV-pt- or OHFV/TBE-ME ($P < 0.01$). Taken together, these results
216 indicated that the structural proteins prM and E are not responsible for the differences in disease
217 phenotype elicited by TBEV and OHFV.

218

219 Major organs (i.e., spleen, liver, lung, and brain) and serum were harvested at various time
220 points to determine viral titers in mice infected with parental and chimeric viruses. Virus was
221 initially detected between 1 and 3 dpi, with virus titers peaking between 3 and 5 dpi in serum and

222 spleen (Fig. 3). No significant differences in viral titer were observed in the serum or peripheral
223 organs of mice infected with parental or chimeric viruses.

224 Similar viral titers were observed in the brains of mice infected with TBEV-pt and
225 TBEV/OHF-ME, with virus first detected at 5 dpi, and peaking at 7 dpi. In contrast, virus was not
226 detected in the brains of OHFV-pt-infected mice until 9 dpi, with viral titers reaching levels similar
227 to TBEV-pt or TBEV/OHF-ME-infected mice at 11 dpi.

228 Despite differences in disease phenotype, viral replication in OHFV/TBE-ME-infected mice was
229 similar to that seen in mice infected with TBEV-pt or TBEV/OHF-ME. These data indicate that the
230 replacement of viral prM and E proteins from OHFV with those of TBEV increased the
231 neuroinvasiveness of the virus, but did not directly affect disease phenotype.

232

233 **The NS3 and NS5 genes determine neurological disease caused by TBEV in mice**

234 To identify specific genetic determinants of disease phenotypes in TBEV and OHFV, we
235 constructed chimeric OHFV viruses with the C protein or each of the NS protein genes replaced
236 with the equivalent gene from TBEV (Fig. 1A). Viable chimeric viruses were recovered and basic
237 replication characteristics were investigated in BHK-21 cells. While growth of OHFV-TBE-NS3
238 was elevated slightly, there were no significant differences between parental OHFV and each
239 chimeric virus (Fig. 2B).

240 The pathogenicity of each chimeric virus was examined in a mouse model (Table 3).
241 Significant lethality was seen for each of the eight chimeric viruses, with most mice exhibiting
242 general signs of disease (i.e., ruffled fur, decreased activity and weight loss). There were no
243 significant differences in disease onset or survival times between groups. Less than 20% of mice
244 infected with OHFV/TBE-C, OHFV/TBE-NS1, OHFV/TBE-NS2A, OHFV/TBE-NS2B, or
245 OHFV/TBE-NS4AB showed evidence of neurological disease. However, approximately half

246 (45.5%) of the mice infected with OHFV/TBE-NS3 exhibited clear neurological signs including
247 loss of balance, paresis, and hind-limb paralysis. Even higher rates of neurological disease were
248 seen in mice infected with OHFV/TBE-NS5, with 88.9% of mice exhibiting significant neurological
249 signs. These data suggested that the NS5 protein of TBEV was critical for development of severe
250 neurological disease in mice, with the NS3 protein of TBEV also affecting the disease phenotype to
251 some extent.

252 To further delineate specific regions within the NS5 protein involved in development of severe
253 neurological disease, we constructed OHFV chimeras in which the NS5 protein was partially
254 substituted with that of TBEV (Fig. 1B). Flavivirus NS5 consists of two principle domains, the
255 methyltransferase (MTase) domain located on the N terminal side of the protein and the RNA
256 dependent RNA polymerase (RdRp) domain on the C terminal side (20, 32). We therefore
257 constructed chimeric OHFV viruses replacing either the MTase or RdRp domains of NS5 with that
258 of TBEV. Each chimeric virus showed replication properties similar to those of the parental OHFV
259 in BHK cells (Fig. 2C). As shown in Fig. 1B and Table 3, replacement of the RdRp domain
260 substantially increased the frequency (75%) at which mice developed signs of neurological disease,
261 while replacement of the MTase domain had only a minimal impact compared with the parental
262 OHFV (16.7% vs. 6.4%, respectively) (Fig. 1 and Table 3).

263 The RdRp domain was further divided into three regions, and each region of OHFV was
264 substituted with that of TBEV (Fig. 1B). As shown in Table 3, replacement of the N-terminus of the
265 RdRp domain (nt 8458 - 9488) had no impact on the development of neurological signs in infected
266 mice (11.1%). Replacement of the middle region of the RdRp domain (nt 9488 - 10295) markedly
267 reduced the virulence, with the majority of infected mice exhibiting no signs of disease and
268 surviving; those animals that did eventually succumb to infection exhibited no neurological signs
269 prior to death. This low morbidity may have been related to the lower replication efficacy compared

270 to parental OHFV observed in BHK-21 cells (Fig. 2C). Replacement of the C-terminus of the RdRp
271 domain (nt 10295 - 10377) resulted in a frequency of severe neurological signs similar to the
272 parental TBEV (80.8% vs. 81%, respectively). This result was surprising given that there are only
273 four amino acid differences between TBEV and OHFV in this region: at amino acids 879 - 881
274 (Lys/Phe/Lys in TBEV vs. Arg/Tyr/Ser in OHFV), and amino acid 891 (Asp in TBEV vs. Glu in
275 OHFV).

276 To determine the effects of the four amino acid differences on the pathogenicity of TBEV and
277 OHFV we made a series of chimeric viruses substituting the 879-881 triplet and residue 891 either
278 individually or in tandem (Fig 1C). Insertion of the TBEV amino acid triplet 879-881 or residue
279 891 into the NS5 protein into OHFV led to only modest increases in the rate of neurological signs
280 when compared with the OHFV parental strain (20% and 22.2%, respectively vs. 6.3%; Fig 1C and
281 Table 3). However, substitution of all four amino acids (879 - 881 and 891) resulted in a
282 significant increase in the frequency of neurological signs, similar to that seen with TBEV (Fig. 1C
283 and Table 3).

284 To further demonstrate the critical roles of these four residues in the development of severe
285 neurological disease, we constructed a recombinant TBEV in which the four amino acids in the
286 C-terminus of NS5 were substituted with those of OHFV. Fewer mice infected with the chimeric
287 TBEV showed neurological signs compared to those infected with the parental TBEV. However as
288 shown in Table 3, the four amino acid substitutions did not impact the morbidity, mortality, or
289 survival curves of TBEV and OHFV. These results indicated that the combined motif (designated
290 as the KFK-D motif) including amino acids Lys₈₇₉/Phe₈₈₀/Lys₈₈₁ and Asp₈₉₁ in the C-terminus of
291 NS5 is a critical determinant of neurological disease in mice infected with TBEV.

292

293 **Effects of the four amino acid substitutions in NS5 on viral characteristics**

294 To determine the relationship between neurological disease development and viral replication,
295 effects of the KFK-D motif on viral growth characteristics were investigated both *in vitro* and *in*
296 *vivo*. BHK or mouse neuroblastoma NA cells were infected with TBEV-pt, TBEV/NS5₈₇₉RYS₈₉₁E,
297 OHFV-pt, or OHFV/NS5₈₇₉KFK₈₉₁D at a multiplicity of infection of 0.01. Virus was harvested 24
298 to 72 h post-infection and quantified by a plaque assay. As shown in Figs. 2D and 4A, although
299 TBEV grew more rapidly than OHFV ($P < 0.05$), similar growth curves were obtained in both BHK
300 and NA cells between parental viruses and the virus with four amino acid substitutions in NS5 (no
301 significant differences), indicating that these mutations did not affect viral replication properties in
302 cultured cells.

303 Viral loads in serum, spleen, and brain of infected mice were compared between mice
304 inoculated with TBEV-pt, TBEV/NS5₈₇₉RYS₈₉₁E, OHFV-pt, or OHFV/NS5₈₇₉KFK₈₉₁D (Fig 4B).
305 Transient viremia and multiplication in the spleen were observed in mice infected with each virus.
306 Detection of virus in the brain was delayed in OHFV-infected mice as compared to those infected
307 with TBEV ($P < 0.05$). However, the four amino acid substitutions in NS5 did not affect viral
308 multiplication in any of these organs (no significant differences). These results indicated that the
309 differences seen in the neuropathogenesis of OHFV and TBEV are not due to alterations in viral
310 replication.

311 To determine the relationship between central nervous system (CNS) pathology and
312 neurological disease, histopathological features of mice were examined following infection with
313 either TBEV-pt, TBEV/NS5₈₇₉RYS₈₉₁E, OHFV-pt, or OHFV/NS5₈₇₉KFK₈₉₁D. At 7 dpi,
314 non-suppurative encephalitis with mild perivascular cuffing and meningitis was observed in the
315 brains of some mice, but there were no significant differences between groups (data not shown).

316 Next, dying mice exhibiting obvious signs of severe illness, including the inability to stand or
317 move, total paralysis, and/or weight loss of $>30\%$, were sacrificed for histopathological

318 examination at the terminal phase of disease (8 - 14 dpi). Mild to severe non-suppurative
319 encephalitis including neuronal degeneration, activation of microglial cells and infiltration of
320 mononuclear cells in the perivascular area was observed throughout the cerebral cortex, cerebellum,
321 and brain stem (Fig. 5A to D). Pathologic lesions accompanied by vacuolation, nuclear pyknosis of
322 neuronal cells and ischemic changes (necrosis) of neurons were prominent in the brains of animals
323 infected with each virus. Using immunohistochemistry, viral antigens were diffusely detected in all
324 groups in the cytoplasm of neurons of the cerebral cortex, hippocampus, and brain stem, as well as
325 in the Purkinje cells and granule cells of the cerebellum (Fig. 5E to 5H). By utilizing TUNEL assays
326 and immunohistochemistry for active caspase-3, apoptotic cells were identified primarily as
327 cerebral neurons and granule cells in cerebellum (data not shown). Although virus-induced
328 encephalitis was confirmed in all groups, no clear differences in histopathological features or virus
329 distribution were observed between parental and chimeric viruses containing the four amino acid
330 KFK-D motif.

331 The inflammatory response following infection with parental or chimeric viruses was also
332 assessed by measuring expression levels of inflammatory cytokines in brains of infected mice at 7
333 to 11 dpi. Although levels of inflammatory cytokines TNF α , IL-1 β , and IL-6 increased after
334 infection with each virus, no significant differences which could be correlated with substitutions in
335 NS5 were observed (Fig 6). Taken together, these results suggest that differences in neurological
336 disease induced by KFK-D motif substitutions were the result of factors other than direct lesions of
337 the brain caused by viral cytopathic effects and inflammatory responses.

338

339 A previous study by Wigerius *et al.* suggested that NS5 was involved in the attenuation of
340 neurite outgrowth using PC12 cells derived from rat pheochromocytoma (48). PC12 cells resemble
341 neurons in many respects, and when grown in the presence of NGF, they differentiate into a

342 neuronal phenotype by developing neurites, becoming electrically excitable and increasing the
343 synthesis of various neurotransmitters (11). We investigated the effects of the KFK-D motif of
344 TBEV on neurite formation and development using PC12 cells.

345 PC12 cells were infected with TBEV-pt, TBEV/NS5₈₇₉RYS₈₉₁E, OHFV-pt, or OHFV/NS5
346₈₇₉KFK₈₉₁D and were then treated with NGF. As shown in Fig. 7A, although TBEV grew faster than
347 OHFV ($P < 0.05$), similar growth curves were obtained between parental viruses and those with
348 four amino acid substitutions in NS5 (no significant differences), as observed in BHK and NA cells.
349 During the experiments, infected cells remained viable up to at least 7 days post-infection. However,
350 significant differences were observed in neurite outgrowth in infected-cells. Neurite length in cells
351 infected with viruses with the KFK-D motif was significantly shorter than that in cells infected with
352 viruses without the KFK-D motif (Figs. 7B and C). These results indicated that the KFK-D motif of
353 TBEV is involved in the attenuation of neurite outgrowth.

354

355 Discussion

356

357 In this study, we utilized infectious clones of two viruses with different disease phenotypes to
358 determine the genetic determinants of neurological disease. We identified four amino acids in the
359 C-terminal region of TBEV non-structural protein NS5 that were critical determinants of
360 neurological disease, but did not affect viral replication or histopathological features.

361

362 Replacement of the prM and E proteins of OHFV with the equivalent proteins from TBEV
363 increased viral neuroinvasiveness. In several reports, amino acid changes in the E protein have been
364 shown to affect the neuroinvasiveness of tick-borne flaviviruses (9, 17, 21, 35), although the
365 detailed mechanism of viral entry into the CNS remains unclear. There are a total of 39 amino acid

366 differences between TBEV strain Oshima and OHFV strain Guriev, but none has been previously
367 reported to be involved in viral neuroinvasiveness. The identification of amino acids responsible for
368 facilitating the rapid neuroinvasiveness of TBEV could lead to new insights into viral pathogenesis
369 and help to clarify the mechanism by which the virus gains entry into the CNS. TBEV/OHF-ME,
370 carrying the prM and E genes of OHFV and the other genes for TBEV entered brain earlier than
371 OHFV. Thus, the regions other than the prM and E proteins are also responsible for the
372 neuroinvasiveness of TBEV. Several reports indicated amino acid changes in the NS proteins affect
373 the neuroinvasiveness of tick-borne flaviviruses (18, 36) and mosquito-borne flaviviruses (6, 25, 28,
374 46, 47). Multiplication of TBEV/OHF-ME in the brain was similar to that of parental TBEV.
375 Regions other than the prM and E proteins may compensate for the effects of replacement of the
376 prM and E proteins on entry and multiplication in the brain because parental TBEV is highly
377 neuroinvasive.

378 Although recombinant chimeric OHFV incorporating the TBEV prM and E proteins multiplied
379 in the brain similar to TBEV, this failed to translate into neurological disease. Therefore, factors
380 other than viral multiplication in the brain are involved in the induction of neurological disease, and
381 these factors are distinct from prM and E.

382

383 Four amino acids in the C-terminus of the NS5 were identified as critical determinants of
384 neurological disease following TBEV infection in mice. The NS5 protein of flaviviruses is a
385 multifunctional protein containing an N-terminal MTase domain and a C-terminal RdRp domain (20,
386 32), separated by an interdomain region with nuclear localization sequences (2, 7). NS5 has also
387 been shown to have interferon antagonist activity in several flavivirus studies (1, 22, 23, 27). The
388 four amino acids identified in this study are located in the C-terminal region of the RdRp domain.
389 Several studies have shown that amino acid changes in the RdRp domain affect flavivirus genome

390 replication and disease development (42, 50). However, the KFK-D motif described here did not
391 affect viral multiplication either *in vitro* or *in vivo*. In addition, no significant differences in
392 histopathological features, such as inflammatory response or viral antigen distribution, were
393 observed in animals infected with OHFV chimeras incorporating the KFK-D motif. Therefore, the
394 differential disease phenotype induced by making KFK-D motif substitutions in OHFV was not a
395 result of alterations in the viral replication properties of NS5, nor induction of viral lesions in the
396 brain directly. Instead, these effects appear to be the result of other factors such as induction of
397 neuronal dysfunction and/or degeneration in virus-infected neurons, resulting in the neurological
398 disease phenotype in mice.

399 Neuronal dysfunction and degeneration have been associated with a number of neurotropic viral
400 infections. It has been suggested that neuronal dysfunction, rather than neuronal death, is likely
401 responsible for the severe neurological symptoms caused by rabies virus, as neuropathological
402 findings are relatively mild. Rabies infection induces electrophysiological alterations including
403 effects on ion channels and neurotransmission which may be the cause of functional impairment (8).
404 Alterations in synaptic function have been also reported in borna disease virus and human
405 immunodeficiency virus (HIV) infections (43, 44), while axonal degeneration is instrumental in the
406 development of neuronal dysfunction during herpesvirus and HIV infections (5, 30, 33, 38). Despite
407 these clear associations between neurotropic viruses and neuronal dysfunction, the majority of
408 research examining neurological diseases caused by tick-borne flaviviruses has focused on
409 virus-induced cytopathic effects or immunopathogenic responses. In this study, we showed that the
410 KFK-D motif was involved in the arrest of neurite outgrowth in PC12 cells. Impaired neurite
411 outgrowth has been linked to various neurological disorders, and it may be involved in the
412 development of neurological disease by TBEV infection. Further studies focusing on the biological
413 activity of the KFK-D motif of NS5, and its role in neuronal dysfunction including neurite

414 differentiation may provide new insight into the molecular mechanisms of the pathogenicity of
415 neurological disease following tick-borne flavivirus infection.

416

417 As shown in Fig. 8, the KFK-D motif is found on the lateral surface of the thumb domain of
418 flavivirus RdRP using the crystal structure of the West Nile virus RdRP domain as a template (PDB
419 code: 2HFZ). Similar results were obtained using that of Japanese encephalitis virus as a template
420 (PDB code: 4K6M). It is possible that individual amino acids play important roles in the interaction
421 with unidentified host factors within neurons. The tick-borne flavivirus NS5 has been shown to
422 possess a C-terminal PDZ binding motif (PBM), Ser/Ile/Ile (45), thereby facilitating binding to a
423 variety of PDZ domain-containing proteins (29). PDZ domains are protein-interaction modules that
424 are often found in multi-domain scaffolding proteins. PDZ-containing scaffolds assemble specific
425 proteins into large molecular complexes involved in maintaining cell polarity and regulation of
426 synaptic plasticity and synaptic vesicle dynamics (16, 19, 34). In a previous study of TBEV, NS5
427 opposed neuronal differentiation by binding to the PDZ domain protein Scribble (48). It was
428 reported that a second anchorage site upstream of the C-terminal PBM supported the interaction
429 between the PDZ domain protein and the PBM (40). Therefore, it is possible that the substitutions
430 of the four amino acids in the C-terminus of NS5 might affect the interaction between NS5 and host
431 proteins, such as PDZ domain containing proteins, resulting in neuronal dysfunction and
432 degeneration, through alteration of synaptic plasticity and axonal degeneration.

433 Interestingly, the amino acids 879 - 881 Lys/Phe/Lys and 891 Asp are highly conserved among
434 TBEV (Fig. 9). Eighty percent of TBEV strains encode these residues, with residues 880 Phe and
435 891 Asp conserved across all strains. In contrast, all reported OHFVs encode for 879 - 881 Arg/Tyr/
436 Ser and 891 Glu. Residues 879 - 880 Arg/Tyr and 891 Glu are also conserved in the other
437 hemorrhagic tick-borne flaviviruses, KFDV and ALKV, despite their phylogenetic distance from

438 OHFV. This conservation of the KFK-D motif among TBEV strains supports the suggestion that
439 this motif is important in the development of neurological disease of TBE, and that the conserved
440 amino acids in hemorrhagic tick-borne flaviviruses play a role in the development of hemorrhagic
441 disease. The amino acid differences between these viruses may also be the result of adaptive
442 evolution within a particular tick species leading to the selection of different virus variants, as
443 tick-borne flaviviruses are maintained predominantly in ticks (10).

444 In conclusion, this study provides the first description of critical viral genetic factors important
445 for the different disease manifestations of tick-borne flaviviruses. Four amino acids near the
446 C-terminus of the viral NS5 were shown to be critical for the development of neurological disease
447 in TBEV infection in mice. Mutation of these amino acids did not directly affect viral replication or
448 histopathological features, including inflammatory responses, suggesting that neuronal dysfunction
449 and degeneration are involved in neurological disease manifestations. These insights may provide
450 important information for identifying the mechanisms of pathogenesis of tick-borne flaviviruses.

451

452

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461

References

- 463 1. **Best, S. M., K. L. Morris, J. G. Shannon, S. J. Robertson, D. N. Mitzel, G. S. Park, E. Boer, J. B.**
 464 **Wolfenbarger, and M. E. Bloom.** 2005. Inhibition of interferon-stimulated JAK-STAT signaling by
 465 a tick-borne flavivirus and identification of NS5 as an interferon antagonist. *Journal of virology*
 466 **79**:12828-12839.
- 467 2. **Brooks, A. J., M. Johansson, A. V. John, Y. Xu, D. A. Jans, and S. G. Vasudevan.** 2002. The
 468 interdomain region of dengue NS5 protein that binds to the viral helicase NS3 contains
 469 independently functional importin beta 1 and importin alpha/beta-recognized nuclear
 470 localization signals. *The Journal of biological chemistry* **277**:36399-36407.
- 471 3. **Burke, D. S., and T. P. Monath.** 2001. Flaviviruses, p. 1043-1125. *In* D. M. Knipe and P. M.
 472 Howley (ed.), *Field's virology*, 4 ed. Lippincott Williams & Wilkins, Philadelphia.
- 473 4. **Chambers, T. J., C. S. Hahn, R. Galler, and C. M. Rice.** 1990. Flavivirus genome organization,
 474 expression, and replication. *Annu Rev Microbiol* **44**:649-688.
- 475 5. **De Regge, N., H. J. Nauwynck, K. Geenen, C. Krummenacher, G. H. Cohen, R. J. Eisenberg, T. C.**
 476 **Mettenleiter, and H. W. Favoreel.** 2006. Alpha-herpesvirus glycoprotein D interaction with
 477 sensory neurons triggers formation of varicosities that serve as virus exit sites. *The Journal of*
 478 *cell biology* **174**:267-275.
- 479 6. **Engel, A. R., A. A. Rumyantsev, O. A. Maximova, J. M. Speicher, B. Heiss, B. R. Murphy, and A.**
 480 **G. Pletnev.** 2010. The neurovirulence and neuroinvasiveness of chimeric tick-borne
 481 encephalitis/dengue virus can be attenuated by introducing defined mutations into the envelope
 482 and NS5 protein genes and the 3' non-coding region of the genome. *Virology* **405**:243-252.
- 483 7. **Forwood, J. K., A. Brooks, L. J. Briggs, C. Y. Xiao, D. A. Jans, and S. G. Vasudevan.** 1999. The
 484 37-amino-acid interdomain of dengue virus NS5 protein contains a functional NLS and
 485 inhibitory CK2 site. *Biochemical and biophysical research communications* **257**:731-737.
- 486 8. **Fu, Z. F., and A. C. Jackson.** 2005. Neuronal dysfunction and death in rabies virus infection.
 487 *Journal of neurovirology* **11**:101-106.
- 488 9. **Goto, A., D. Hayasaka, K. Yoshii, T. Mizutani, H. Kariwa, and I. Takashima.** 2003. A BHK-21 cell
 489 culture-adapted tick-borne encephalitis virus mutant is attenuated for neuroinvasiveness.
 490 *Vaccine* **21**:4043-4051.
- 491 10. **Gould, E. A., X. de Lamballerie, P. M. Zanutto, and E. C. Holmes.** 2001. Evolution, epidemiology,
 492 and dispersal of flaviviruses revealed by molecular phylogenies. *Advances in virus research*
 493 **57**:71-103.
- 494 11. **Greene, L. A., and A. S. Tischler.** 1976. Establishment of a noradrenergic clonal line of rat

adrenal pheochromocytoma cells which respond to nerve growth factor. Proceedings of the National Academy of Sciences of the United States of America **73**:2424-2428.

12. **Gritsun, T. S., V. A. Lashkevich, and E. A. Gould.** 2003. Tick-borne encephalitis. Antiviral Res **57**:129-146.
13. **Gritsun, T. S., K. Venugopal, P. M. Zanotto, M. V. Mikhailov, A. A. Sall, E. C. Holmes, I. Polkinghorne, T. V. Frolova, V. V. Pogodina, V. A. Lashkevich, and E. A. Gould.** 1997. Complete sequence of two tick-borne flaviviruses isolated from Siberia and the UK: analysis and significance of the 5' and 3'-UTRs. Virus research **49**:27-39.
14. **Hayasaka, D., T. S. Gritsun, K. Yoshii, T. Ueki, A. Goto, T. Mizutani, H. Kariwa, T. Iwasaki, E. A. Gould, and I. Takashima.** 2004. Amino acid changes responsible for attenuation of virus neurovirulence in an infectious cDNA clone of the Oshima strain of tick-borne encephalitis virus. The Journal of general virology **85**:1007-1018.
15. **Holbrook, M. R., J. F. Aronson, G. A. Campbell, S. Jones, H. Feldmann, and A. D. Barrett.** 2005. An animal model for the tickborne flavivirus--Omsk hemorrhagic fever virus. The Journal of infectious diseases **191**:100-108.
16. **Hung, A. Y., and M. Sheng.** 2002. PDZ domains: structural modules for protein complex assembly. The Journal of biological chemistry **277**:5699-5702.
17. **Hurrelbrink, R. J., and P. C. McMinn.** 2003. Molecular determinants of virulence: the structural and functional basis for flavivirus attenuation. Advances in virus research **60**:1-42.
18. **Kentaro, Y., S. Yamazaki, K. Mottate, N. Nagata, T. Seto, T. Sanada, M. Sakai, H. Kariwa, and I. Takashima.** 2013. Genetic and biological characterization of tick-borne encephalitis virus isolated from wild rodents in southern Hokkaido, Japan in 2008. Vector Borne Zoonotic Dis **13**:406-414.
19. **Kim, E., and M. Sheng.** 2004. PDZ domain proteins of synapses. Nature reviews. Neuroscience **5**:771-781.
20. **Koonin, E. V.** 1993. Computer-assisted identification of a putative methyltransferase domain in NS5 protein of flaviviruses and lambda 2 protein of reovirus. The Journal of general virology **74** (Pt 4):733-740.
21. **Kozlovskaya, L. I., D. I. Osolodkin, A. S. Shevtsova, L. Romanova, Y. V. Rogova, T. I. Dzhivanian, V. N. Lyapustin, G. P. Pivanova, A. P. Gmyl, V. A. Palyulin, and G. G. Karganova.** 2010. GAG-binding variants of tick-borne encephalitis virus. Virology **398**:262-272.
22. **Laurent-Rolle, M., E. F. Boer, K. J. Lubick, J. B. Wolfenbarger, A. B. Carmody, B. Rockx, W. Liu, J. Ashour, W. L. Shupert, M. R. Holbrook, A. D. Barrett, P. W. Mason, M. E. Bloom, A. Garcia-Sastre, A. A. Khromykh, and S. M. Best.** 2010. The NS5 protein of the virulent West Nile virus NY99 strain is a potent antagonist of type I interferon-mediated JAK-STAT signaling. Journal of virology **84**:3503-3515.

23. **Lin, R. J., B. L. Chang, H. P. Yu, C. L. Liao, and Y. L. Lin.** 2006. Blocking of interferon-induced Jak-Stat signaling by Japanese encephalitis virus NS5 through a protein tyrosine phosphatase-mediated mechanism. *Journal of virology* **80**:5908-5918.
24. **Lindquist, L., and O. Vapalahti.** 2008. Tick-borne encephalitis. *Lancet* **371**:1861-1871.
25. **Liu, W. J., X. J. Wang, D. C. Clark, M. Lobigs, R. A. Hall, and A. A. Khromykh.** 2006. A single amino acid substitution in the West Nile virus nonstructural protein NS2A disables its ability to inhibit alpha/beta interferon induction and attenuates virus virulence in mice. *Journal of virology* **80**:2396-2404.
26. **Mandl, C. W., S. L. Allison, H. Holzmann, T. Meixner, and F. X. Heinz.** 2000. Attenuation of tick-borne encephalitis virus by structure-based site-specific mutagenesis of a putative flavivirus receptor binding site. *Journal of virology* **74**:9601-9609.
27. **Mazzon, M., M. Jones, A. Davidson, B. Chain, and M. Jacobs.** 2009. Dengue virus NS5 inhibits interferon-alpha signaling by blocking signal transducer and activator of transcription 2 phosphorylation. *The Journal of infectious diseases* **200**:1261-1270.
28. **Melian, E. B., E. Hinzman, T. Nagasaki, A. E. Firth, N. M. Wills, A. S. Nouwens, B. J. Blitvich, J. Leung, A. Funk, J. F. Atkins, R. Hall, and A. A. Khromykh.** 2010. NS1' of flaviviruses in the Japanese encephalitis virus serogroup is a product of ribosomal frameshifting and plays a role in viral neuroinvasiveness. *Journal of virology* **84**:1641-1647.
29. **Melik, W., K. Ellencrona, M. Wigerius, C. Hedstrom, A. Elvang, and M. Johansson.** 2012. Two PDZ binding motifs within NS5 have roles in Tick-borne encephalitis virus replication. *Virus research* **169**:54-62.
30. **Michaud, J., R. Fajardo, G. Charron, A. Sauvageau, F. Berrada, D. Ramla, H. Dilhuydy, Y. Robitaille, and A. Kessous-Elbaz.** 2001. Neuropathology of NFHgp160 transgenic mice expressing HIV-1 env protein in neurons. *Journal of neuropathology and experimental neurology* **60**:574-587.
31. **Proutski, V., E. A. Gould, and E. C. Holmes.** 1997. Secondary structure of the 3' untranslated region of flaviviruses: similarities and differences. *Nucleic Acids Res* **25**:1194-1202.
32. **Rice, C. M., E. M. Lenches, S. R. Eddy, S. J. Shin, R. L. Sheets, and J. H. Strauss.** 1985. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. *Science* **229**:726-733.
33. **Robinson, B., Z. Li, and A. Nath.** 2007. Nucleoside reverse transcriptase inhibitors and human immunodeficiency virus proteins cause axonal injury in human dorsal root ganglia cultures. *Journal of neurovirology* **13**:160-167.
34. **Roche, J. P., M. C. Packard, S. Moeckel-Cole, and V. Budnik.** 2002. Regulation of synaptic plasticity and synaptic vesicle dynamics by the PDZ protein Scribble. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **22**:6471-6479.

- 567 35. **Rumyantsev, A. A., B. R. Murphy, and A. G. Pletnev.** 2006. A tick-borne Langat virus mutant that
568 is temperature sensitive and host range restricted in neuroblastoma cells and lacks
569 neuroinvasiveness for immunodeficient mice. *Journal of virology* **80**:1427-1439.
- 570 36. **Ruzek, D., T. S. Gritsun, N. L. Forrester, E. A. Gould, J. Kopecky, M. Golovchenko, N. Rudenko,**
571 **and L. Grubhoffer.** 2008. Mutations in the NS2B and NS3 genes affect mouse neuroinvasiveness
572 of a Western European field strain of tick-borne encephalitis virus. *Virology* **374**:249-255.
- 573 37. **Ruzek, D., V. V. Yakimenko, L. S. Karan, and S. E. Tkachev.** 2010. Omsk haemorrhagic fever.
574 *Lancet* **376**:2104-2113.
- 575 38. **Soffer, D., and J. R. Martin.** 1989. Axonal degeneration and regeneration in sensory roots in a
576 genital herpes model. *Acta neuropathologica* **77**:605-611.
- 577 39. **Takano, A., K. Yoshii, Y. Omori-Urabe, K. Yokozawa, H. Kariwa, and I. Takashima.** 2011.
578 Construction of a replicon and an infectious cDNA clone of the Sofjin strain of the Far-Eastern
579 subtype of tick-borne encephalitis virus. *Arch Virol* **156**:1931-1941.
- 580 40. **Terrien, E., A. Chaffotte, M. Lafage, Z. Khan, C. Prehaud, F. Cordier, C. Simenel, M. Delepierre,**
581 **H. Buc, M. Lafon, and N. Wolff.** 2012. Interference with the PTEN-MAST2 Interaction by a Viral
582 Protein Leads to Cellular Relocalization of PTEN. *Science signaling* **5**:ra58.
- 583 41. **Tigabu, B., T. Juelich, J. Bertrand, and M. R. Holbrook.** 2009. Clinical evaluation of highly
584 pathogenic tick-borne flavivirus infection in the mouse model. *J Med Virol* **81**:1261-1269.
- 585 42. **Van Slyke, G. A., A. T. Ciota, G. G. Willsey, J. Jaeger, P. Y. Shi, and L. D. Kramer.** 2012. Point
586 mutations in the West Nile virus (Flaviviridae; Flavivirus) RNA-dependent RNA polymerase
587 alter viral fitness in a host-dependent manner in vitro and in vivo. *Virology*.
- 588 43. **Volmer, R., C. Monnet, and D. Gonzalez-Dunia.** 2006. Borna disease virus blocks potentiation of
589 presynaptic activity through inhibition of protein kinase C signaling. *PLoS pathogens* **2**:e19.
- 590 44. **Volmer, R., C. M. Prat, G. Le Masson, A. Garenne, and D. Gonzalez-Dunia.** 2007. Borna disease
591 virus infection impairs synaptic plasticity. *Journal of virology* **81**:8833-8837.
- 592 45. **Werme, K., M. Wigerius, and M. Johansson.** 2008. Tick-borne encephalitis virus NS5 associates
593 with membrane protein scribble and impairs interferon-stimulated JAK-STAT signalling.
594 *Cellular microbiology* **10**:696-712.
- 595 46. **Whiteman, M. C., J. A. Wicker, R. M. Kinney, C. Y. Huang, T. Solomon, and A. D. Barrett.** 2011.
596 Multiple amino acid changes at the first glycosylation motif in NS1 protein of West Nile virus are
597 necessary for complete attenuation for mouse neuroinvasiveness. *Vaccine* **29**:9702-9710.
- 598 47. **Wicker, J. A., M. C. Whiteman, D. W. Beasley, C. T. Davis, C. E. McGee, J. C. Lee, S. Higgs, R. M.**
599 **Kinney, C. Y. Huang, and A. D. Barrett.** 2012. Mutational analysis of the West Nile virus NS4B
600 protein. *Virology* **426**:22-33.
- 601 48. **Wigerius, M., W. Melik, A. Elvang, and M. Johansson.** 2010. Rac1 and Scribble are targets for the
602 arrest of neurite outgrowth by TBE virus NS5. *Molecular and cellular neurosciences* **44**:260-271.

- 603 49. **Yoshii, K., and M. R. Holbrook.** 2009. Sub-genomic replicon and virus-like particles of Omsk
604 hemorrhagic fever virus. *Arch Virol* **154**:573-580.
- 605 50. **Yoshii, K., M. Igarashi, K. Ito, H. Kariwa, M. R. Holbrook, and I. Takashima.** 2011. Construction
606 of an infectious cDNA clone for Omsk hemorrhagic fever virus, and characterization of mutations
607 in NS2A and NS5. *Virus research* **155**:61-68.
- 608 51. **Yoshii, K., A. Konno, A. Goto, J. Nio, M. Obara, T. Ueki, D. Hayasaka, T. Mizutani, H. Kariwa,**
609 **and I. Takashima.** 2004. Single point mutation in tick-borne encephalitis virus prM protein
610 induces a reduction of virus particle secretion. *The Journal of general virology* **85**:3049-3058.
611

Figure legends

Figure 1.

Neurological disease in mice infected with chimeric viruses.

The coding regions for OHFV and TBEV proteins are shown in white and black, respectively.

The percentages of mice exhibiting signs of neurological disease are shown on the right. (A)

Chimeric viruses were constructed by replacement of the coding region for each viral protein. (B)

The coding region of OHFV non-structural protein NS5 was partially replaced with that of TBEV; nucleotide positions and restriction enzyme sites used for this replacement are indicated.

(C) The amino acids at positions 879, 880, 881, and/or 891 of NS5 were substituted. Normal and bold letters indicate the amino acid sequences of OHFV and TBEV, respectively.

Figure 2. Growth curves of chimeric viruses.

A Monolayer of BHK-21 cells was infected with wild-type and chimeric viruses at a multiplicity of infection (MOI) of 0.01. Media were harvested at each time point, and viral titers determined by plaque assay in BHK-21 cells. The chimeric viruses showed no significant differences from parental virus at any time point examined.

(A) Chimeric viruses with replacement of viral envelope proteins (prM/E). * indicates significant difference between TBEV-pt and OHFV-pt, and between TBEV-pt and OHFV/TBE-ME ($P < 0.05$).

(B) Chimeric OHFV with replacement of the C or each NS protein.

(C) Chimeric OHFV with partial replacement in the NS5 protein.

(D) Chimeric viruses with substitutions in the KFK-D motif. * indicates a significant difference between TBEV-pt and all chimeric OHFV ($P < 0.05$).

Figure 3.

Multiplication in organs of chimeric viruses following replacement of the envelope proteins.

Mice were infected with 10,000 pfu of each virus (TBEV-pt, TBEV/OHF-ME, OHFV-pt, and OHFV/TBE-ME). Viral titers in serum, spleen, and brain were determined by plaque assays on the days indicated. Error bars represent the SD ($n = 4$). By 11 dpi, all mice inoculated with TBEV/OHF-ME had died. * indicates a significant difference between TBEV-pt and OHFV-pt ($P < 0.05$). No significant differences were observed between TBEV-pt, TBEV/OHF-ME and OHF/TBE/ME.

Figure 4.

Growth properties of chimeric viruses containing NS5 amino acid substitutions.

A. Monolayers of BHK-21 or NA cells were infected with wild-type and chimeric viruses at a multiplicity of infection (MOI) of 0.01. Media were harvested at each time point, and viral titers were determined by plaque assay in BHK-21 cells.

B. Mice were infected with 10,000 pfu of each virus. Viral titers in serum, spleen, and brain were determined by plaque assays on the days indicated. Error bars represent the SD ($n = 3$). * indicates significant difference between TBEV-pt and OHFV-pt, and between TBEV-pt and OHFV/NS5₈₇₉KFK₈₉₁D ($P < 0.05$). No significant differences were observed between TBEV-pt and TBEV/NS5₈₇₉RYS₈₉₁E, or between OHFV-pt and OHFV/NS5₈₇₉KFK₈₉₁D at any time-points.

Figure 5.

Histopathological features of the brains of mice infected with chimeric viruses containing NS5 amino acid substitutions.

Mice were inoculated with 10,000 pfu of TBEV-pt (A and E), TBEV/NS5₈₇₉RYS₈₉₁E (B and F), OHFV-pt (C and G), or OHFV/NS5₈₇₉KFK₈₉₁D (D and H), and sacrificed at terminal stages of disease (7 - 13 dpi). Brain histopathology consisted of marked non-suppurative encephalitis in all mice. Severe tissue damage with degeneration and necrosis of Purkinje cells, and pyknosis of granule cells in the cerebellum (A - D, original magnification $\times 400$). Viral antigens (arrows) were detected in the cytoplasm of various neuronal cells in the brains of virus-infected mice (E - H, bars = 50 μ m).

Figure 6.

Expression of inflammatory cytokines in the brains of mice infected with chimeric viruses.

Mice were inoculated with 10,000 pfu of TBEV-pt, TBEV/NS5₈₇₉RYS₈₉₁E, OHFV-pt, or OHFV/NS5₈₇₉KFK₈₉₁D, and the expression of inflammatory cytokines in the brain was measured by real-time PCR. The levels of TNF- α , IL-1 β , and IL-6 mRNA expression were measured at the time points indicated, and normalized against GAPDH. Expression levels are shown relative to uninfected controls. * indicates a significant difference ($P < 0.05$).

Figure 7.

Neurite formation by PC12 cells infected with chimeric viruses containing NS5 amino acid substitutions.

PC12 cells were infected with TBEV-pt, TBEV/NS5₈₇₉RYS₈₉₁E, OHFV-pt, or OHFV/NS5₈₇₉KFK₈₉₁D at a multiplicity of infection (MOI) of 10, and were treated with 150 ng/mL NGF at 24 hours post-infection.

A. Media were harvested at each time point, and viral titers were determined by plaque assay in BHK-21 cells. * indicates a significant difference between TBEV-pt and OHFV-pt, and between TBEV-pt and OHFV/NS5₈₇₉KFK₈₉₁D ($P < 0.05$). No significant differences were observed

between TBEV-pt and TBEV/NS5₈₇₉RYS₈₉₁E, or between OHFV-pt and OHFV/NS5₈₇₉KFK₈₉₁D at any time point.

B. Typical images of the cells 72 h after NGF-treatment are shown. Scale bar, 50 μ m.

C. Average of neurite length was quantified 72 h after NGF-treatment. * indicates significant difference ($P < 0.01$).

Figure 8.

Amino acids involved in the neurological disease caused by tick-borne flaviviruses.

A three-dimensional model of the tick-borne flavivirus RNA-dependent RNA polymerase domain in NS5 was constructed based on the crystal structure of West Nile virus polymerase (PDB code: 2HFZ). The structure of tick-borne flavivirus polymerase is shown in ribbon (upper) and surface (lower) representations. Amino acid positions 879, 880, 881, and 891 are colored blue.

Figure 9.

An Alignment of C-terminal amino acids (841-900 in TBEV) of NS5 for flaviviruses.

The KFK-D motifs of TBEV are shown in bold type and are shaded.

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<http://www.textcheck.com/certificate/zJ82DN>

Table 3. Pathogenicity of the chimeric viruses in mouse model.

	No. of mice	Morbidity	Onset of disease (days) ¹	Mortality ²	Survival time (days) ¹	Neurological disease ³	Neurological score ⁴
OHFV-pt	32	96.9% (31/32)	9.7 ± 2.6	96.9% (31/32)	11.9 ± 3.3	6.4% (2/31)	0.11 ± 0.07
TBEV-pt	31	100% (31/31)	9.0 ± 1.4	67.7% (21/31)	14.2 ± 3.6	81.0% (17/21)	1.71 ± 0.27^{**}
OHFV/TBE-ME	15	93.3% (14/15)	10.1 ± 2.2	93.3% (14/15)	12.2 ± 2.9	14.2% (2/14)	0.27 ± 0.18
TBEV/OHF-ME	15	100% (15/15)	8 ± 0.93	100% (15/15)	10.3 ± 1.5	93.3% (14/15)	2.27 ± 0.25^{**}
OHFV/TBE-C	12	100% (12/12)	7.3 ± 0.87	83.3% (10/12)	9.1 ± 2.0	10% (1/10)	0.17 ± 0.17
OHFV/TBE-NS1	12	75% (9/12)	10.2 ± 3.2	75% (9/12)	14.5 ± 5.7	11.1% (1/9)	0.08 ± 0.08
OHFV/TBE-NS2A	12	100% (12/12)	9.8 ± 2.6	100% (12/12)	13.1 ± 3.5	16.7% (2/12)	0.33 ± 0.22
OHFV/TBE-NS2B	12	91.7% (11/12)	10.3 ± 3	83.3% (10/12)	13.4 ± 3.7	18.2% (2/11)	0.42 ± 0.23
OHFV/TBE-NS3	12	91.7% (11/12)	10.5 ± 4.0	91.7% (11/12)	13.5 ± 4.1	45.5% (5/11)	1 ± 0.37
OHFV/TBE-NS4AB	12	91.7% (11/12)	10.7 ± 3.	91.7% (11/12)	13.7 ± 3.1	9.1% (1/11)	0.17 ± 0.17
OHFV/TBE-NS5	18	100% (18/18)	8.6 ± 2.1	100% (18/18)	11.6 ± 3.1	88.9% (16/18)	2.17 ± 0.31^{**}
OHFV/TBE-Mtase	12	100% (12/12)	10.6 ± 3.8	100% (12/12)	13.3 ± 4.1	16.7% (2/12)	0.25 ± 0.18
OHFV/TBE-RdRp	18	88.9% (16/18)	11.2 ± 3.7	88.9% (16/18)	14.1 ± 4.0	75% (12/16)	1.78 ± 0.38^{**}
OHFV/TBE-8458-9488	10	90% (9/10)	11.3 ± 4.2	90% (9/10)	13.7 ± 4.3	11.1% (1/9)	0.4 ± 0.27
OHFV/TBE-9488-10295	10	20% (2/10)	9.0 ± 1.4	20% (2/10) [*]	13.5 ± 2.1	0% (0/2)	0
OHFV/NS5 ₈₇₉ KFK ₈₉₁ D	27	96.2% (26/27)	9 ± 3.7	96.2% (26/27)	11.4 ± 4.3	80.8% (21/26)	1.65 ± 0.22^{**}
OHFV/NS5 ₈₇₉ KFK	10	100% (10/10)	8.1 ± 1.5	100% (10/10)	11.4 ± 2.5	20% (2/10)	0.5 ± 0.34
OHFV/NS5 ₈₉₁ D	10	100% (10/10)	9.1 ± 2.4	90% (10/10)	13 ± 3.2	22.2% (2/9)	0.4 ± 0.27
TBEV/NS5 ₈₇₉ RYS ₈₉₁ E	15	100% (15/15)	9.3 ± 1.4	66.7% (10/15)	14 ± 2.8	30% (3/10)	0.33 ± 0.19

¹ There were no statically significant differences in average of the onset of disease and the survival time in each groups.

² Survival of the mice was analyzed by Kaplan-Meier method. * indicated the significant difference from OHFV-pt (P < 0.001)

³ The percentage of mice showing signs of neurological symptoms in dead mice.

⁴ The neurological scores for the severity of neurological signs were quantified as described in Methods. ** indicates the significant difference from the score of OHFV-pt

Table 2. Primer sets used in the quantitative RT-PCR

Primer		Sequence
TNF α	Sense	5' -CAAATGGCCTCCCTCTCATC- 3'
	Anti-sense	5' -CTCCAGCTGCTCCTCCACTT- 3'
IL-1 β	Sense	5' -CCTTCCAGGATGAGGACATGA- 3'
	Anti-sense	5' -CAGCACGAGGCTTTTTTGTTG- 3'
IL-6	Sense	5' -GGGACTGATGCTGGTGACAA- 3'
	Anti-sense	5' -TCCACGATTTCCCAGAGAACA- 3'
GAPDH	Sense	5' -GCACCACCACTGCTTAGCC- 3'
	Anti-sense	5' -GGATGCAGGGATGATGTTCTG- 3'