A Molecularly Cloned, Live-Attenuated Japanese Encephalitis Vaccine SA14-14-2 Virus: A Conserved Single Amino Acid in the "ij" Hairpin of the Viral E Glycoprotein Determines Neurovirulence in Mice

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Abstract

Japanese encephalitis virus (JEV), a mosquito-borne flavivirus that causes fatal neurological disease in humans, is one of the most important emerging pathogens of public health significance. JEV represents the JE serogroup, which also includes West Nile, Murray Valley encephalitis, and St. Louis encephalitis viruses. Within this serogroup, JEV is a vaccine-preventable pathogen, but the molecular basis of its neurovirulence remains unknown. Here, we constructed an infectious cDNA of the most widely used live-attenuated JE vaccine, SA14-14-2, and rescued from the cDNA a molecularly cloned virus, SA14-14-2MCV, which displayed in vitro growth properties and in vivo attenuation phenotypes identical to those of its parent, SA14-14-2. To elucidate the molecular mechanism of neurovirulence, we selected three independent, highly neurovirulent variants (LD50 <1.5 PFU) from SA14-14-2MCV (LD50 >1.5×10^5 PFU) by serial intracerebral passage in mice. Complete genome sequence comparison revealed a total of eight point mutations, with a common single G1709A—A substitution replacing a Gly with Glu at position 244 of the viral E glycoprotein. Using our infectious SA14-14-2 cDNA technology, we showed that this single Gly-to-Glu change at E-244 is sufficient to confer lethal neurovirulence in mice, including rapid development of viral spread and tissue inflammation in the central nervous system. Comprehensive site-directed mutagenesis of E-244, coupled with homology-based structure modeling, demonstrated a novel essential regulatory role in JEV neurovirulence for E-244, within the "ij" hairpin of the E dimerization domain. In both mouse and human neuronal cells, we further showed that the E-244 mutation altered JEV infectivity in vitro, in direct correlation with the level of neurovirulence in vivo, but had no significant impact on viral RNA replication. Our results provide a crucial step toward developing novel therapeutic and preventive strategies against JEV and possibly other encephalitic flaviviruses.

Introduction

Japanese encephalitis virus (JEV) is the most common cause of viral encephalitis in Asia and parts of the Western Pacific, with ~60% of the world’s population at risk of infection [1]. Within the family Flaviviridae (genus Flavivirus), JEV belongs to the JE serological group, which also includes medically important human pathogens found on every continent except Antarctica [2,3]: West Nile virus (WNV), St. Louis encephalitis virus (SLEV), and Murray Valley encephalitis virus (MVEV). Historically, the JE serological group members have clustered in geographically distinct locations, but the recent emergence and spread of JEV in Australia [4] and WNV in North America [5,6] have caused growing concern that these viruses can spread into new territory, posing a significant challenge for global public health [3,7]. In the US, where WNV and SLEV are endemic, the situation is particularly problematic because the likelihood of JEV being introduced is considerable [8,9]. Worldwide, ~50,000–175,000 clinical cases of JEV are estimated to occur annually [10]; however, this incidence is undoubtedly a considerable underestimate because surveillance and reporting are inadequate in most endemic areas, and only ~0.1–4% of JEV-infected people develop clinical disease [11,12]. On average, ~20–30% of patients die, and ~30–50% of survivors suffer from irreversible neurological and/or psychiatric sequelae.
Author Summary

A group of mosquito-borne flaviviruses that cause fatal encephalitis in humans is among the most important of all emerging human pathogens of global significance. This group includes Japanese encephalitis (JE), West Nile, St. Louis encephalitis, and Murray Valley encephalitis viruses. In this work, we have developed a reverse genetics system for SA14-14-2, a live JE vaccine that is most commonly used in JE-endemic areas, by constructing an infectious bacterial artificial chromosome that contains the full-length SA14-14-2 cDNA. Using this infectious SA14-14-2 cDNA, combined with a mouse model for JEV infection, we have identified a key viral neurovirulence factor, a conserved single amino acid in the γ hairpin adjacent to the fusion loop of the viral E glycoprotein, which regulates viral infectivity into neurons within the central nervous system in vivo and neuronal cells of mouse and human in vitro. Thus, our findings elucidate the molecular basis of the neurovirulence caused by JE and other closely related encephalitic flaviviruses, a major step in understanding their neuro-pathogenesis. From a clinical perspective, the discovery of the viral neurovirulence factor and its role will have direct application to the design of a novel class of broad-spectrum antivirals to treat and prevent infection of JEV and other taxonomically related neurotropic flaviruses.

JEV is maintained in an enzootic cycle involving multiple species of mosquito vectors (primarily Culex species) and vertebrate hosts/reservoirs (mainly domestic pigs/wading birds). Humans become infected incidentally when bitten by an infected mosquito [40]. In the absence of antiviral therapy, active immunization is the only strategy for sustainable long-term protection. Four types of JE vaccines are used in different parts of the world [49,50]: (i) the mouse brain-derived inactivated vaccine based on the Nakayama or Beijing-1 strain, (ii) the cell culture-derived inactivated vaccine based on the Beijing-3 or SA14-14-2 strain, (iii) the cell culture-derived live-attenuated vaccine based on the SA14-14-2 strain, and (iv) the live chimeric vaccine developed on a yellow fever virus (YFV) 17D genetic background that carries two surface proteins of JEV SA14-14-2. Of the four vaccines, the only one that is available internationally is the mouse-brain-derived inactivated Nakayama [11]. Unfortunately, the production of this vaccine was discontinued in 2006 [51] because of vaccine-related adverse events, short-term immunity, and high production cost [13,52]. To date, the most commonly used vaccine in Asia is the live-attenuated SA14-14-2 [53], but this vaccine is not recommended by the WHO for global immunization [13,54]. In addition to the dependence of the duration of immunity on the number of doses received, there is at least a theoretical risk of virus mutation and reversion of the vaccine virus to high virulence. Recently, the SA14-14-2 vaccine virus has been utilized to produce a new Vero cell-derived inactivated vaccine that has been approved in the US, Europe, Canada, and Australia since 2009 [51,55,56]. In the US, this vaccine is recommended for adults aged ≥17 years travelling to JEV-endemic countries and at risk of JEV exposure [51,57], but no vaccine is currently available for children under 17 [58]. More recently, the prM and E genes of JEV SA14-14-2 have been used to replace the corresponding genes of YFV 17D [59], creating a live chimeric vaccine [60] that is now licensed in Australia and Thailand [61,62]. Thus, the application of JEV SA14-14-2 to vaccine development and production is continuously expanding, but the viral factors and fundamental mechanisms responsible for its loss of virulence are still elusive.

The virulence of JEV is defined by two properties: (i) neuroinvasiveness, the ability of the virus to enter the central nervous system (CNS) when inoculated by a peripheral route; and (ii) neurovirulence, the ability of the virus to replicate and cause damage within the CNS when inoculated directly into the brain of a host. Over the past 20 years, many investigators have sought to understand the molecular basis of JEV virulence, by using cell and animal infection model systems to compare the nucleotide sequences of the genomes of several JEV strains that differ in virological properties [63–74]. These studies have identified a large number of mutations scattered essentially throughout the entire viral genome. Because of the complexity of the mutations, however, the major genetic determinant(s) critical for either JEV neurovirulence or neuroinvasiveness remains unclear. In particular, the situation is more complicated for the live-attenuated SA14-14-2 virus, which has been reported to have a number of different mutations, i.e., 47–64 nucleotide changes (17–27 amino acid substitutions), when compared to its virulent parental strain SA14; the exact number depends on both the passage history of the viruses and the type of cell substrate used for virus cultivation [63–65]. A more comprehensive sequence comparison with another SA14-derivated attenuated vaccine strain, SA14-2-8, together with two other virulent strains, has suggested seven common amino acid substitutions that may be involved in virus attenuation: 4 in E, 1 in NS2B, 1 in NS3, and 1 in NS4B [64]. However, the genetic component directly responsible for the attenuation of SA14-14-2 is...
still unknown. Given that SA14-14-2 has been administered to >300 million children for >20 years in China and recently in other Asian countries [33], it is striking that there is a fundamental gap in our knowledge at the molecular level about how SA14-14-2 is attenuated.

Here we report the development of an infectious cDNA-based reverse genetics system for JEV SA14-14-2 that has enabled the analysis of molecular aspects of its attenuation in neurovirulence. By in vitro passage of a molecularly defined, cDNA-derived SA14-14-2 virus, we generated three isogenic variants, each displaying lethal neurovirulence in mice, with a common single G\(^{3708}\)A substitution that corresponds to a Gly\(\rightarrow\)Glu change at position 244 of the viral E glycoprotein. By in vitro site-directed mutagenesis of the infectious SA14-14-2 cDNA, coupled with conventional virologic and experimental pathologic methods and homology-based structure modeling, we have demonstrated a novel regulatory role in JEV neurovirulence of a conserved single amino acid at position E-244 in the \(\alpha\) hairpin adjacent to the fusion loop of the E dimerization domain. These findings offer new insights into the molecular mechanism of JEV neurovirulence and will directly aid the development of new approaches to treating and preventing JEV infection.

Results

Development of a full-length infectious cDNA of SA14-14-2, a live JE vaccine virus

As an initial step in investigating the molecular basis for the virulence attenuation of SA14-14-2, we generated a full-length infectious SA14-14-2 cDNA to serve as a template for genetic manipulation of the viral genome (Fig. 1A). The 10,977-nucleotide genomic SA14-14-2 (GenBank accession number JN604986) was first cloned as four contiguous cDNAs into the bacterial artificial chromosome (BAC) designated pBAC/SA14-14-2 (Fig. 1B). pBAC/SA14-14-2 was modified to have an SP6 promoter immediately upstream of the viral 5'-end, allowing \(\text{in vitro}\) run-off transcription in the presence of the m7G(5'-ppp(5')ppp(5')A cap structure analog. Transcription of the synthetic RNAs into BHK-21 cells gave specific infectivities of 6.0–7.5 \(\times\) 10\(^6\) PFU/mg; the virus titers recovered from the RNA-transfected cells were 3.0–4.5 \(\times\) 10\(^6\) PFU/ml at 22 h post-transfection (hpt) and increased \(\sim\)10-fold to 2.9–3.7 \(\times\) 10\(^6\) PFU/ml at 40 hpt (Fig. 1D). Unequivocally, the recovered virus contained the marker mutation (A\(^{9134}\)T) that had been introduced in pBAC/SA14-14-2 (data not shown). Our results show that the synthetic RNAs generated from the full-length SA14-14-2 cDNA are highly infectious in BHK-21 cells, producing a high titer of molecularly defined, infectious virus.

In cell cultures [75, 76], we assessed the \(\text{in vitro}\) growth properties of the molecularly cloned virus (SA14-14-2\(^{\text{SA14-2MCV}}\)) rescued from the infectious cDNA, as compared to those of the uncloned parental virus (SA14-14-2) used for cDNA construction. In hamster kidney BHK-21 cells, which are used most frequently for JEV propagation in laboratories, SA14-14-2\(^{\text{SA14-2MCV}}\) replicated as efficiently as SA14-14-2, with no noticeable difference in the accumulation of viral genomic RNA (Fig. 2A) and proteins (Fig. 2B) over the first 24 h after infection at a multiplicity of infection (MOI) of 1 plaque-forming unit (PFU) per cell. These observations were consistent with their growth kinetics, which were essentially identical for 4 days following infection at three different MOIs: 0.1, 1, and 10 PFU/cell (Fig. 2C and data not shown). Similarly, there was no difference in focus/plaque morphology between SA14-14-2\(^{\text{SA14-2MCV}}\) and SA14-14-2 at 4 days post-infection (dpi) (Fig. 2D); as expected, their foci/plaques were \(\sim\)30% smaller than those produced by CNU/LP2, a virulent JEV strain used as a reference (Fig. S1). Also, their growth properties were equivalent in two other cell lines, human neuroblastoma SH-SY5Y and mosquito C6/36 cells, which are potentially relevant to JEV pathogenesis and transmission, respectively (Fig. S2). These data suggest that the uncloned parental and molecularly cloned viruses are indistinguishable in viral replication and spread in both mammalian and insect cells.

In mice [75, 77], we evaluated \(\text{in vivo}\) the attenuation phenotypes of SA14-14-2\(^{\text{SA14-2MCV}}\) and SA14-14-2, with a virulent JEV CNU/LP2 [78] in parallel. Groups of 3-week-old ICR mice (\(n = 20\)) were infected with various doses (1.5 to 1.5 \(\times\) 10\(^8\) PFU/mouse) of each virus, via three different inoculation routes: intracerebral (IC) for neurovirulence, and intramuscular (IM) and intraperitoneal (IP) for neuroinvasiveness. As with SA14-14-2, the 50% lethal doses (LD\(_{50}\)) of SA14-14-2\(^{\text{SA14-2MCV}}\), regardless of the route of inoculation, were all 
\(\sim 1.5 \times 10^5\) PFU (Figs. 2E and S3). Specifically, all mice infected with SA14-14-2\(^{\text{SA14-2MCV}}\) or SA14-14-2 remained healthy and displayed no clinical signs of JEV infection (e.g., ruffled fur, hunched posture, tremors, or hindlimb paralysis) after IM or IP inoculation with any of the tested doses; on the other hand, a small fraction of the mice infected with SA14-14-2\(^{\text{SA14-2MCV}}\) (5–20%) or SA14-14-2 (5–10%) developed typical symptoms, and death after the IC inoculation with a relatively high dose of \(\geq 1.5 \times 10^5\) PFU/mouse, but not the low dose of \(\leq 1.5 \times 10^6\) PFU/mouse (Fig. S3). In all dead or surviving mice, virus titration confirmed the presence (1.8–4.1 \(\times\) 10\(^6\) PFU/brain) or absence, respectively, of viral replication in their brain tissues. As expected [75, 77], the LD\(_{50}\) values of CNU/LP2 [78], irrespective of the inoculation route, were always \(< 1.5\) PFU (Figs. 2E and S3); the control groups of mock-infected mice all survived with no signs of disease (Fig. S3). Thus, our data indicate that SA14-14-2\(^{\text{SA14-2MCV}}\) displays a variety of biological properties identical to those of SA14-14-2, both \(\text{in vitro}\) and \(\text{in vivo}\).

Generation of three highly neurovirulent variants derived from SA14-14-2\(^{\text{SA14-2MCV}}\)

As was true for SA14-14-2, direct inoculation of a relatively high dose of SA14-14-2\(^{\text{SA14-2MCV}}\) into mouse brains initiated a productive infection in the CNS and caused lethal encephalitis, albeit at a very low frequency (Fig. S3). Intrigued by this observation, we decided to generate isogenic neurovirulent variants from SA14-14-2\(^{\text{SA14-2MCV}}\) by serial brain-to-brain passage in mice (Fig. 3A). At passage 1 (P1), the cDNA-derived SA14-14-2\(^{\text{SA14-2MCV}}\) was directly inoculated into the brains of 3-week-old ICR mice at 1.5 \(\times\) 10\(^8\) PFU/mouse (three groups, \(n = 10\) per group); one or two infected mice per group exhibited clinical symptoms of JEV infection. At the onset of hindlimb paralysis
(6–10 dpi), virus was harvested from the brain of a moribund mouse in each group (3 total); in each case, a brain homogenate was prepared for plaque titration and used as an inoculum for the next round of passage. Serial intracerebral passage was continued for three additional rounds, with a gradually decreasing inoculum in order to ensure the stability of selected mutations and a sufficiently pure population of viruses: 1,500 (P2), to 15 (P3), to 1.5 PFU/mouse (P4). Using this approach, we obtained three independently selected variants, SA14-14-2MCV/V1 to V3 (Fig. 3A).

We first compared the biological properties of the three SA14-14-2MCV variants, both in vitro and in vivo, to those of the parental SA14-14-2MCV. In three cell cultures (BHK-21, SH-SY5Y, and C6/36), all three variants exhibited characteristics of viral replication identical to the parent, as demonstrated by (i) quantitative real-time RT-PCRs to measure the level of viral genomic RNA production, (ii) immunoblotting with a panel of JEV-specific rabbit polyclonal antisera to probe the profile and level of viral structural and nonstructural protein accumulation, and (iii) one-step growth analyses to assess the yield of progeny virions produced during a single round of infection (data not shown). In 3-week-old ICR mice, however, there was a clear difference between the parent and the three variants in both phenotype and virulence level (Fig. 3B). When peripherally inoculated (i.e., IM and IP), neither the parent nor its three variants caused any symptoms or death at a maximum dose of \(1.5 \times 10^5\) PFU/mouse. In contrast, when inoculated IC, the three variants, unlike the parent (IC LD50, \(>1.5 \times 10^5\) PFU), were all highly neurovirulent (IC LD50, <1.5 PFU) (Fig. 3B and Table S1). Our findings show that all three variants still lacked a detectable level of neuroinvasiveness but gained a high level of neurovirulence after serial IC passage in mice.

Next, we determined the complete nucleotide sequence of the genome of the three SA14-14-2MCV variants to identify the nucleotide(s) and/or amino acid(s) in specific viral loci/genes that is(are) potentially responsible for the drastic increase in neurovirulence. According to our protocol [77], the consensus genome sequence of each variant was generated by direct sequencing of three overlapping, uncloned cDNA amplicons covering the entire viral RNA genome except the 5’- and 3’-termini; the remaining consensus sequences of the 5’- and 3’-terminal regions were obtained by 5’- and 3’-RACE reactions, each followed by cDNA cloning and sequencing of 10–15 independent clones. In all three variants, when the consensus genome sequence was compared to that of the parent, a single nucleotide G-to-A transition was always found at nucleotide 1708, changing a Gly (GGG) to Glu (GAG) codon at amino acid 244 of the viral E glycoprotein (Fig. 3C). In addition, each of the three variants also contained a small number of unique silent point mutations scattered over the genome, confirming they were indeed independent variants (Fig. 3C): one in SA14-14-2MCV/V1 (U2580C), two in SA14-14-2MCV/V2 (G317A and U8588C), and four in SA14-14-2MCV/V3 (U419C, C3215U, C5987U, and G6551A). These results suggest that the G1708A substitution, the only mutation observed in all three variants, may contribute to the viral neurovirulence in mice.

Identification of a single Gly-to-Glu change at position E-244 that is responsible for the reversion to neurovirulence

To identify a key point mutation(s) in three variants of SA14-14-2MCV that leads to the acquisition of neurovirulence, we generated eight derivatives of SA14-14-2MCV, each containing one of the
eight point mutations found in our three variants, by cloning them individually into the infectious SA 14-14-2 cDNA and transfecting the synthetic RNAs derived from each mutant cDNA into BHK-21 cells. In all cases, the mutant RNA was as infectious as the parent RNA, with a specific infectivity of 6.4–8.3 $\times 10^5$ PFU/mg; the sizes of the foci/plaques produced by each mutant RNA were indistinguishable from those generated by the parent RNA, paralleling their levels of virus production, with an average yield of 2.1–4.5 $\times 10^5$ PFU/ml at 22 hpt (Fig. 4A). In agreement with these results, no difference was observed in the profile or expression level of the viral proteins, i.e., three structural (C, prM, and E) and one nonstructural (NS1), as determined by immunoblotting of RNA-transfected cells at 18 hpt (Fig. 4B). All the mutant viruses grew as efficiently as did the parental virus over the course of 96 h after infection at an MOI of 0.1 in BHK-21 cells (Fig. 4C). Thus, there was no apparent effect of any of the eight introduced genetic changes on virus replication.

In mice, we examined the neurovirulence of these eight mutant viruses. Groups of 3-week-old ICR mice ($n = 10$ per group) were infected by IC inoculation with various doses (1.5 to $1.5 \times 10^5$ PFU/mouse) of the parent or each mutant virus. One of the eight mutants containing the G 1708A substitution had an IC LD50 of $1.5$ PFU, making it capable of killing all mice within $7$ dpi with a minimum dose of $1.5$ PFU/mouse; the other seven mutants had IC LD50 values all $>1.5 \times 10^5$ PFU and behaved like the parental virus, with only $20\%$ of infected mice developing clinical symptoms and death at a maximum dose of $1.5 \times 10^5$ PFU/mouse (Fig. 4D and Table S2). In all dead or surviving mice, virus titration confirmed the presence (1.4–3.5 $\times 10^6$ PFU/brain) or absence, respectively, of productive viral replication in the brain tissues; as expected, all mock-infected mice survived with no signs of disease (data not shown). Thus, our findings showed that of the eight point mutations, a single G1708A substitution, replacing a Gly with Glu at amino acid residue 244 of the viral E glycoprotein, is sufficient to confer lethal neurovirulence in mice.

To determine whether the mutant G 1708A, unlike the parent SA14-14-2 MCV, is able to replicate and spread in the CNS, we immunohistochemically stained for JEV NS1 antigen in mouse
brains after IC inoculation (Fig. 4E shows hippocampal slides, and Fig. S4 presents slides of other brain areas, i.e., amygdala, cerebral cortex, thalamus, hypothalamus, and brainstem): (i) In brains infected with a virulent JEV CNU/LP2 (control) [75,77,78], a large number of NS1-positive neurons were observed at 3 dpi in all areas we stained; this number was increased significantly at 5 dpi. In the hippocampus, most infected neurons were found in the CA2/3 region at 3 dpi and had spread to the CA1 region by 5 dpi. (ii) In brains infected with the parent SA14-14-2MCV, almost no NS1-positive cells were found in any brain region during the entire 7-day course of the experiment. In a few atypical cases, a small number of NS1-positive neurons were noted at 5–7 dpi in the hippocampal CA2/3 region, but not the CA1 region (data not shown). In brains infected with the mutant G 1708A, a considerable number of NS1-positive neurons were observed at 3 dpi, mainly in the hippocampal CA2/3 region, and only a few in other areas (amygdala, cerebral cortex, thalamus, and brainstem); overall, the number of infected neurons was much lower than in brains infected with JEV CNU/LP2. At 5–7 dpi, the number of NS1-positive neurons was noticeably increased in the hippocampus (now in the CA1) and amygdala, but not in other brain regions. Our findings show that, in mice, a single G 1708A substitution changing a Gly with Glu at position E-244 promotes susceptibility to SA14-14-2MCV infection of neurons.

Understanding the novel regulatory role in neurovirulence of E-244, located in the hairpin of the viral E glycoprotein

To probe the functional importance of the amino acid side chain at position E-244 for the viral replication and neurovirulence of SA14-14-2MCV, we performed site-directed mutagenesis, replacing G 244 with 14 other amino acids of six different classes: (I) aliphatic A, V, and L; (2) hydroxyl S and T; (3) cyclic P; (4) aromatic F and W; (5) basic R and K; and (6) acidic and their amides D, E, N, and Q. We first tested the viability of synthetic RNAs transcribed in vitro from the corresponding mutant cDNAs by measuring their infectivity after transfection of BHK-21 cells. In all cases, the mutant RNA was as viable as the parent RNA, with a specific infectivity of 6.5–8.2×10⁶ PFU/µg (Fig. 5A, RNA infectivity). However, three mutants (G 244K, G 244F, and G 244W) were noticeably different from the parent and the other 11 mutants, as demonstrated by a ~10-fold decrease in the yield of progeny virions released into culture medium during the first 22 hpt (Fig. 5A, virus yield) and a ~2-2.5-fold reduction in the size of foci/plaques produced at 96 hpt (Fig. 5A, foci/plaques), although no significant difference was observed in the level of viral proteins (i.e., C, prM, E, and NS1) accumulated in RNA-transfected cells at 18 hpt (Fig. S5). As compared to G 244K, the mutant G 244R exhibited a barely marginal decrease in focus/plaque size and no detectable change in virus production (Fig. 5A). Overall, these findings were more evident when all mutant viruses were evaluated in multistep growth assays over the course of 96 h after infection at an MOI of 0.1, assessing their ability to grow and establish a productive infection (Fig. 5B). Our findings indicate that in BHK-21 cells, the amino acid side chain at position E-244 has no effect on the viability of the mutant RNAs, although it has a negative impact on the production and spread of infectious virions in the case of the three mutants G 244K, G 244F, and G 244W.

In mice, we determined the neurovirulence of our 14 mutant viruses by IC inoculating groups of 3-week-old ICR mice (n = 10 per group) with various doses ranging from 1.5 to 10⁶ PFU/mouse of the parent or each mutant virus. According to their IC LD₅₀ values, the 14 mutant viruses are classified into three groups (Fig. 5G and Table S3): (i) group 1 (six mutants), neurovirulent, with an IC LD₅₀ of ≤1.5 to 31 PFU, exemplified by substituting G 244 with E, D, T, S, Q, and P; (ii) group 2 (six mutants), non-neurovirulent or neuroattenuated, with an IC LD₅₀ of 10⁴ or 10⁵ PFU, exemplified by substituting G 244 with R, K, F, W, N, and L; and (iii) group 3 (two mutants), with an intermediate phenotype and an IC LD₅₀ of 1.2–31 PFU, exemplified by substituting G 244 with A and V. We confirmed the presence or absence of viral
replication in the brain tissues of all dead or surviving mice, respectively; all mock-infected mice survived with no signs of disease (data not shown). Also, the mutation and phenotype relationship was corroborated by sequence analysis of recovered viruses from brain tissues of moribund or dead mice following IC inoculation. We analyzed all of the 14 mutants except for four group 2 mutants (G244R, G244F, G244W, and G244L), which failed to produce a lethal infection. In each case, the complete 2,001-nucleotide coding region of the prM and E genes was amplified from each of four randomly selected brain samples, followed by cloning and sequencing of at least seven independent clones per brain sample. In all six group 1 and two group 3 mutants, we found that the initial mutations introduced at the G244 codon were maintained with no second-site mutations, consistent with the high and intermediate levels of their neurovirulent phenotype (Table 1). In the remaining two group 2 mutants (G244K and G244N), however, a majority of the sequenced clones contained a point mutation in the same codon that led to an amino acid substitution (i.e., K→E/T and N→D, respectively), converting both mutants into neuroviral viruses and highlighting the biological importance of the amino acid at position E-244 for neurovirulence (Table 1).

We next performed homology modeling to gain insight into the structural basis of E-244 function. The 3D model of the E monomer of JEV SA14-14-2 was constructed using the 3.0-Å crystal structure of the E monomer of WNV NY99 [79] as a template, with 75.5% sequence identity. The model was then fitted into the outer layer of the cryo-electron microscopy (EM) structure of WNV NY99 [18], thereby visualizing three monomers placed into an icosahedral asymmetric unit on the viral membrane. In each E monomer of SA14-14-2 containing three domains (DI, DII, and DIII), we noted that E-244 lies within the ij hairpin adjacent to the fusion loop at the tip of DII, with its amino acid side chain pointing toward the viral membrane (Fig. 5D). We also confirmed the location of E-244 in the crystal structure of the E ectodomain of JEV SA14-14-2 [80] that has been described recently (Fig. S6).

Elucidation of the functional role of E-244 during JEV infection of neuronal cells in vitro

We hypothesized that E-244, located at the ij hairpin of the viral E glycoprotein, plays an important role in JEV infection of neuronal cells. To test this hypothesis, we performed multistep
growth assays in two neuronal cells, NSC-34 (mouse motor neuron) and SH-SY5Y (human neuroblastoma), by infecting at an MOI of 0.1 with the non-neurovirulent parent SA 14-14-2MCV and each of the four representative E-244 mutant viruses, i.e., two neurovirulent (G 244E and G 244D) and two non-neurovirulent (G244R and G 244K). In parallel, the non-neuronal BHK-21 cells were also infected for comparison with the same set of five viruses. In NSC-34 cells, while the two neurovirulent viruses grew rapidly and reached their maximum titers of \(1.8 \times 10^6\) PFU/ml at 72–96 hours post-infection (hpi), the three non-neurovirulent viruses, including the parent, all replicated poorly, with peak titers only approaching \(1.0 \times 10^3\) PFU/ml, \(100\)-fold lower than those of the two neurovirulent viruses (Fig. 6A). In SH-SY5Y cells, a similar defect in viral growth was also observed, with a \(\sim 50\)–\(100\)-fold difference in maximum virus titers between the neurovirulent and the non-neurovirulent viruses. In addition, we noted a differential growth defect in the three non-neurovirulent viruses, with G 244R replicating more poorly than the parent but better than G244K (Fig. 6B). In contrast to the pattern of viral growth observed in NSC-34 and SH-SY5Y cells, we found that in BHK-21 cells, only G244K had a noticeable defect in viral growth, with \(\sim 20\)-fold lower peak titers than those of the other four viruses that grew well to maximum titers of \(0.8 \times 10^6\) PFU/ml at 48–72 hpi (Fig. 6C). These data indicate that E-244 plays a crucial role in the productive infection of JEV in neuronal cells.

Subsequently, we examined the infectivity/replicability of the parent and its four E-244 mutant viruses/RNAs in NSC-34 and SH-SY5Y cells, in parallel with BHK-21 cells for comparison. First, virus infectivity was quantified using flow cytometry by infecting the three cell types at an MOI of 1 with each of the five viruses and counting the number of cells stained with a mouse \(\alpha\)-JEV antiserum at 12–15 hpi. In NSC-34 cells, the two non-neurovirulent mutants (G 244R and G 244K) exhibited infectivities nearly identical to that of the non-neurovirulent parent (Fig. 6D), whereas the two neurovirulent mutants (G244E and G 244K) showed infections nearly identical to that of the non-neurovirulent parent (Fig. 6D), whereas the two non-neurovirulent mutants (G244E and G 244D) showed infectivities \(\sim 16\)–\(20\)-fold higher than that of the non-neurovirulent parent. Similarly, the E-244 mutation also altered virus infectivity in SH-SY5Y cells. Specifically, the two neurovirulent mutants had \(\sim 3\)-\(4\)-fold higher infectivities than the non-neurovirulent parent; on the other hand, the two non-neurovirulent mutants displayed even lower infectivities than the parent (G 244R, \(3\)-fold; G244K, \(10\)-fold) (Fig. 6E). In contrast, no significant difference in virus infectivity was observed among all five viruses in BHK-21 cells (Fig. 6F).

Next, the replication efficiency of the viral genomic RNA was quantified by directly transfecting the three cell types with each of the five synthetic RNAs transcribed in vitro from the respective JEV cDNAs and estimating the number of infectious foci stained with the mouse \(\alpha\)-JEV antiserum at 4 days post-transfection (dpi). In each of the three cell types, there was no detectable difference in
the specific infectivity of the five RNAs (NSC-34, Fig. 6G; SH-SY5Y, Fig. 6H; and BHK-21, Fig. 6I). Also, quantitative real-time RT-PCRs indicated that the level of the viral genomic RNAs accumulated in the RNA-transfected cells over the first 15 h of transfection was indistinguishable between the parent and the four different E-244 mutants (data not shown). Regardless of cell type, however, the G244K mutant was different from the parent and the other three E-244 mutants, as demonstrated by a ~1-log decrease in the yield of infectious virions released into culture medium during the first 20 hpt (NSC-34, Fig. 6J; SH-SY5Y, Fig. 6K; and BHK-21, Fig. 6L). Overall, these results show that the E-244 mutation alters JEV infectivity in a neuronal cell-specific manner, in agreement with the neurovirulence phenotype observed in mice, and it also affects infectious particle production in a cell type-nonspecific manner.

**Comparison of the amino acid sequences of the ij hairpin in encephalitic and non-encephalitic flaviviruses**

We initially generated a multiple sequence alignment using all 154 full-length JEV genomes available from the GenBank sequence database. Of note is the fact that SA14 and SA14-14-2 have been fully sequenced by three and four independent groups, respectively; their nucleotide and deduced amino acid sequences have been fully sequenced by three and four independent groups, and it also affects infectious particle production in a cell type-nonspecific manner.

**Table 1. Genetic stability of 14 E-244 mutants recovered from mouse brains after IC inoculation.**

| Input virus | Initial codon | Recovered virus | Nucleotide change (amino acid change) | No. of independent clones |
|-------------|---------------|----------------|--------------------------------------|--------------------------|
| A           | G244A         | AAC            | None                                 | 28/28                    |
| N           | G244E         | GAG            | None                                 | 30/30                    |
| A           | G244F         | UUC            | None                                 | 30/30                    |
| A           | G244G         | GAC            | None                                 | 30/30                    |
| A           | G244H         | AGA            | None                                 | 30/30                    |
| A           | G244I         | AAG            | None                                 | 30/30                    |
| A           | G244J         | UUC            | None                                 | 30/30                    |
| A           | G244K         | UUC            | None                                 | 30/30                    |
| A           | G244L         | CAG            | None                                 | 30/30                    |
| A           | G244M         | CAG            | None                                 | 30/30                    |
| A           | G244N         | CAG            | None                                 | 30/30                    |
| A           | G244O         | CUG            | None                                 | 30/30                    |
| A           | G244P         | CAC            | None                                 | 30/30                    |
| A           | G244Q         | CAG            | None                                 | 30/30                    |
| A           | G244R         | AAC            | None                                 | 30/30                    |
| A           | G244S         | AAC            | None                                 | 30/30                    |
| A           | G244T         | AAC            | None                                 | 30/30                    |
| A           | G244U         | AAC            | None                                 | 30/30                    |
| A           | G244V         | AAC            | None                                 | 30/30                    |
| A           | G244W         | AAC            | None                                 | 30/30                    |
| A           | G244X         | AAC            | None                                 | 30/30                    |
| A           | G244Y         | AAC            | None                                 | 30/30                    |
| A           | G244Z         | AAC            | None                                 | 30/30                    |

A, Attenuated; I, Intermediate; N, Neurovirulent.
N.A., Not available.

Discussion

In this work, we have developed a reverse genetics system for SA14-14-2, a live-attenuated JE vaccine [53,82], by constructing an infectious cDNA and rescuing molecularly cloned virus from the cDNA. This reverse genetics system offers us a unique opportunity to elucidate the genetic and molecular basis of JEV neurovirulence. Using our infectious SA14-14-2 cDNA technology, we (i) generated three isogenic SA14-14-2 variants that unlike its parent, displayed lethal neurovirulence in mice; (ii) identified a single point mutation, G244K, causing a Gly→Glu change at position 244 of the viral E glycoprotein that is sufficient to confer a full neurovirulence by promoting viral infection into neurons in the mouse CNS in vivo and mouse/human neuronal cells in vitro;
and (iii) demonstrated the structure-function relationship for neurovirulence of E-244 in the α-hairpin adjacent to the fusion loop at the tip of the viral E DII. Thus, our findings reveal fundamental insights into the neurotropism and neurovirulence of JEV and other taxonomically related encephalitic flaviviruses, including WNV, SLEV, and MVEV. Intriguingly, our results also provide a new target, the α-hairpin, for the development of novel antivirals for the prevention and treatment of infection with the encephalitic flaviviruses.

The flavivirus glycoprotein E mediates receptor-mediated endocytosis and low pH-triggered membrane fusion [33,83,84]. On the viral membrane, 180 E monomers are packed into 30 protein “rafts”, each composed of three E head-to-tail homodimers [17–19]. Each E monomer is composed of three parts: (i) an elongated ectodomain that directs receptor binding and membrane fusion; (ii) a “stem” region containing two amphipathic α-helices that lies flat on the viral membrane underneath the ectodomain; and (iii) a membrane “anchor” region containing two transmembrane antiparallel coiled-coils. The E ectodomain folds into three β-barrel domains [85]: (i) DI, a structural domain centrally located in the molecule; (ii) DII, an elongated dimerization domain containing the highly conserved fusion loop at its tip [86]; and (iii) DIII, an Ig-like domain implicated in receptor binding [20,87,88] and antibody neutralization [89–92]. Based on pre- and post-fusion crystal structures of the ectodomain [28,30,31,47,79,80,85] and biochemical analyses [29,32,93], a current, detailed model for flavivirus membrane fusion has been developed. In this model, the fusion is initiated by a low pH-induced dissociation of the antiparallel E homodimers that leads to the exposure of the fusion loops and their insertion into the host membrane, followed by a large-scale structural rearrangement into a parallel E homotrimer [33,83,84,94]. In the parallel conformation, DIII folds back toward

Figure 6. E-244: A major determinant of viral infectivity in mouse and human neuronal cells in vitro. (A-C) Viral growth. NSC-34 (A), SH-SY5Y (B), or BHK-21 (C) cells were infected at an MOI of 0.1 with the parent or one of the following four E-244 mutant viruses: G244E, G244D, G244R, and G244K. At the indicated time points, culture supernatants were harvested for virus titration on BHK-21 cells. (D-F) Viral particle infectivity. NSC-34 (D), SH-SY5Y (E), or BHK-21 (F) cells were infected at an MOI of 1 with each of the five viruses, as indicated. At 12–15 hpi, the number of infected cells was measured by flow cytometry using mouse α-JEV antiserum. The results are the average of two independent experiments, presented as -fold changes in infectivity relative to the parent (infectivity value of 1). (G-I) Viral RNA infectivity/replicability. NSC-34 (G), SH-SY5Y (H), or BHK-21 (I) cells were transfected with RNAs transcribed from the parent or each mutant cDNA, as indicated. At 4 dpt, RNA infectivity was quantified by infectious center assay, coupled with staining of cell monolayers using an α-JEV antiserum. (J-L) Virus yield. At 20 h after RNA transfection, culture supernatants from RNA-transfected NSC-34 (J), SH-SY5Y (K), or BHK-21 (L) cells were harvested for virus titration on BHK-21 cells.

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DII, presumably with the stem extended from the C-terminus of DIII along DII and toward the fusion loop (“zipping”), driving the fusion of the viral and host membranes [95–98]. Despite our detailed knowledge about the fusion process, there is little available structural information about how flaviviruses bind to their cellular receptors. In encephalitic flaviviruses, the presence of an RGD motif in DIII and carbohydrate moieties on the viral surface suggests a mechanism involving interaction with the RGD motif-recognizing integrins and sugar-binding lectins on the cell surface, respectively. However, blocking/alteration of either the RGD motif or glycan does not abolish viral entry [22,99–101]. Thus, the viral factors and the interacting cellular counterparts required for viral entry are still elusive.

In flaviviruses, the $ij$ hairpin is a structural motif that is closely associated with the fusion loop at the tip of the viral E DII, but its role is thus far unknown. In JEV, we now report that a single amino acid in the $ij$ hairpin, E-244, serves as a key regulator to control the level of neurovirulence of SA14-14-2 in mice. This amino acid was also correlated with a differential ability to infect neurons, the primary target cells in the CNS. Consistent with this finding, we found that site-directed mutagenesis of the codon for the $ij$ hairpin acts as a viral factor that elevates the neurovirulence of the ChimeriVax-JE virus in vivo and/or neuroinvasiveness of JEV [63–74]. Due to the complexity and variation of the properties of its amino acid side chain: (i) charge (R/K vs. E/D); (ii) size (N vs. Q and L vs. A/V); and (iii) functional group (N vs. D). Our data suggest that the $ij$ hairpin acts as a viral factor that promotes JEV infection of neurons within the CNS, likely through its role in one of three major steps involved in viral entry: binding, endocytosis, or membrane fusion [33,83,84]. Alternatively, it is possible that the late steps in the virus life cycle in neurons, such as assembly, maturation, and release, could be affected. For JEV, WNV, and tick-borne encephalitis virus, the assembly/release of infectious virions or virus-like particles has been shown to be affected by the N-glycosylation of the viral prM and/or E protein in non-neuronal cells [44,75,102–104]. Moreover, a conserved single N-glycosylation site in the JEV prM protein has been shown to be important for viral pathogenicity in mice [75].

Over the years, the virulence of JEV has been an active area of research. Initially, comparison of the genomic sequences of several JEV strains with a different degree of pathogenicity had predicted a number of potential loci in the viral genome that are involved in virulence [63–74]. Due to the complexity and variation of the mutations, however, the identity of the major viral factor that is critical for JEV virulence remains unclear. In particular, SA14-14-2 has been reported to have a total of 47–64 nucleotide changes (17–27 amino acid substitutions) when compared to its virulent parental strain SA14; the number of mutations varies and depends on the cultivation history of the viruses [63–65]. Of the ten viral proteins, the E protein has been the primary target of genetic studies in virulence, mainly because it is involved in cell/tissue tropism and pathogenesis. Several amino acid residues in the E protein have been suggested to contribute to the neurovirulence and/or neuroinvasiveness of JEV $m$ vivo: (i) E-123, illustrated by an S123R substitution that is capable of enhancing the neuroinvasiveness of the Mic/41/2002 strain in 3-week-old ddY mice [105]; (ii) E-279, exemplified by an M279K mutation that is able to increase the neurovirulence of ChimeriVax-JE (a chimeric virus that carries the prM and E genes of JEV SA14-14-2 on a YFV 17D genetic background) in suckling mice and rhesus monkeys [106]; and (iii) E-138, indicated by two reciprocal mutations: (1) a K138E substitution, when combined with at least two other mutations, which elevates the neurovirulence of the ChimeriVax-JE virus in 4-week-old ICR mice [107], and (2) an E138K substitution, which

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**Figure 7. Structure-based, $ij$-hairpin amino acid sequence alignment for six representative flaviviruses (14 strains total): JEV, WNV, SLEV, MVEV, YFV, and DENV.** All sequence alignments were performed using ClustalX [116]. Highlighted are the 15-aa $ij$-hairpin (yellow), the 3-aa DENV-specific motif (pink), and the 4-aa YFV-specific motif (green). The consensus sequence of the $ij$-hairpin and its flanking region is presented on top, and only differences from that sequence are shown. Deletions are indicated by hyphens. The amino acid residue is numbered based on the JEV SA14-14-2 (GenBank accession no. JN604986).

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lowers the neurovirulence and neuroinvasiveness of three different JEVs (the JLaArS902 strain in 2- to 3-week-old Swiss ICR mice [73] and the AT31 and NT109 strains in 3-week-old BALB/c mice [108,109]). These data indicate that multiple amino acid residues in the E protein of JEV function in a more coordinated way to achieve the maximal level of neurovirulence and/or neuroinvasiveness [53,107]. This notion is consistent with our finding that although a single G244E mutation in the E protein of SA14-14-2 is sufficient to confer lethal neurovirulence in 3-week-old ICR mice, the spread of the virus in the brains is still slow and limited, as compared to the highly virulent CNU/LP2 strain. These and previous findings suggest that in addition to E-244, other amino acid residues in the E protein play a role in determining the neurovirulence of SA14-14-2. In addition, JEV NS1' (a product of ribosomal frameshifting [110,111]) is reported to be produced in cells infected with SA14 but not with SA14-14-2, and its lack of expression is shown to contribute to the attenuation phenotype of SA14-14-2 in mice [112]. Similarly, the expression of NS1 is also suggested to be associated with the neuroinvasiveness of WNV [110].

The neuroattenuation phenotype of SA14-14-2 has been tested in several laboratory animals, including mice and monkeys [53]. In 2- to 4-week-old ICR and ddY mice, no morbidity or mortality has been observed after subcutaneous and intracerebral inoculations with 10^4-10^6 PFU of SA14-14-2 [53,63,113]; in a rare case, however, the virus was found to be able to cause the death of the mice following IC inoculation [113]. In line with these previous results, we also found that none of the 3-week-old ICR mice inoculated IM or IP with up to ~10^5 PFU of SA14-14-2 showed clinical signs or death; on the other hand, although there was some variability among the groups of mice and the doses of virus inoculum, ~5–30% of the mice inoculated IC with a dose of 10^3–10^5 PFU developed JEV-specific symptoms and death. This low but unexpected morbidity and mortality after the IC inoculation of SA14-14-2 is likely caused by a combination of factors and conditions imposed on our infection experiments, particularly the age and strain of mice: (i) Age-dependent susceptibility of flaviviruses, including JEV, in the murine model has been documented previously, although its molecular mechanisms remain unclear [106,114,115]. (ii) A noticeable variability in mortality has also been reported when two different lineages of the age-matched outbred ICR mice are inoculated IC with a mutant of ChimeriVax-JE that contains two amino acid substitutions (F107L and K138E) in the SA14-14-2 E protein-coding region, suggesting that differences in the genetic background of mice may account for the variable neurovirulence [107]. More importantly, it should be pointed out that in our study, all the revertants recovered from the mice inoculated IC with SA14-14-2 appeared to have the G244E mutation, which is sufficient to confer lethal neurovirulence to the virus, corroborating that the parental SA14-14-2 virus is highly attenuated in neurovirulence. Furthermore, it is intriguing to note that the G244E substitution has been introduced into ChimeriVax-JE, in which no mortality occurs when eight 4-week-old ICR mice are injected IC with 10^5 PFU of the mutant virus [107]; therefore, it appears that neurovirulence may depend on the genetic background of the pathogen. Further investigation is needed to fully elucidate the neurovirulence and neuroinvasiveness of JEV.

In summary, we show for the first time that the E-244 in the ij hairpin of the viral E DII is a key regulator determining the neurovirulence of SA14-14-2, and we also provide direct evidence that viral E can contribute to the neurovirulence of JEV and possibly other closely related encephalitic flaviviruses via its role in the early or late stage of viral replication in neurons. A detailed, complete understanding of the evolutionarily conserved viral ij hairpin and its function in the virus life cycle will have direct application to the design of a novel and promising class of broad-spectrum antivirals (e.g., ligands and small molecules) to expand the currently available preventive and therapeutic arsenal against infection with encephalitic flaviviruses.

**Materials and Methods**

**Viruses and cells**

An original stock of JEV SA14-14-2 was retrieved directly from a batch of commercial vaccine vials (Chengdu Institute of Biological Products, China) for viral genome sequencing and cDNA construction, to avoid any potential concern that its adaptation could occur during propagation in cell culture. This virus stock was propagated twice in BHK-21 cells to generate high-titer viral preparations for cell and mouse infection experiments. Stocks of JEV CNU/LP2 were derived from the infectious cDNA pBAC965/JVFttx/Xba1 [78]. BHK-21 cells were grown in alpha minimal essential medium (α-MEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, vitamins, and penicillin-streptomycin at 37°C in 5% CO2 [78]. SH-SY5Y cells were cultivated in a 1:1 mixture of MEM and Ham’s F-12 nutrient mix supplemented with 10% FBS, 0.1 mM nonessential amino acids, and penicillin-streptomycin at 37°C in 5% CO2 [75]. NSC-34 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% FBS and penicillin-streptomycin at 37°C in 5% CO2.

**JEV reverse genetics**

As a vector, we used the BAC plasmid pBeloBAC11 [78]. First, four cDNA fragments covering the entire viral genome were cloned into the vector individually, then joined sequentially at three natural restriction sites (BsrGI, BamHI, and XbaI) to generate a single BAC clone that contained the full-length SA14-14-2 cDNA, named pBAC/SA14-14-2 (Fig. 1). The SP6 promoter sequence was positioned just upstream of the viral 5’-end, and an artificial XbaI run-off site was engineered just downstream of the viral 3’-end. A pre-existing XbaI site at nucleotide 9131 was removed by introducing a silent point mutation (A9131G→T); this mutation also served as a rescue marker to identify the cDNA-derived SA14-14-2. All mutations were created by overlap extension PCR. All PCR-generated fragments were sequenced. Detailed cloning procedures are described in Supporting Information.

**RNA transcription and transfection**

All BAC plasmids were purified by centrifugation using CsCl-ethidium bromide equilibrium density gradients. The closed circular plasmids were linearized by XbaI and mung bean nuclease digestion to produce DNA templates for in vitro run-off transcription. RNA was transcribed from a linearized plasmid with SP6 RNA polymerase as described [78]. The resulting RNA was stored at −80°C until needed. RNA yield was measured on the basis of the incorporation rate of [3H]UTP, and RNA integrity was evaluated by agarose gel electrophoresis. RNA was transfected by electroporation into cells under our optimized conditions (980 V, a 99-μs pulse length, and five pulses for BHK-21 cells; and 760 V, a 99-μs pulse length, and five pulses for NSC-34 and SH-SY5Y cells) [78]. RNA infectivity was determined by infectious center assay as reported [78]. The infectious centers of foci were detected by decorating of cells with a mouse α-JEV antibody (American Type Culture Collection [ATCC], 1:500) and a horseradish peroxidase-conjugated goat α-mouse IgG (Jackson
ImmunoResearch, 1:1,000), followed by staining with 3,3’-diaminobenzidine (Vector).

**Northern blots**

Total RNA was extracted with TRIzol reagent [Invitrogen]. Northern blot analysis was performed as described [78]. JEV genomic RNA was detected with an antisense riboprobe that binds to a 209-bp region (nt 9143–9351) in the NS5 protein-coding region. The probe was synthesized with [α-32P]CTP by using the T7-MEGAscript kit (Ambion). The blots were prehybridized, hybridized, and washed at 55°C. Autoradiographs were obtained by exposure to film for 24–48 h.

**Immunoblots**

Cells were lysed in sample buffer (80 mM Tri-HCl [pH 6.8], 2.0% SDS, 10% glycerol, 0.1 M dithiothreitol, 0.2% bromophenol blue). Equal amounts of the lysates were run on SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and subjected to immunoblotting as described [78]. The following polyclonal antisera were used as primary antibodies [75,76]: α-JEV (mouse, 1:1,000), α-C (rabbit, 1:1,000), α-pr (rabbit, 1:4,000), α-E (rabbit, 1:500), α-NS1 (rabbit, 1:1,000), and α-GAPDH (rabbit, 1:10,000). An alkaline phosphatase-conjugated goat α-mouse or α-rabbit IgG (Jackson ImmunoResearch, 1:5,000) was used for the secondary antibody, as appropriate. The specific signals were visualized by chromogenic membrane staining with a mixture of 3-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium (Sigma-Aldrich).

**Flow cytometry**

Cells (5×10^6) were harvested by trypsinization, washed with phosphate-buffered saline (PBS), and collected by centrifugation at 1,000×g for 5 min. The cells were resuspended in 250 μl of Cytofix/Cytoperm solution (BD Biosciences) and incubated at 4°C for 20 min in the dark. All subsequent wash and staining steps were performed in Perm/Wash buffer (BD Biosciences). The cells were washed twice and incubated in 200 μl of mouse α-JEV antiserum (ATCC, 1:500) for 1 h at 4°C. The cells were then washed twice and resuspended in 200 μl of Perm/Wash buffer. The samples were analyzed on a FACSAriaII cell sorter with Diva 6.1.3 software (BD Biosciences). For each sample, 50,000 events were collected within the linear range of detection.

**Sequence analysis**

The full genome sequences of SA14-14-2 and its neurovirulent variants were determined as described [77]. Sequencing of the prM-E coding region of the E-244 mutants was done as follows: (i) amplification of a 2,069-bp cDNA by RT-PCR using a set of three primers (prMEFrt, prMEFw, and prMEFv; see Table S1); (ii) cloning of a 2,057-bp Xhol-SacII fragment into the pRS2 vector; and (iii) sequencing of ~30 randomly picked independent clones containing the insert. Multiple sequence alignments were performed using ClustalX [116].

**Mouse infection**

Female 3-week-old ICR mice (Charles River) were used. Groups of 10 or 20 mice were inoculated IC (20 μl), IM (50 μl), or IP (50 μl) with 10-fold serial dilutions of virus stock in α-MEM. Mice were monitored for any JEV-induced clinical signs or death every 12 h for 24 days. The LD₅₀ values were determined as described [75,77]. In all mice, viral replication in brain tissue was confirmed by plaque titration and/or RT-PCR [75].

**Ethics statement**

All animal studies were conducted in strict accordance with the regulations in the Guide for the Care and Use of Laboratory Animals issued by the Ministry of Health and Welfare of the Republic of Korea. The protocol was approved by the Institutional Animal Care and Use Committee of the Chungbuk National University Medical School ( Permit Number: LML08-73). All mice were housed in our animal facility located at the Chungbuk National University Medical School, and every effort was made to minimize suffering.

**Immunohistochemistry**

Groups of 3-week-old female ICR mice (n = 15 per group) were infected IC with 10⁵ PFU of virus in 20 μl of α-MEM; 10 control mice were inoculated IC with an equivalent volume of supernatant from uninfected control BHK-21 cell cultures at comparable dilution. At 3, 5, and 7 dpi, five randomly selected mice were transcardially perfused with ice-cold PBS, followed by 4% paraformaldehyde (PFA). Brains were fixed in 4% PFA, embedded in paraffin, and cut into 6-μm sections. Brain sections were treated in microwave for antigen retrieval and incubated with 1% H₂O₂ in ice-cold methanol for 30 min to block endogenous peroxidase. They were then blocked with 1% normal goat serum and incubated with rabbit α-NS1 antiserum (1:200) for 12 h at 4°C, followed by incubation with biotinylated α-rabbit IgG plus the avidin-biotin-peroxidase complex (Vector). Signals were visualized by staining with 3,3’-diaminobenzidine solution containing 0.003% H₂O₂ and counterstaining with hematoxylin.

**Homology modeling**

The sequence and structure of the E ectodomain of WNV NY99 (PDB accession code 2HG0) was used as template for the homology modeling. The sequence alignment was done using the online version of ClustalW2 [117]. Protein structure homology modeling was performed using the SWISS-MODEL Workspace, accessible via the ExPASy web server [118]. The generated model was visualized using UCSF Chimera 1.5.3. The model is in agreement with a recent crystal structure of the E ectodomain of JEV SA14-14-2 (PDB accession code 3PS4) [80].

**Supporting Information**

**Figure S1** Representative focus/plaque morphologies of SA14-14-2MCV. BHK-21 cells were mock-infected or infected with one of the following three JEVs: SA14-14-2MCV, SA14-14-2, or CNU/LP2 (a virulent strain used as a reference). After infection, cells were overlaid with agarose to examine focus/plaque morphologies. At 4 dpi, cell monolayers were first immunostained with a mouse α-JEV antiserum to visualize the infectious foci, and the same monolayers were then restained with crystal violet to observe the infectious plaques. The average plaque sizes (mean ± SD) were determined by counting 10 representative plaques.

**Figure S2** Viral growth properties of SA14-14-2MCV in SH-SY5Y and C6/36 cells. Cells were infected at an MOI of 1 with the molecularly cloned virus (SA14-14-2MCV) rescued from the full-length infectious cDNA or the original parental virus (SA14-14-2) used for cDNA construction. Culture supernatants...
were collected at the hour postinfection (hpi) indicated, and virus titers were determined by plaque assays on BHK-21 cells. (PPT)

Figure S3 Virological properties of SA14-14-2MCV in mice. Groups of 3-week-old female ICR mice (n = 20 per group) were mock-infected or infected intracerebrally (IC), intramuscularly (IM), or intraperitoneally (IP) with serial 10-fold dilutions of SA14-14-2MCV, SA14-14-2, or CNU/LP2 (a virulent JEV strain used as a reference). Mice were observed for any JEV-induced clinical signs and death every 12 h for 24 days. Survival curves were plotted by the Kaplan-Meier method. (PPT)

Figure S4 A single point mutation promotes susceptibility to SA14-14-2MCV infection of neurons in the CNS. Groups of 3-week-old female ICR mice (n = 15 per group) were mock-infected or infected IC with 10^5 PFU of SA14-14-2MCV (Parent), G1708A, or CNU/LP2 (a virulent JEV strain used as a reference). On the indicated days after infection, five mice were subjected for immunostaining of JEV NS1 antigen in fixed brain slices with an α-NS1 antiserum. Presented are representative slides of amygdala, cerebral cortex, thalamus, hypothalamus, and brainstem (note that hippocampal slides are shown in Fig. 4E). Arrowheads indicate the NS1-positive cells. (PPT)

Figure S5 Levels of JEV protein accumulation in BHK-21 cells transfected with 14 E-244 mutant RNAs. BHK-21 cells were mock-transfected or transfected with RNAs transcribed from SA14-14-2MCV (Parent) or each of the 14 E-244 mutant cDNAs as indicated. At 18 hpt, viral protein accumulation was analyzed by immunoblotting of cell lysates with a panel of JEV-specific antisera. In parallel, GAPDH protein was used as a loading and transfer control. (PPT)

Figure S6 The location of E-244 on the crystal structure of the E ectodomain of JEV SA14-14-2. The E ectodomain of JEV SA14-14-2: DI (colored red), DII (yellow), DIII (blue), and the fusion loop (green). The critical residue Gly at E-244 in the ij hairpin adjacent to the fusion loop of the viral E DI is shown. The crystal structure of the E ectodomain of JEV SA14-14-2 was retrieved from the RCSB Protein Data Bank (PDB accession code 3P54). (PPT)

Figure S7 Amino acid sequence alignment of 154 fully sequenced JEV strains at the conserved ij hairpin of viral E glycoprotein. Multiple sequence alignments were performed using the amino acid sequence of 154 fully sequenced JEV genomes, including SA14 (red), SA14-14-2 (green), and two other SA14-derived attenuated strains, SA14-2-8 (orange) and SA14-12-1-7 (blue). Note that SA14 and SA14-14-2 have been sequenced by three and four independent research groups, respectively. The consensus sequence of the ij hairpin and its flanking region is presented on top, and only differences from that sequence are shown. Highlighted are the ~15-aa ij-hairpin and the position E-244 in that hairpin. (PPT)

Table S1 Neurovirulence and neuroinvasiveness of SA14-14-2MCV and its three variants in 3-week-old ICR mice. (PPT)

Table S2 Neurovirulence of SA14-14-2MCV and its eight mutants in 3-week-old ICR mice. (PPT)

Table S3 Neurovirulence of SA14-14-2MCV and its 14 E-244 mutants in 3-week-old ICR mice. (PPT)

Table S4 Oligonucleotides used for ligation, cDNA synthesis, and PCR amplification. (PPT)

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Author Contributions
Conceived and designed the experiments: SIY BHS YML. Performed the experiments: SIY BHS JKK GNY EYL. Analyzed the data: SIY BHS YML. Contributed reagents/materials/analysis tools: LL RJK MGR JDM. Wrote the paper: SIY BHS YML.

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