



Title	The variable region of the 3' untranslated region is a critical virulence factor in the Far-Eastern subtype of tick-borne encephalitis virus in mouse model
Author(s)	Sakai, Mizuki; Yoshii, Kentaro; Sunden, Yuji; Yokozawa, Kana; Hirano, Minato; Kariwa, Hiroaki
Citation	Journal of General Virology, 95(4), 823-835 https://doi.org/10.1099/vir.0.060046-0
Issue Date	2014-04
Doc URL	http://hdl.handle.net/2115/58282
Type	article (author version)
File Information	J.Gen.Virol_submission.pdf



[Instructions for use](#)

1 **The variable region of the 3' untranslated region is a critical virulence**
2 **factor in the Far-Eastern subtype of tick-borne encephalitis virus in**
3 **mouse model**

4

5 Running title: Role of variable region as a virulence factor in TBEV

6 The Content Category: Animal Viruses-Positive-strand RNA

7

8 Mizuki Sakai¹, Kentaro Yoshii¹, Yuji Sunden², Kana Yokozawa¹, Minato Hirano¹,
9 Hiroaki Kariwa¹

10

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

14

15 Corresponding author: Kentaro YOSHII, PhD., D.V.M.,

16 Postal address: Laboratory of Public Health, Graduate School of Veterinary Medicine,

17 Hokkaido University, kita-18 nishi-9, kita-ku, Sapporo, Hokkaido 060-0818, Japan

18 Tel/fax: +81-11-7-6-5213

19 Email: kyoshii@vetmed.hokudai.ac.jp

20

21 Word count for summary: 236

22 Word count for text: 4787

23

24 **Summary**

25 Tick-borne encephalitis virus (TBEV) is a major arbovirus that causes thousands of cases of severe
26 neurological illness in humans annually. However, virulence factors and pathological mechanisms of
27 TBEV remain largely unknown. To identify the virulence factors, we constructed chimeric viruses
28 between two TBEV strains of the Far-Eastern subtype, Sofjin-HO (highly pathogenic) and Oshima
29 5-10 (low pathogenic). The replacement of the coding region for the structural and non-structural
30 proteins from Sofjin into Oshima showed a partial increase of the viral pathogenicity in a mouse
31 model. Oshima-based chimeric viruses with the variable region of the 3' untranslated region
32 (3'-UTR) of Sofjin, which had a deletion of 207 nucleotides, killed 100% of mice and showed almost
33 same virulence with Sofjin. Replacement of the variable region of 3'-UTR from Sofjin into Oshima
34 did not increase viral multiplication in cultured cell and a mouse model at the early phase of viral
35 entry into the brain. At the terminal phase of viral infection in mice, the virus titer of the
36 Oshima-based chimeric virus with the variable region of the 3'-UTR of Sofjin reached a level
37 identical to that of Sofjin, and showed a similar histopathological change in the brain tissue. This is
38 the first report to show that the variable region of the 3'-UTR is a critical virulence factor in mice.
39 These findings encourage further study to understand the mechanisms of the pathogenicity of TBEV
40 and develop preventative and therapeutic strategies for TBE.

41

42 **Introduction**

43 Tick-borne encephalitis (TBE) virus, which is a member of the genus *Flavivirus* in the family
44 *Flaviviridae*, causes fatal encephalitis in humans. It is a major arbovirus that causes thousands of
45 cases of severe neurological illness annually. TBE is a significant public health problem in endemic
46 areas of European and Asian countries (Bazan & Fletterick, 1989).

47 TBE virus (TBEV) is a positive-stranded RNA virus with a genome of ~11 kb that encodes a long
48 polyprotein in a single open reading frame (ORF), flanked by 5' and 3'-untranslated regions (UTRs).
49 The corresponding polyprotein is processed into structural proteins, i.e., capsid (C), pre-Membrane
50 (prM), envelope (E) protein, as well as non-structural proteins NS1, NS2A, NS2B, NS3, NS4A,
51 NS4B, and NS5 (Heinz & Allison, 2003). The genome contains the 5' and 3'-UTR. The C protein is
52 associated with the genome RNA packaging of TBEV (Kofler *et al.*, 2002; Kofler *et al.*, 2003). The
53 M protein, which is initially translated as a precursor protein known as prM (Lobigs & Mullbacher,
54 1993), forms a heterodimer with E protein, adding folding and maturation of the E protein. It is
55 known that E protein is responsible for binding to cellular receptors (Kopecky *et al.*, 1999;
56 Kozlovskaya *et al.*, 2010; Navarro-Sanchez *et al.*, 2003). The non-structural proteins play roles in
57 genome replication and the processing of viral proteins. NS3 functions as a protease (Bazan &
58 Fletterick, 1989; Fischl *et al.*, 2008), and helicase (Matusan *et al.*, 2001), and NS5 functions as a
59 methyltransferase (Egloff *et al.*, 2002), RNA-dependent RNA polymerase (Park *et al.*, 2007). The 5'
60 and 3'-UTR are believed to be associated with the viral genome replication (Khromykh *et al.*, 2001;
61 Kofler *et al.*, 2006).

62 Based on phylogenetic analysis, TBEV can be divided into three subtypes: Far-Eastern, European
63 and Siberian subtype. Each subtype causes different symptoms and mortality (Gritsun *et al.*, 2003).
64 The Far-Eastern subtype is also known as Russian spring summer encephalitis virus, and is prevalent
65 in Far-Eastern Russia. This subtype causes severe neural disorders such as encephalitis and

66 meningoencephalitis with a higher mortality rate up to 30 % (Bredenbeek *et al.*, 2003; Ecker *et al.*,
67 1999). The European subtype produces biphasic febrile illness and milder encephalitis, and the
68 mortality rate is lower than 2% (Dumpis *et al.*, 1999). The Siberian subtype also causes less severe
69 disease (case mortality rate, 7 to 8%) than the Far-Eastern subtype and is often associated with
70 chronic disease (Gritsun *et al.*, 2003). It remains unknown about viral factors to determine the
71 difference of the pathogenicities among the subtypes.

72 The virus strain Sofjin-HO was isolated from a patient in Russia in 1937 and has been used as a
73 prototype of the Far-Eastern subtype (Barkhash *et al.*, 2010). It is also known to be highly
74 pathogenic in a mouse model. The strain Oshima 5-10 was isolated from a sentinel dog in 1995 in
75 the area in which a human case of TBE was reported in Japan, and was classified as the Far-Eastern
76 subtype of TBEV. Oshima 5-10 is less virulent than Sofjin-HO in a mouse model (Bredenbeek *et al.*,
77 2003; Chiba *et al.*, 1999; Goto *et al.*, 2002). The nucleotide homology between Oshima 5-10 and
78 Sofjin-HO is high (96%) with differences of only 44 amino acids and a deletion of 207 nucleotides
79 in the 3'-UTR of Sofjin-HO (Supplementary table 1 and 2). However, no information exists
80 concerning the detailed mechanisms of different virulence in the two closely related strains although
81 they exhibit a high homology. Identifying the genetic factors associated with the different virulence
82 is expected to facilitate elucidation of the mechanism of pathogenicity of TBEV.

83 Infectious cDNA clones are useful in investigating the genetic determinants of flavivirus
84 replication and pathogenicity. Infectious cDNA clones have been generated for multiple flaviviruses,
85 including yellow fever virus, West Nile virus, Dengue virus, Japanese encephalitis virus, Omsk
86 hemorrhagic fever virus and TBEV (Bredenbeek *et al.*, 2003; Mandl *et al.*, 1997; Puri *et al.*, 2000;
87 Shi *et al.*, 2002; Yoshii *et al.*, 2011; Yun *et al.*, 2003). In previous studies, we constructed full-length
88 infectious cDNA clones of the Far-Eastern subtype Oshima 5-10 and Sofjin-HO strains (Hayasaka *et al.*
89 *et al.*, 2004a; Hayasaka *et al.*, 2004b; Takano *et al.*, 2011).

90 In the present study, we constructed chimeric viruses between the infectious cDNA clones of the
91 Far-Eastern subtype Sofjin-HO and Oshima 5-10 strains. The virulence of the chimeric viruses was
92 subsequently investigated in a mouse model. We showed that the 3'-UTR is an important factor that
93 determines the virulence of the Far-Eastern subtype of TBEV.

94

95 **Results**

96 *Replacement of the coding region for the structural proteins had no effect on virulence*

97 The structural proteins of flaviviruses, especially the E proteins, have been reported to be
98 important for virulence (Kopecky *et al.*, 1999; Kozlovskaya *et al.*, 2010; Navarro-Sanchez *et al.*,
99 2003). To examine whether the structural proteins are determinants of virulence in mice,
100 Sofjin-IC/OshimaCME and Oshima-IC/sofjinCME were constructed by replacement of the coding
101 region for most of the structural proteins (nucleotides 240-2291) with that of Oshima-IC-pt and
102 Sofjin-IC-pt, respectively (Fig.1(a)). Although relatively lower growth was observed in the chimeric
103 viruses, intact viruses were recovered (Fig.1(b)).

104 The pathogenicity of the recombinant viruses was examined in a mouse model. C57BL/6 mice
105 were infected subcutaneously with 1,000 pfu of Sofjin-IC-pt, Oshima-IC-pt, Sofjin-IC/OshimaCME
106 or Oshima-IC/sofjinCME virus and survival was recorded for 28 days. The mice inoculated with
107 each virus showed general signs of illness, such as reduced body weight, ruffled fur and neurologic
108 signs of trembling and hind-limb paralysis, however, the survival time was longer and the mortality
109 rate was lower in the mice infected with Oshima-IC-pt than in those infected with Sofjin-IC-pt
110 (Fig.1(b) and Table 1). The viruses in which the coding region for the structural protein were
111 replaced (Sofjin-IC/OshimaCME or Oshima-IC/sofjinCME) showed virulence similar to that of
112 parental Sofjin-IC-pt or Oshima-IC-pt, regarding the survival curve, average of survival time and
113 mortality in mice (Fig.1(b) and Table 1). The results suggested that the difference in virulence

114 between Sofjin and Oshima was not due to the structural proteins.

115

116 *The C-terminus of NS5 and the 3'-UTR are associated with the difference in virulence between*
117 *Sofjin and Oshima*

118 Because the replacement of the coding region for structural proteins did not affect the virulence,
119 the other regions were next investigated. Recombinant Oshima-IC viruses were generated by partial
120 replacement of the regions except the coding sequence for structural proteins, as shown in Fig.2 (a).
121 The growth of each chimeric virus was higher than that of the parental Oshima strains (Fig.2(b)).
122 Mice were then infected with these recombinant viruses. Compared with the Oshima-IC-pt virus,
123 mice infected with each recombinant virus showed a higher mortality and shorter survival time.
124 However, only mice infected with the Oshima-IC/NS5^C-3'UTR virus, in which the coding regions
125 for the C-terminus of NS5 and 3'-UTR were replaced with those of Sofjin-IC, showed a similar
126 virulence to that of mice infected with Sofjin-IC-pt, regarding the survival curve, days of onset,
127 average survival time (significantly shorter than that of Oshima-IC-pt) and mortality (Fig.2(b) and
128 Table 1). These results suggested that the C-terminus of NS5 and/or 3'-UTR was important for the
129 difference in virulence between the Sofjin-HO and Oshima 5-10 strains.

130 Because Oshima-IC/sofjinNS2A^C-4B^N also showed a high pathogenicity with a short survival
131 time, recombinant Oshima-IC viruses with replacement of the genes for NS2A, the N-terminal or
132 C-terminal region of NS3, or the N-terminus of NS4B were constructed (Fig.3). No difference was
133 noted in the amino acids of NS2B and NS4A between the Sofjin-HO and Oshima 5-10 strains. The
134 growth of each chimeric virus was almost similar to that of the parental-Oshima strain (Fig.3(b)).
135 The mortality of mice was 100% following infection of the chimeric virus with the N-terminus of
136 NS3. However, compared with Sofjin-IC-pt, the days to onset and survival time were longer in mice
137 infected with the chimeric virus. The mice infected with the other viruses showed survival curves

138 similar to the mice infected with Oshima-IC-pt, and no significant difference was found in the
139 average survival time between each virus and the parental Oshima-IC-pt. These results indicated that
140 the difference in virulence between the Sofjin and Oshima strains could also be attributed to the
141 N-terminus of NS3, which encodes a serine protease.

142

143 ***Partial deletion of the variable region of the 3'-UTR affects virulence***

144 There are four amino acid differences in the C-terminus of NS5 and the nucleotide differences in the
145 3'-UTR. The 3'-UTR can be divided into two regions: the “variable region” which varies among
146 TBEV strains, and the “core element” which is highly conserved in its sequence. In the variable
147 region, there are two nucleotide differences between the Sofjin and Oshima strains. A deletion of
148 207 nucleotides is present in the variable region of Sofjin, as shown in Fig.4 (a). The virus titer of
149 the supernatant of Sofjin-IC-pt infected cells was significantly higher than that of each chimeric
150 virus or Oshima-IC-pt infected cells. No significant difference in each chimeric virus and
151 Oshima-IC-pt viral titers was found (Fig.4(b)). In the core element, 12 nucleotide differences are
152 evident between the two strains. To identify the factor (s) that affects the virulence in the coding
153 regions for the C-terminus of NS5 and 3'-UTR, we constructed recombinant Oshima-IC viruses with
154 single amino acid substitution in NS5, and replacement of the variable region or core element of the
155 3'-UTR, as described in Fig.4 (a). Mice were then infected with each recombinant virus. Only the
156 Oshima-IC/3'-UTR_vari virus, in which the variable region was replaced with that of Sofjin-IC-pt,
157 killed 100% of mice and showed almost identical virulence to that of Sofjin-IC-pt virus, in terms of
158 the survival curve, days of onset and mortality. Conversely, the other recombinant viruses showed a
159 similar virulence to that of the mice infected with Oshima-IC-pt (Fig. 4(b) and Table 1). These
160 results suggested that the deletion in the variable region of the 3'-UTR is an important determinant of
161 the difference in virulence between the Sofjin and Oshima strains.

162

163 ***Effect of the deletion in the variable region of the 3'-UTR on viral multiplication and***

164 ***pathogenicity***

165 We investigated the effect of the deletion in the variable region on viral multiplication in mouse
166 neuroblastoma (NA) cells (Fig.5). The virus titer of the supernatant of Sofjin-IC-pt infected cells was
167 significantly higher than that of Oshima-IC/Sofjin 3'-UTR_vari or Oshima-IC-pt infected cells. No
168 significant difference in the Oshima-IC/Sofjin 3'-UTR_vari and Oshima-IC-pt viral titers was found.
169 This result suggested that the deletion in the variable region did not affect viral multiplication in
170 cultured cells. To examine the correlation between disease development and viral replication in
171 organs, the viral loads in the blood, spleen, and brain were compared in mice inoculated with the
172 Sofjin-IC-pt, Oshima-IC/3'-UTR_vari or Oshima-IC-pt viruses (Fig.6). Transient viremia was
173 observed in the mice infected with each virus, which almost disappeared by 5 to 7 days post
174 infection. Increases in viral replication were observed in the spleen after viremia (from 3 days post
175 infection).

176 The virus was detected in the brain by 7 days post infection. The titer was reached 1.4×10^7 pfu
177 ml^{-1} at 7 days post infection in the mice inoculated with Sofjin-IC-pt, and was significantly higher
178 than that in the mice infected with the Oshima-IC/Sofjin 3'-UTR_vari or Oshima-IC-pt virus
179 ($P < 0.05$). No significant difference between in titer in brains infected with
180 Oshima-IC/Sofjin3'-UTR_vari and Oshima-IC-pt was found. However, at 9 days post infection, the
181 virus titer in the brain infected with Oshima-IC/Sofjin3'-UTR_vari reached 8.3×10^7 pfu ml^{-1} , a level
182 almost identical to that in the brain infected with Sofjin-IC-pt, and significantly higher than that in
183 the mouse brain infected with Oshima-IC-pt.

184 Histopathological features in mice infected with Oshima-IC-pt, Sofjin-IC-pt or
185 Oshima/3'-UTR_vari at 7 and 9 days post infection were investigated (Fig.7). At day 7, there were

186 few pathological changes in mice infected with each virus (data not shown). However, at day 9,
187 abundant viral antigens and pathological changes, such as inflammatory infiltrations, small
188 hemorrhages, and necrotic or degenerated neurons were observed throughout the brains of mice
189 infected with Sofjin-IC-pt and Oshima/3'-UTR_vari. Compared with Sofjin-IC-pt and
190 Oshima/3'-UTR_vari infected mice brains, there were fewer virus-antigen-positive cells and mild
191 pathological changes in the brains of mice infected with Oshima-IC-pt.

192 Taken together, these data suggested that the deletion in the variable region of the 3'-UTR
193 enhanced virus multiplication and pathogenicity in the mouse brain.

194

195 **Discussion**

196 In the present study, the important determinants of virulence were identified between the
197 Far-Eastern subtype strains of TBEV Sofjin and Oshima. We showed that multiple viral factors
198 affected the virulence cumulatively, and that the variable region of the 3'-UTR was a critical
199 virulence determinant.

200 The E protein is thought to play a key role in determining the virulence of TBEV (Mandl, 2005).
201 The E protein is expressed on the surface of mature virions and mediates virus entry into the host
202 cell by binding to cell surface molecules (Heinz & Allison, 2003). The E protein has been suggested
203 to be a crucial determinant of tissue tropism and neuropathogenesis during flavivirus infection.
204 Amino acid changes in the E protein have been reported to affect the neurovirulence and
205 neuroinvasiveness of tick-borne Flaviviruses (Goto *et al.*, 2003; Kozlovskaya *et al.*, 2010; Mandl *et*
206 *al.*, 2001; Rumyantsev *et al.*, 2006). However, the structural proteins, including the E protein, were
207 not associated with the different virulence between the Sofjin-HO and Oshima 5-10 strains.

208 Replacement of the N-terminus of NS3 increased virulence in mice. The Flavivirus NS3 encodes
209 a serine protease domain at its N-terminal that is required for cleavage of the polyprotein during

210 viral replication (Lescar *et al.*, 2008). It combines with NS2B and forms the NS2B-NS3 protease
211 complex as the activated serine protease (Bazan & Fletterick, 1989; Chambers *et al.*, 1990). Several
212 amino acid substitutions in the protease domain of NS3 can influence the activity of the enzyme
213 and the virulence of TBEV (Chiba *et al.*, 1999; Ruzek *et al.*, 2008). Seven amino acid differences in
214 the N-terminus of NS3 exist between Sofjin-HO and Oshima 5-10. A previous report suggested that
215 the serine to phenylalanine substitution at amino acid position 45 affects TBEV pathogenicity
216 (Chiba *et al.*, 1999). An identical substitution was also observed between Sofjin-HO and Oshima
217 5-10 strains. Therefore, this substitution might be associated with partially affecting the difference
218 in virulence between Sofjin-HO and Oshima 5-10 we report here.

219 Replacement of the variable region of the 3'-UTR of Oshima with that of Sofjin resulted in a
220 marked increase in virulence. The 3'-UTR of TBEV consists of two distinct domains, the 5'-terminal
221 variable region and 3'-terminal core element (Gritsun *et al.*, 1997; Wallner *et al.*, 1995). The core
222 element shows a high degree of sequence conservation among TBEV strains, and contains sequences
223 necessary for viral genome replication, such as cyclization sequence (Kofler *et al.*, 2006). The
224 sequence of variable region varies among the strains of TBEV strains, and the role of this region is
225 unclear. In a study of European TBEV subtype strains, deletion of the entire 3'-UTR variable region
226 did not affect the viral multiplication in cultured cells or virulence in mice (Mandl *et al.*, 1998). The
227 discrepant results obtained in the present study might be due to use of different strains. Because the
228 Neudoerfl strain used in the study of Mandl *et al.* (Mandl *et al.*, 1998) was highly virulent in the
229 mouse model ($LD_{50} < 10$), it is possible that deletion of the whole variable region did not result in an
230 increase in virulence. Additionally, the Neudoerfl strain contains an insertion of a poly-A sequence in
231 the variable region (Mandl *et al.*, 1998) that is not present in most of other TBEV strains. It is also
232 possible that the addition of the poly-A sequence might affect the function of the 3'-UTR, as
233 observed for the partial deletion of the 3'-UTR in Sofjin, resulting in increased virulence.

234 Nevertheless, the deletion of the variable region in the 3'-UTR of the Far-Eastern subtype of TBEV
235 resulted in an increased virulence in mice. This result suggested an unidentified role of the variable
236 region in the viral pathogenicity.

237 Replacement of the variable region of the 3'-UTR did not affect viral replication in cell culture.
238 It also did not increase viral multiplication in the mouse brain by 7 days post infection. However,
239 by 9 days post infection, the viral titer of the chimeric virus with the variable region of the Sofjin
240 strain increased markedly to level identical to that of the Sofjin strain as evidenced by severe
241 pathological changes in the brain. These data suggested involvement of the variable region in
242 regulation of the host response, which in turn affected the viral replication in the brain. A recent
243 study of West Nile virus and Japanese encephalitis virus reported that the subgenomic flavivirus
244 RNA (sfRNA) was mediated from the 3'-UTR as a product of the genomic RNA degradation by
245 host exoribonuclease, and that sfRNA mediated pathogenicity by interfering with host protective
246 responses, such as the RNAi machinery and type I interferon response (Pijlman *et al.*, 2008;
247 Schnettler *et al.*, 2012). Therefore, the deletion in the 3'-UTR of Sofjin-HO may affect the function,
248 amount, or stability of sfRNA.

249 The sequence of the 3'-UTR variable region varies among TBEV strains; however, the role of
250 this region remains unknown. Strains freshly-isolated from ticks and wild rodents do not have a
251 deletion in the variable region, and this region is considered to be essential for the natural
252 transmission cycle of TBEV (Bredenbeek *et al.*, 2003). Conversely, deletions in the variable region
253 of 3'-UTR were found in many Far-Eastern subtype isolates from human patients (Leonova *et al.*,
254 2013). Mandl *et al* reported that the deletion in the 3'-UTR occurred during passage in mammalian
255 cell culture or in mice (Mandl *et al.*, 1998). Together, these reports suggest that the deletion caused
256 by adaptation or selection in mammalian cells affected replication, resulting in an increased
257 virulence in mammals.

258 In conclusion, we report here that the different virulence between Sofjin and Oshima is
259 determined by multiple viral factors cumulatively, and the variable region of the 3'-UTR is an
260 important determinant of pathogenicity in mice. Deletion in the region affected multiplication in the
261 brain, resulting in the severe pathological changes associated with the Far-Eastern subtype TBEV.
262 These findings encourage further research to identify the pathogenic mechanisms of TBEV and
263 develop prevention and therapeutic strategies for TBE, such as development of an attenuated live
264 vaccine and design of targets of anti- viral drugs.

265

266 **Methods**

267 **Cells.** Baby hamster kidney (BHK-21) cells and mouse neuroblastoma (NA) cells were grown in
268 Eagle's minimal essential medium (MEM), supplemented with 8% and 10% fetal calf serum (FCS),
269 respectively.

270

271 **Viruses.** Viruses were prepared from infectious cDNA clones. Infectious cDNA plasmids of parental
272 Sofjin-IC and Oshima-IC (Sofjin-IC-pt and Oshima-IC-pt), which encode the full-length cDNA of
273 the TBEV Sofjin-HO (accession no. AB062064) and Oshima 5-10 (accession no. AB062063) strains,
274 respectively, were prepared as described previously (Hayasaka *et al.*, 2004a; Hayasaka *et al.*, 2004b;
275 Takano *et al.*, 2011).

276 Infectious cDNA plasmids of the recombinant viruses listed in Figs.1 and 2 were constructed by
277 the replacement of the indicated regions between Sofjin-IC-pt and Oshima-IC-pt using the indicated
278 restriction enzyme sites. To construct infectious cDNA plasmid of the recombinant viruses listed in
279 Fig. 3(a), the DNA fragment with the indicated nucleotides of Sofjin was amplified by
280 fused-polymerase chain reaction (PCR) and was inserted into Oshima-IC using the *AgeI* and *AatII*
281 restriction enzyme sites. Oshima-IC/sofjinNS2A^C was constructed by site-directed mutagenesis as

282 described below. To construct infectious cDNA plasmids of recombinant Oshima-IC viruses with
283 substitutions of single amino acids, the site-directed mutations were introduced into the amino acid
284 position 225 of NS2A, and the amino acid positions 778, 827, 832 and 862 of NS5 using the Quick
285 Change II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, U.S.) as shown
286 in Figs. 3(a) and 4(a).

287 To construct infectious cDNA plasmids of the recombinant virus Oshima-IC/sofjin3'-UTR-vari
288 and Oshima-IC/sofjin3'-UTR-core, the fragment (nucleotides 9830-11100 of Oshima-IC) with the
289 variable region (nucleotides 10377-10551) and the core element (nucleotides 10552-10894) of
290 Sofjin-IC were amplified by fused PCR, and inserted into Oshima-IC using *AscI* and *SpeI*, as shown
291 in Fig.4(a). The differences of nucleotide and amino acids between Sofjin, Oshima and each
292 recombinant virus were shown in Supplementary table 1 and 2.

293 The infectious cDNA plasmids were linearized with the *SpeI* and transcribed into RNA using the
294 mMESAGE mMACHINE SP6 Kit (Life Technology, Carlsbad, CA, USA), as described previously
295 (Gritsun & Gould, 1995). The mRNA samples were treated with DNase I and precipitated with LiCl.
296 The precipitated RNA was dissolved in 30 μ l of diethylpyrocarbonate-treated water. BHK-21 cells
297 were transfected with mRNA using a trans IT-mRNA Transfection Kit (Mirus Bio LLC, Madison,
298 WI, USA) as described previously (Hayasaka *et al.*, 2004a). Two days post transfection, recombinant
299 viruses in the supernatant of the RNA-transfected cells was harvested and stored at -80 °C.

300

301 **Virus titration.** Plaque assays were carried out with BHK-21 cells using 12-well plates. The cells
302 were inoculated with the serial 10-fold dilutions of organ suspensions or culture medium from
303 infected cells (100 μ l), and they were incubated for 1 h at 37°C before 1.5% carboxy methyl
304 cellulose in MEM (1 ml well⁻¹) was added. Incubation was continued for 3-4 days, and the
305 monolayers were stained with 0.1% crystal violet in 10% formalin neutral buffer solution. Plaques

306 were counted, and infectivity titers were expressed as plaque-forming units (pfu) ml⁻¹.

307

308 **Growth curve in cell culture.** Subconfluent NA cells were grown in 24-well plates. Cells were
309 inoculated with each virus at a multiplicity of infection (MOI) of 1. Cells were incubated at 37°C in
310 5% CO₂. The supernatant was harvested at 24 and 48 h post-inoculation and stored in aliquots at
311 -80°C.

312

313 **Animal model.** Five-week-old female C57BL6 mice (Jackson Immuno Research, West Grove, PA,
314 USA) were inoculated subcutaneously with 1,000 pfu of viruses. Morbidity was defined as the
315 appearance of 10% weight loss. Surviving mice were monitored for 28 days post infection to obtain
316 survival curves and mortality rates. For the analysis of viral distribution in tissues, serum, brain, and
317 spleen were collected from the mice on days 1, 3, 5, 7, and 9 post infection. Organs were
318 individually weighed and homogenized, and prepared as 10% suspensions (w/v) in
319 phosphate-buffered saline with 10% FCS. The suspensions were clarified by centrifugation (4,000
320 rpm for 5 min, 4°C), and the supernatants were titrated by plaque assay on BHK-21 cells. All
321 procedures were performed according to the guidelines of the Animal Care and Use Committee of
322 the Hokkaido University.

323

324 **Histopathological examination.** Three mice infected with 10³ pfu of TBEV were killed at 7 and 9
325 days post infection, and formalin fixed brains were routinely processed and embedded in paraffin,
326 sectioned and stained with haematoxylin and eosin as described previously (Sunden *et al.*, 2010).
327 Immunohistochemical detection of TBEV antigens was performed using rabbit polyclonal antibodies
328 against E protein to detect TBEV antigens (Yoshii *et al.*, 2004).

329

330 **Statistical analysis.** P-values of differences in virus titers were calculated using an unpaired
331 Student's t-tests.

332

333 **Acknowledgements**

334 This work was supported by Grants-in-Aid for Scientific Research (25-1563, 24780293, 22780268
335 and 21405035) and the Global COE Program from the Ministry of Education, Culture, Sports,
336 Sciences and Technology of Japan, and Health Sciences Grants for Research on Emerging and
337 Re-emerging Infectious Disease from Ministry of Health, Labour and Welfare of Japan.

338

339 **References**

- 340 **Bazan, J. F. & Fletterick, R. J. (1989).** Detection of a trypsin-like serine protease domain in
341 flaviviruses and pestiviruses. *Virology* **171**, 637-639
- 342 **Barkhash, A. V., Perelygin, A. A., Babenko, V. N., Myasnikova, N. G., Pilipenko, P. I.,**
343 **Romaschenko, A. G., Voevoda, M. I. & Brinton, M. A. (2010).** Variability in the
344 2'-5'-oligoadenylate synthetase gene cluster is associated with human predisposition
345 to tick-borne encephalitis virus-induced disease. *J Infect Dis* **202**, 1813-1818.
- 346 **Chambers, T. J., Weir, R. C., Grakoui, A., McCourt, D. W., Bazan, J. F., Fletterick, R. J. &**
347 **Rice, C. M. (1990).** Evidence that the N-terminal domain of nonstructural protein
348 NS3 from yellow fever virus is a serine protease responsible for site-specific
349 cleavages in the viral polyprotein. *Proceedings of the National Academy of Sciences*
350 *of the United States of America* **87**, 8898-8902.
- 351 **Dumpis, U., Crook, D. & Oksi, J. (1999).** Tick-borne encephalitis. *Clinical infectious*
352 *diseases : an official publication of the Infectious Diseases Society of America* **28**,
353 882-890.
- 354 **Ecker, M., Allison, S. L., Meixner, T. & Heinz, F. X. (1999).** Sequence analysis and genetic
355 classification of tick-borne encephalitis viruses from Europe and Asia. *J Gen Virol* **80**
356 (Pt 1), 179-185.
- 357 **Egloff, M. P., Benarroch, D., Selisko, B., Romette, J. L. & Canard, B. (2002).** An RNA cap
358 (nucleoside-2'-O-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal
359 structure and functional characterization. *The EMBO journal* **21**, 2757-2768.
- 360 **Fischl, W., Elshuber, S., Schrauf, S. & Mandl, C. W. (2008).** Changing the protease specificity

361 for activation of a flavivirus, tick-borne encephalitis virus. *Journal of virology* **82**,
362 8272-8282.

363 **Gorbalenya, A. E., Donchenko, A. P., Koonin, E. V. & Blinov, V. M. (1989)**. N-terminal
364 domains of putative helicases of flavi- and pestiviruses may be serine proteases.
365 *Nucleic Acids Res* **17**, 3889-3897

366 **Goto, A., Hayasaka, D., Yoshii, K., Mizutani, T., Kariwa, H. & Takashima, I. (2002)**. Genetic
367 and biological comparison of tick-borne encephalitis viruses from Hokkaido and
368 far-eastern Russia. *Jpn J Vet Res* **49**, 297-307.

369 **Goto, A., Hayasaka, D., Yoshii, K., Mizutani, T., Kariwa, H. & Takashima, I. (2003)**. A
370 BHK-21 cell culture-adapted tick-borne encephalitis virus mutant is attenuated for
371 neuroinvasiveness. *Vaccine* **21**, 4043-4051.

372 **Gritsun, T. S. & Gould, E. A. (1995)**. Infectious transcripts of tick-borne encephalitis virus,
373 generated in days by RT-PCR. *Virology* **214**, 611-618.

374 **Gritsun, T. S., Lashkevich, V. A. & Gould, E. A. (2003)**. Tick-borne encephalitis. *Antiviral*
375 *research* **57**, 129-146.

376 **Gritsun, T. S., Venugopal, K., Zanotto, P. M., Mikhailov, M. V., Sall, A. A., Holmes, E. C.,**
377 **Polkinghorne, I., Frolova, T. V., Pogodina, V. V., Lashkevich, V. A. & Gould, E. A.**
378 **(1997)**. Complete sequence of two tick-borne flaviviruses isolated from Siberia and
379 the UK: analysis and significance of the 5' and 3'-UTRs. *Virus research* **49**, 27-39.

380 **Hayasaka, D., Goto, A., Yoshii, K., Mizutani, T., Kariwa, H. & Takashima, I. (2001a)**.
381 Evaluation of European tick-borne encephalitis virus vaccine against recent
382 Siberian and far-eastern subtype strains. *Vaccine* **19**, 4774-4779.

383 **Hayasaka, D., Gritsun, T. S., Yoshii, K., Ueki, T., Goto, A., Mizutani, T., Kariwa, H., Iwasaki,**
384 **T., Gould, E. A. & Takashima, I. (2004a)**. Amino acid changes responsible for
385 attenuation of virus neurovirulence in an infectious cDNA clone of the Oshima
386 strain of tick-borne encephalitis virus. *The Journal of general virology* **85**,
387 1007-1018.

388 **Hayasaka, D., Ivanov, L., Leonova, G. N., Goto, A., Yoshii, K., Mizutani, T., Kariwa, H. &**
389 **Takashima, I. (2001b)**. Distribution and characterization of tick-borne encephalitis
390 viruses from Siberia and far-eastern Asia. *The Journal of general virology* **82**,
391 1319-1328.

392 **Hayasaka, D., Yoshii, K., Ueki, T., Iwasaki, T. & Takashima, I. (2004b)**. Sub-genomic
393 replicons of Tick-borne encephalitis virus. *Archives of virology* **149**, 1245-1256.

394 **Heinz, F. X. & Allison, S. L. (2003)**. Flavivirus structure and membrane fusion. *Advances in*
395 *virus research* **59**, 63-97.

396 **Khromykh, A. A., Meka, H., Guyatt, K. J. & Westaway, E. G. (2001)**. Essential role of

397 cyclization sequences in flavivirus RNA replication. *Journal of virology* **75**,
398 6719-6728.

399 **Kofler, R. M., Heinz, F. X. & Mandl, C. W. (2002)**. Capsid protein C of tick-borne encephalitis
400 virus tolerates large internal deletions and is a favorable target for attenuation of
401 virulence. *Journal of virology* **76**, 3534-3543.

402 **Kofler, R. M., Hoenninger, V. M., Thurner, C. & Mandl, C. W. (2006)**. Functional analysis of
403 the tick-borne encephalitis virus cyclization elements indicates major differences
404 between mosquito-borne and tick-borne flaviviruses. *Journal of virology* **80**,
405 4099-4113.

406 **Kofler, R. M., Leitner, A., O'Riordain, G., Heinz, F. X. & Mandl, C. W. (2003)**. Spontaneous
407 mutations restore the viability of tick-borne encephalitis virus mutants with large
408 deletions in protein C. *Journal of virology* **77**, 443-451.

409 **Kopecky, J., Grubhoffer, L., Kovar, V., Jindrak, L. & Vokurkova, D. (1999)**. A putative host
410 cell receptor for tick-borne encephalitis virus identified by anti-idiotypic antibodies
411 and virus affinoblotting. *Intervirology* **42**, 9-16.

412 **Kozlovskaya, L. I., Osolodkin, D. I., Shevtsova, A. S., Romanova, L., Rogova, Y. V.,
413 Dzhivanian, T. I., Lyapustin, V. N., Pivanova, G. P., Gmyl, A. P., Palyulin, V. A. &
414 Karganova, G. G. (2010)**. GAG-binding variants of tick-borne encephalitis virus.
415 *Virology* **398**, 262-272.

416 **Leonova, G. N., Belikov, S. I., Kondratov, I. G. & Takashima, I. (2013)**. Comprehensive
417 assessment of the genetics and virulence of tick-borne encephalitis virus strains
418 isolated from patients with inapparent and clinical forms of the infection in the
419 Russian Far East. *Virology* **443**, 89-98.

420 **Lescar, J., Luo, D., Xu, T., Sampath, A., Lim, S. P., Canard, B. & Vasudevan, S. G. (2008)**.
421 Towards the design of antiviral inhibitors against flaviviruses: the case for the
422 multifunctional NS3 protein from Dengue virus as a target. *Antiviral research* **80**,
423 94-101.

424 **Lindquist, L. & Vapalahti, O. (2008)**. Tick-borne encephalitis. *Lancet* **371**, 1861-1871.

425 **Lobigs, M. & Mullbacher, A. (1993)**. Recognition of vaccinia virus-encoded major
426 histocompatibility complex class I antigens by virus immune cytotoxic T cells is
427 independent of the polymorphism of the peptide transporters. *Proceedings of the
428 National Academy of Sciences of the United States of America* **90**, 2676-2680.

429 **Mandl, C. W. (2005)**. Steps of the tick-borne encephalitis virus replication cycle that affect
430 neuropathogenesis. *Virus research* **111**, 161-174.

431 **Mandl, C. W., Ecker, M., Holzmann, H., Kunz, C. & Heinz, F. X. (1997)**. Infectious cDNA
432 clones of tick-borne encephalitis virus European subtype prototypic strain Neudoerfl

433 and high virulence strain Hypr. *The Journal of general virology* **78** (Pt 5),
434 1049-1057.

435 **Mandl, C. W., Holzmann, H., Meixner, T., Rauscher, S., Stadler, P. F., Allison, S. L. & Heinz,**
436 **F. X. (1998).** Spontaneous and engineered deletions in the 3' noncoding region of
437 tick-borne encephalitis virus: construction of highly attenuated mutants of a
438 flavivirus. *Journal of virology* **72**, 2132-2140.

439 **Mandl, C. W., Kroschewski, H., Allison, S. L., Kofler, R., Holzmann, H., Meixner, T. & Heinz,**
440 **F. X. (2001).** Adaptation of tick-borne encephalitis virus to BHK-21 cells results in
441 the formation of multiple heparan sulfate binding sites in the envelope protein and
442 attenuation in vivo. *Journal of virology* **75**, 5627-5637.

443 **Mansfield, K. L., Johnson, N., Phipps, L. P., Stephenson, J. R., Fooks, A. R. & Solomon, T.**
444 **(2009).** Tick-borne encephalitis virus - a review of an emerging zoonosis. *The Journal*
445 *of general virology* **90**, 1781-1794.

446 **Matusan, A. E., Pryor, M. J., Davidson, A. D. & Wright, P. J. (2001).** Mutagenesis of the
447 Dengue virus type 2 NS3 protein within and outside helicase motifs: effects on
448 enzyme activity and virus replication. *Journal of virology* **75**, 9633-9643.

449 **Navarro-Sanchez, E., Altmeyer, R., Amara, A., Schwartz, O., Fieschi, F., Virelizier, J. L.,**
450 **Arenzana-Seisdedos, F. & Despres, P. (2003).** Dendritic-cell-specific ICAM3-grabbing
451 non-integrin is essential for the productive infection of human dendritic cells by
452 mosquito-cell-derived dengue viruses. *EMBO reports* **4**, 723-728.

453 **Park, G. S., Morris, K. L., Hallett, R. G., Bloom, M. E. & Best, S. M. (2007).** Identification of
454 residues critical for the interferon antagonist function of Langat virus NS5 reveals a
455 role for the RNA-dependent RNA polymerase domain. *Journal of virology* **81**,
456 6936-6946.

457 **Pijlman, G. P., Funk, A., Kondratieva, N., Leung, J., Torres, S., van der Aa, L., Liu, W. J.,**
458 **Palmenberg, A. C., Shi, P. Y., Hall, R. A. & Khromykh, A. A. (2008).** A highly
459 structured, nuclease-resistant, noncoding RNA produced by flaviviruses is required
460 for pathogenicity. *Cell host & microbe* **4**, 579-591.

461 **Potapova, U. V., Feranchuk, S. I., Potapov, V. V., Kulakova, N. V., Kondratov, I. G., Leonova,**
462 **G. N. & Belikov, S. I. (2012).** NS2B/NS3 protease: allosteric effect of mutations
463 associated with the pathogenicity of tick-borne encephalitis virus. *Journal of*
464 *biomolecular structure & dynamics* **30**, 638-651.

465 **Puri, B., Polo, S., Hayes, C. G. & Falgout, B. (2000).** Construction of a full length infectious
466 clone for dengue-1 virus Western Pacific,74 strain. *Virus genes* **20**, 57-63.

467 **Rumyantsev, A. A., Murphy, B. R. & Pletnev, A. G. (2006).** A tick-borne Langat virus mutant
468 that is temperature sensitive and host range restricted in neuroblastoma cells and

469 lacks neuroinvasiveness for immunodeficient mice. *Journal of virology* **80**,
470 1427-1439.

471 **Ruzek, D., Gritsun, T. S., Forrester, N. L., Gould, E. A., Kopecky, J., Golovchenko, M.,**
472 **Rudenko, N. & Grubhoffer, L. (2008).** Mutations in the NS2B and NS3 genes affect
473 mouse neuroinvasiveness of a Western European field strain of tick-borne
474 encephalitis virus. *Virology* **374**, 249-255.

475 **Schnettler, E., Sterken, M. G., Leung, J. Y., Metz, S. W., Geertsema, C., Goldbach, R. W.,**
476 **Vlak, J. M., Kohl, A., Khromykh, A. A. & Pijlman, G. P. (2012).** Noncoding flavivirus
477 RNA displays RNA interference suppressor activity in insect and Mammalian cells.
478 *Journal of virology* **86**, 13486-13500.

479 **Shi, P. Y., Tilgner, M., Lo, M. K., Kent, K. A. & Bernard, K. A. (2002).** Infectious cDNA clone
480 of the epidemic west nile virus from New York City. *Journal of virology* **76**,
481 5847-5856.

482 **Sunden, Y., Yano, S., Ishida, S., Ochiai, K. & Umemura, T. (2010).** Intracerebral vaccination
483 suppresses the spread of rabies virus in the mouse brain. *Microbes and infection /*
484 *Institut Pasteur* **12**, 1163-1169.

485 **Takano, A., Yoshii, K., Omori-Urabe, Y., Yokozawa, K., Kariwa, H. & Takashima, I. (2011).**
486 Construction of a replicon and an infectious cDNA clone of the Sofjin strain of the
487 Far-Eastern subtype of tick-borne encephalitis virus. *Arch Virol* **156**, 1931-1941.

488 **Wallner, G., Mandl, C. W., Kunz, C. & Heinz, F. X. (1995).** The flavivirus 3'-noncoding region:
489 extensive size heterogeneity independent of evolutionary relationships among
490 strains of tick-borne encephalitis virus. *Virology* **213**, 169-178.

491 **Yoshii, K., Igarashi, M., Ito, K., Kariwa, H., Holbrook, M. R. & Takashima, I. (2011).**
492 Construction of an infectious cDNA clone for Omsk hemorrhagic fever virus, and
493 characterization of mutations in NS2A and NS5. *Virus research* **155**, 61-68.

494 **Yoshii, K., Konno, A., Goto, A., Nio, J., Obara, M., Ueki, T., Hayasaka, D., Mizutani, T.,**
495 **Kariwa, H. & Takashima, I. (2004).** Single point mutation in tick-borne encephalitis
496 virus prM protein induces a reduction of virus particle secretion. *J Gen Virol* **85**,
497 3049-3058.

498 **Yun, S. I., Kim, S. Y., Rice, C. M. & Lee, Y. M. (2003).** Development and application of a
499 reverse genetics system for Japanese encephalitis virus. *Journal of virology* **77**,
500 6450-6465.

501

Table 1 Morbidity and mortality of mice infected with Sofjin-IC-pt, Oshima-IC-pt or the recombinant viruses †.

	Onset of disease (days)	Survival time (days)	Morbidity (%)	Mortality (%)
Sofjin-IC-pt	7.7±0.8**	9.0±1.5**	100	100
Oshima-IC-pt	11.9±1.7	18.4±5.3	90	50
Sofjin-IC/oshimaCME	8.8±0.4**	8.4±0.5**	100	100
Oshima-IC/sofjinCME	9.6±1.8	15.0±6.2	100	60
Oshima-IC/sofjin5'UTR-C ^N	8.7±1.3**	13.5±4.4	100	100
Oshima-IC/NS1-2A ^N	9.3±1.5**	13.7±3.2	100	90
Oshima-IC/NS2A ^C -4B ^N	8.4±0.7**	11.0±2.4**	100	90
Oshima/sofjinNS2A ^C	11.5±2.6	16	80	20
Oshima/sofjinNS3 ^N	8.2±1.1**	12.9±4.7	100	100
Oshima/sofjinNS3 ^C	9.8±2.0	13.3±1.5	90	30
Oshima/sofjinNS4B ^N	9.5±1.6	18.0±5.7	100	80
Oshima-IC/NS4B ^C -5 ^N	9.3±0.7**	13.9±2.5	100	70
Oshima-IC/NS5 ^C -3'UTR	7.7±0.7**	8.7±1.2**	100	100
Oshima-IC/NS5-778L	10.0±0.8	17.8±5.0	80	40
Oshima-IC/NS5-827S	8.9±1.0**	14.9±4.9	100	80
Oshima-IC/NS5-832A	9.6±1.5*	15.5±4.7	100	60
Oshima-IC/NS5862K	9.8±1.8*	17.4±5.7	100	50
Oshima-IC/3'-UTR-variable	8.6±0.5**	10.4±1.6*	100	100
Oshima-IC/3'-UTR-core	9.6±1.8	13.7±4.5	100	60

† Five adult mice C57BL/6 were infected with Sofjin-IC/oshimaCME and Oshima-IC/sofjinCME, and ten mice were infected with the others.

* or ** denotes a significant difference between Oshima-IC and the other viruses ($P < 0.05$ or 0.01), respectively.

503 **Figure Legends**

504 **Fig.1** Effect of replacement of the TBEV region encoding most of the structural proteins on
505 pathogenicity in mice. **(a)** Schematic representation of the genomes of recombinant Sofjin-IC and
506 Oshima-IC viruses. Sofjin-IC/OshimaCME and Oshima-IC/sofjinCME were constructed by
507 replacement of the coding region for most structural proteins (nucleotides 240-2291) with that of
508 Oshima-IC-pt and Sofjin-IC-pt, respectively. Sofjin-IC and Oshima-IC regions are shown in gray
509 and white, respectively. **(b)** Growth curve of each virus in NA cells. NA cells were infected with
510 each virus at multiplication of infection (MOI) of 1. Viral titers at each time point were determined
511 in BHK-21 cells. The data is the average of three independent experiments. * At 24h post-infection,
512 Sofjin-IC/oshimaCME and Oshima-IC/sofjinCME showed significant differences from
513 Oshima-IC-pt and Sofjin-IC-pt ($P < 0.05$). † At 48h post-infection, a significant difference was
514 observed between Sofjin-IC-pt and both chimeric viruses, and Oshima-IC/sofjinCME showed
515 significant difference from Oshima-IC-pt ($P < 0.05$). **(c)** Survival of mice inoculated with Sofjin-IC,
516 Oshima-IC and the chimeric viruses. Mice were inoculated subcutaneously with 1,000 pfu of
517 Sofjin-IC-pt (closed square), Sofjin-IC/oshimaCME (open square), Oshima-IC/sofjinCME (open
518 circle) and Oshima-IC-pt (closed circle).

519

520 **Fig.2** Effect of replacement of TBEV untranslated regions and the region encoding non-structural
521 proteins on the pathogenicity in mice. **(a)** Schematic representation of the genomes of the chimeric
522 viruses. Each Oshima-based virus was constructed by replacement of the 5'-UTR and the N-terminus
523 of C ($5'UTR-C^N$), the C-terminal region of E and NS1 and the N-terminal region of NS2A
524 ($NS1-2A^N$), the C-terminus of NS2A and NS3 and the N-terminus of NS4B ($NS2A^C-4B^N$), the
525 C-terminus of NS4B and the N-terminus of NS5 ($NS4B^C-5^N$), or the C-terminus of NS5 and the
526 3'-UTR ($NS5^C-3'UTR$) with the respective region regions of Sofjin-IC. The Sofjin-IC- and

527 Oshima-IC regions are shown in gray and white, respectively. **(b)** Growth curve of each virus in NA
528 cells. NA cells were infected with each virus at multiplication of MOI of 1. Viral titers at each time
529 point were determined in BHK-21 cells. The data is the average of three independent experiments. *
530 At 24h post-infection, Oshima-IC/sofjin NS1-2A^N, 5'UTR-C^N and NS4B^C-5^N showed significant
531 difference from Oshima-IC-pt, and Oshima-IC/sofjin NS5^C-3'UTR, NS4B^C-5^N showed it from
532 Sofjin-IC-pt (P<0.05). † At 48h post-infection, a significant difference was observed between
533 Sofjin-IC-pt and Oshima-IC/sofjin 5'UTR-C^N or NS5^C-3'UTR, and between Oshima-IC-pt and the
534 other viruses (P<0.05). **(c)** Survival of mice inoculated with Sofjin-IC-pt, Oshima-IC-pt, and the
535 chimeric viruses. Mice were inoculated subcutaneously with 1,000 pfu of Sofjin-IC-pt (closed
536 square), Oshima-IC/sofjin5'UTR-C^N (closed triangle), Oshima-IC/sofjinNS1-2A^N (open triangle),
537 Oshima-IC/sofjinNS2A^C-4B^N (closed diamond), Oshima-IC/sofjinNS4B^C-5^N (open diamond),
538 Oshima-IC/sofjin NS5^C-3'UTR (open square), or Oshima-IC-pt (closed circle).

539

540

541 **Fig.3** Effect of replacement of the TBEV region encoding non-structural proteins (NS2A, NS3 and
542 NS4B) on the pathogenicity in mice. (a) Schematic representation of the genomes of the chimeric
543 viruses. Each Oshima-based virus was constructed by replacement of the NS2A, the N- and
544 C-terminal region of NS3 and the C-terminal region of NS4B with each of Sofjin-IC. The Sofjin-IC
545 and Oshima-IC regions are shown in gray and white, respectively. **(b)** Growth curve of each virus in
546 NA cells. NA cells were infected with each virus at multiplication of MOI of 1. Viral titers at each
547 time point were determined in BHK-21 cells. The data is the average of three independent
548 experiments. * At 24h post-infection, Oshima-IC/sofjin NS3^C and NS4B^N showed a significant
549 difference from Oshima-IC-pt, and Oshima-IC/sofjin NS2A^C, NS3^N, NS4B^N showed it from
550 Sofjin-IC-pt (P<0.05). † At 48h post-infection, a significant difference was observed between

551 Sofjin-IC-pt and the other viruses ($P < 0.05$). No significant differences between Oshima-IC-pt and
552 each chimeric virus was observed. (c) Survival of mice inoculated with Sofjin-IC-pt, Oshima-IC-pt
553 and the chimeric viruses. Mice were inoculated subcutaneously with 1,000 pfu Sofjin-IC-pt (closed
554 square), Oshima-IC/sofjin NS2A^C (closed triangle), Oshima-IC /sofjin NS3^N (open triangle),
555 Oshima-IC/sofjin NS3^C (closed diamond), Oshima-IC/sofjin NS4B^N (open diamond), or
556 Oshima-IC-pt (closed circle).

557

558

559 **Fig.4** Effect of substitutions of TBEV amino acids in NS5 and replacement of the 3'-UTR. (a)
560 Schematic representation of the genome of recombinant viruses. Single-amino acid substitutions
561 were introduced at NS5 positions 778 (NS5-778L), 827 (NS5-827S), 832 (NS5-832A), and 862
562 (NS5-862K) of Oshima-IC. The gray and white arrowheads indicate amino acids derived from
563 Sofjin-IC-pt and Oshima-IC-pt, respectively. Oshima-IC/sofjin3'-UTR_vari and
564 Oshima-IC/sofjin3'-UTR_core are Oshima-IC chimeric viruses in which the variable region and core
565 element of the 3'-UTR were replaced with those of Sofjin-IC. The gray lines indicate the regions
566 derived from the 3'-UTR of Sofjin-IC. The broken line indicates the region lacking in Sofjin-IC-pt.
567 (b) Growth curve of each virus in NA cells. NA cells were infected with each virus at multiplication
568 of MOI of 1. Viral titers at each time point were determined in BHK-21 cells. The data is the average
569 of three independent experiments. * At 24h post-infection, the chimeric viruses except for
570 Oshima-IC/sofjin 3'-UTR_core showed a significant difference from Sofjin-IC-pt ($P < 0.05$). † At 48h
571 post-infection, a significant difference was observed between Sofjin-IC-pt and the other viruses
572 ($P < 0.01$). No significant difference between Oshima-IC-pt and each chimeric virus was observed at
573 24h and 48h post-infection. (c) Survival of mice inoculated with Sofjin-IC-pt, Oshima-IC-pt and the
574 chimeric viruses. Mice were inoculated subcutaneously with 1,000 pfu of Sofjin-IC-pt (closed

575 square), Oshima-IC/NS5-778L (closed triangle), Oshima-IC/NS5-827S (open triangle),
576 Oshima-IC/NS5-832A (closed diamond), Oshima-IC/NS5-862K (open diamond),
577 Oshima-IC/sofjin3'-UTR_vari (open square), Oshima-IC/sofjin3'-UTR_core (open circle), and
578 Oshima-IC-pt (closed circle).

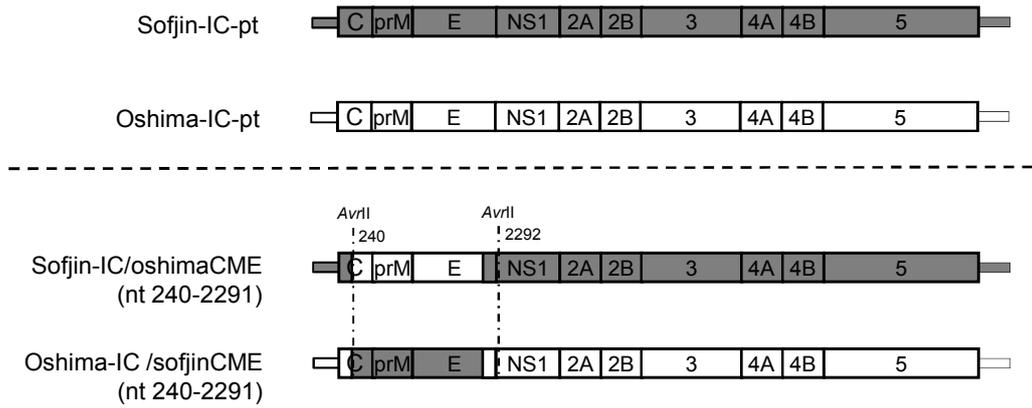
579

580 **Fig. 5** Effects of the replacement of the variable region on viral multiplication in organs. Mice were
581 infected with 1,000 pfu of Sofjin-IC-pt, Oshima/sofjin_3'-UTR vari, and Oshima-IC-pt. Virus titers
582 in the blood (a), spleen (b), and brain (c) at the indicated days after infection were determined by
583 plaque assays. The horizontal dashed lines indicate the limits of detection for the assay (100 pfu
584 mL⁻¹). Error bars represent the SD (n=3). An asterisk (*) or dagger (†) denotes a significant
585 difference compared with Oshima-IC-pt or Sofjin-IC-pt, respectively (P <0.05).

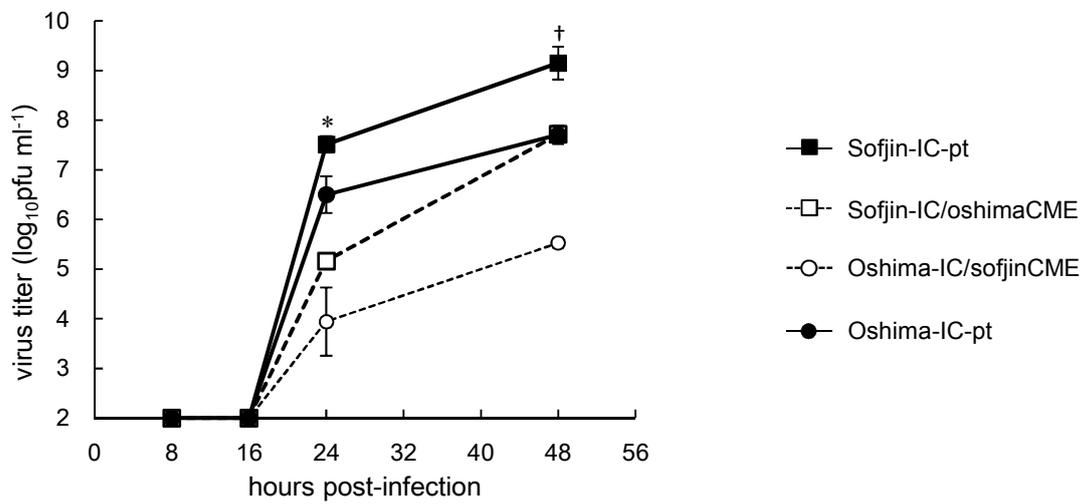
586

587 **Fig. 6** Histopathological features in the brain of mice at 9 days after subcutaneous infection. Mice
588 were infected with 10³pfu of Oshima-IC-pt ((a), (b)) or Sofjin-IC-pt ((c), (d)) and
589 Oshima/3'-UTR_vari ((e), (f)). TBEV antigens were detected using E-protein-specific antibodies
590 (brown signal in left columns). Non-suppurative encephalitis including perivascular cuffing
591 (arrowhead) was observed in mice infected with each virus (right columns).

(a)



(b)



(c)

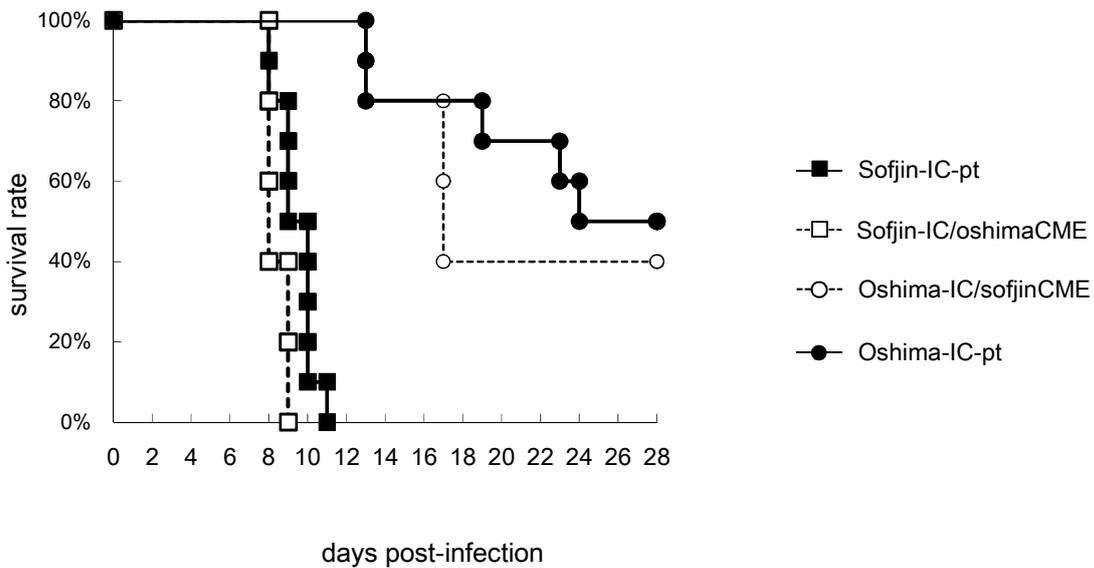
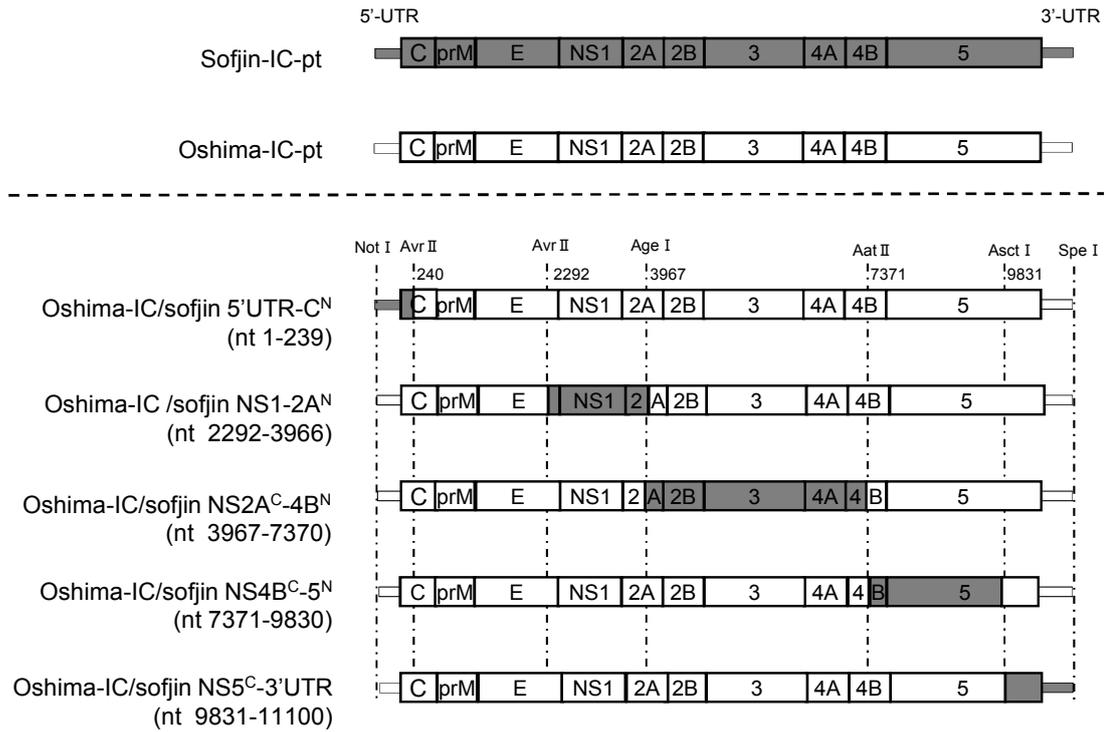
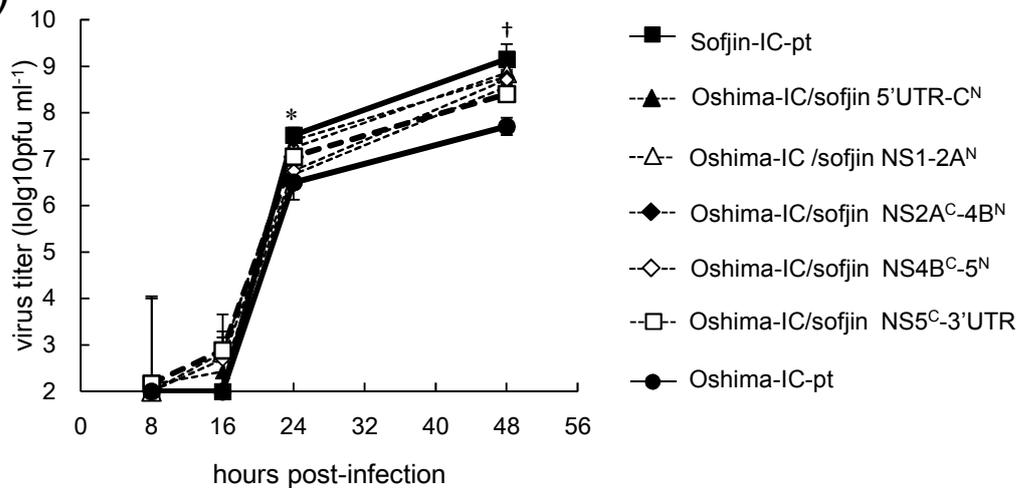


Fig. 1

(a)



(b)



(c)

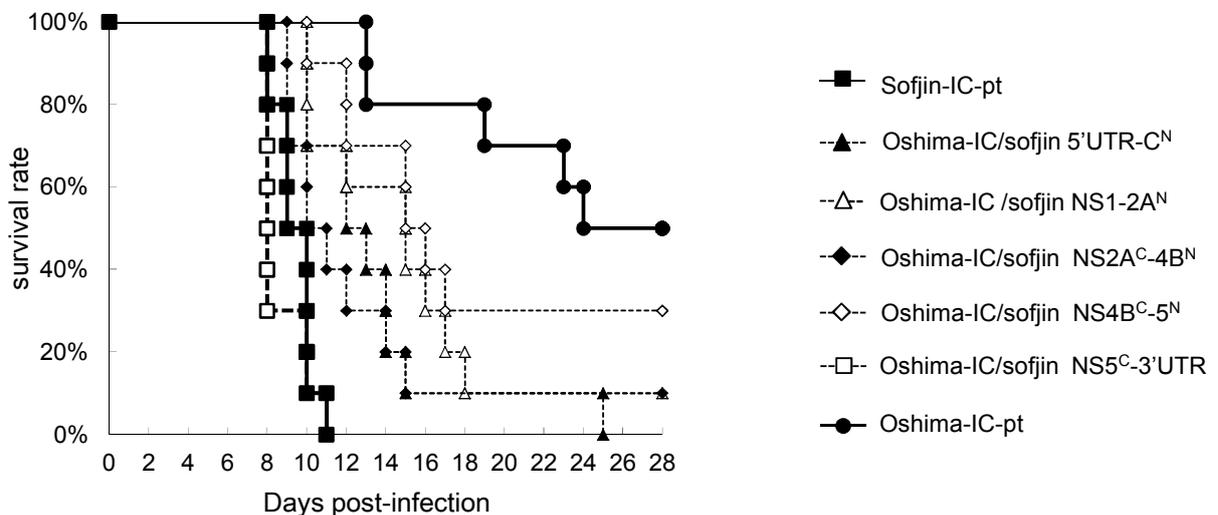
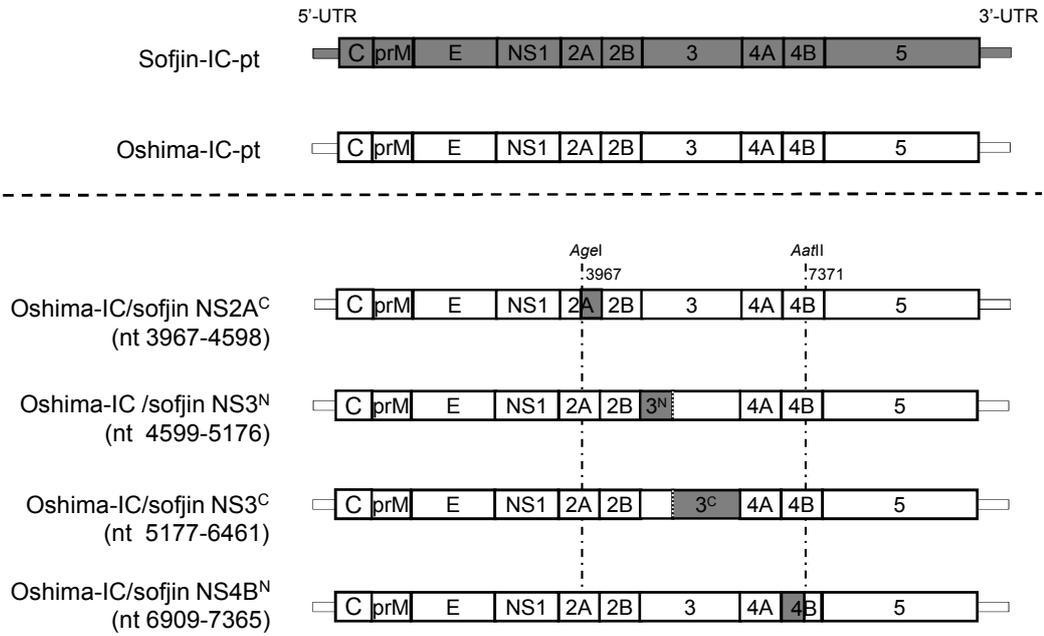
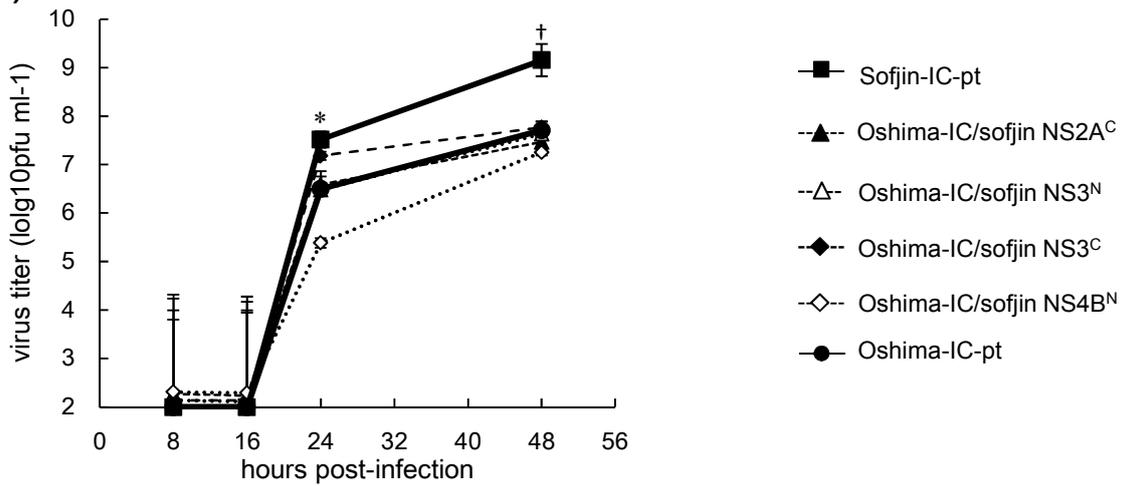


Fig.2

(a)



(b)



(c)

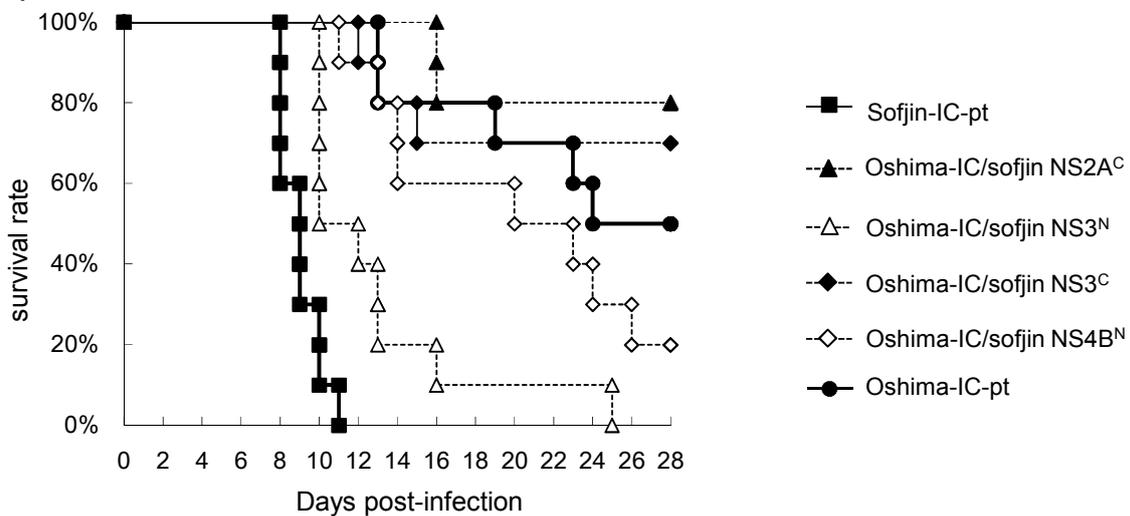


Fig.3

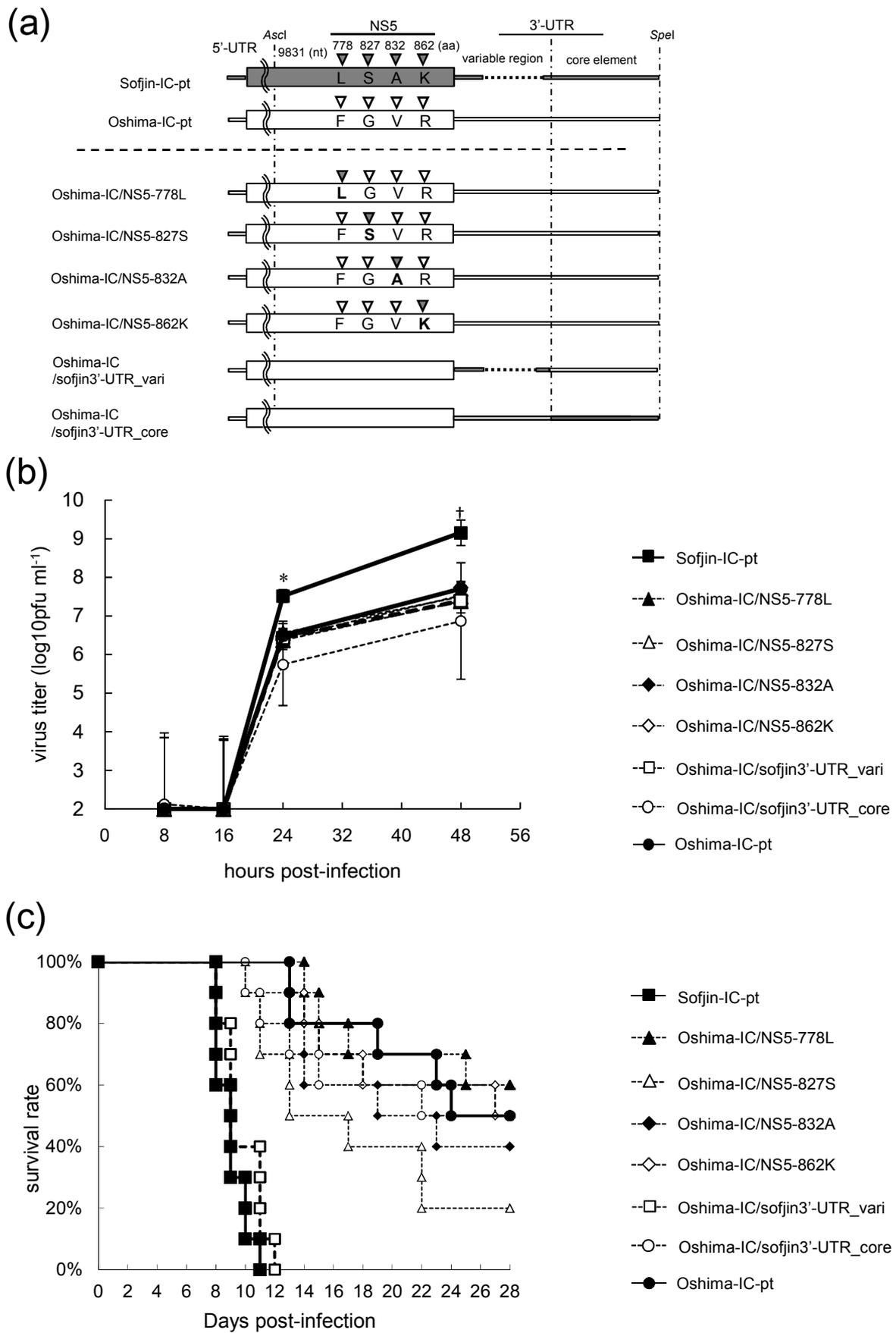


Fig.4

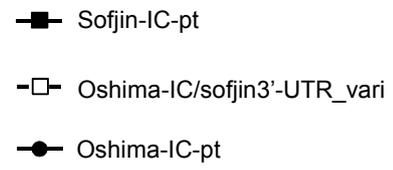
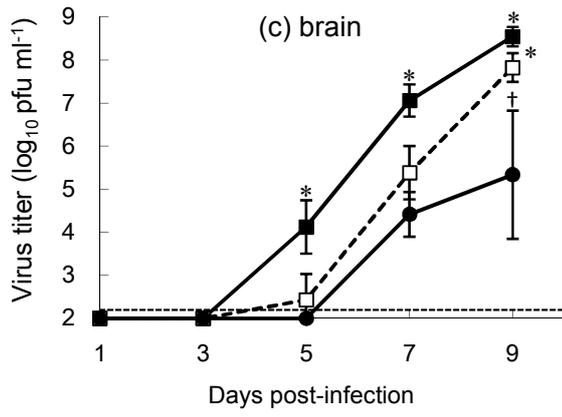
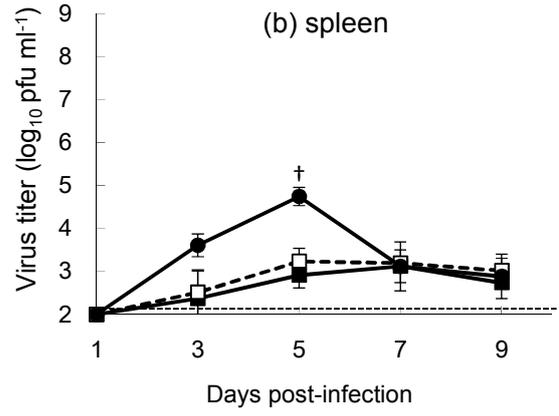
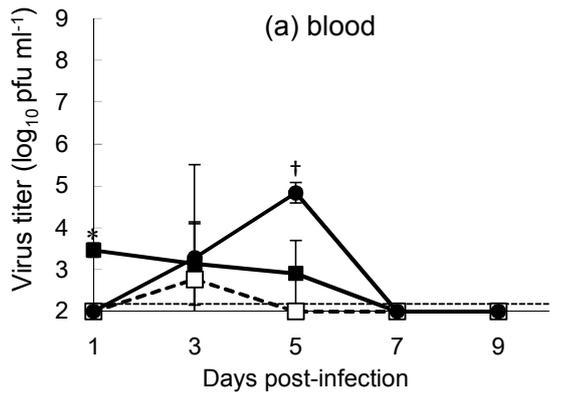


Fig.5

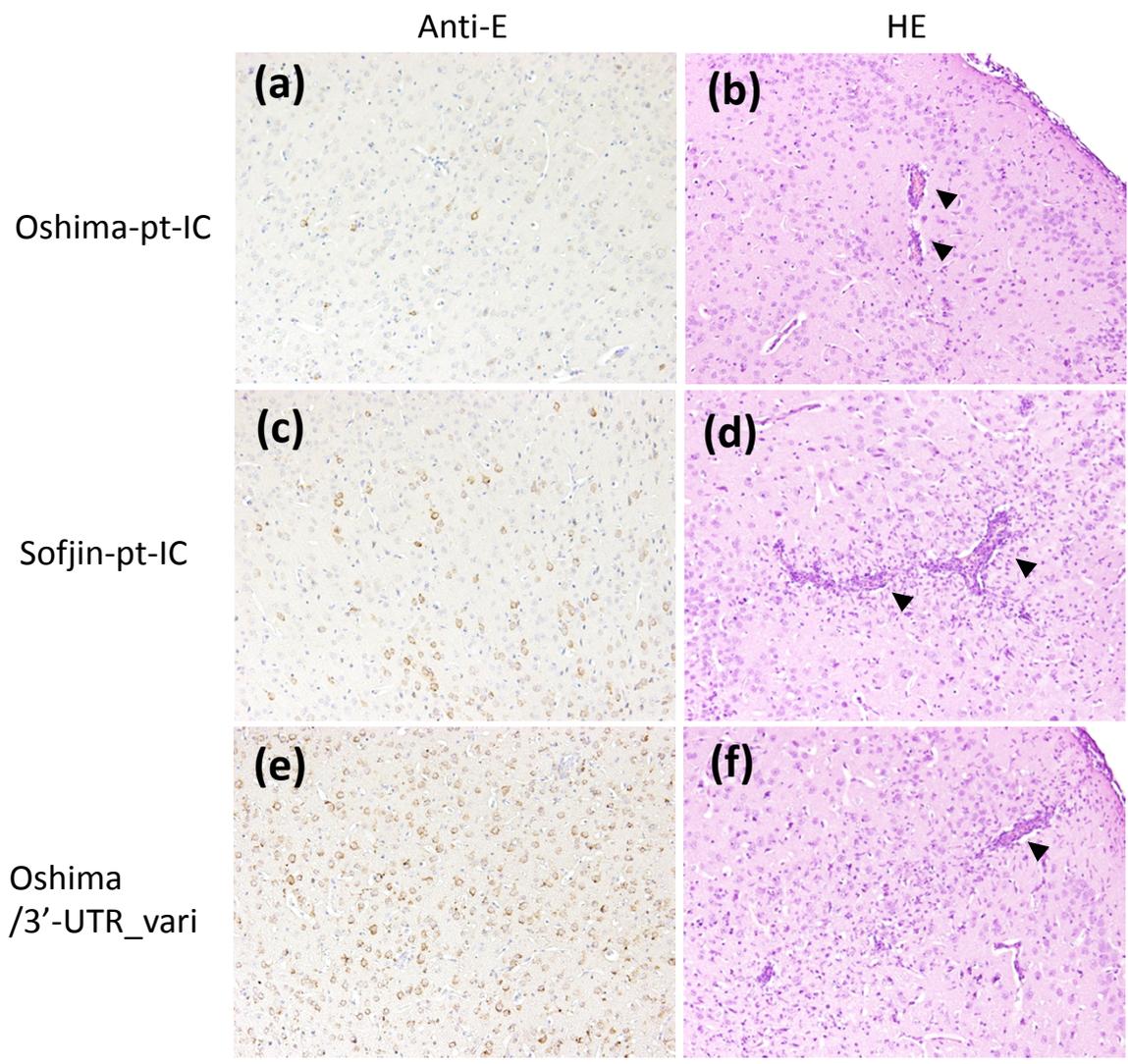


Fig.6