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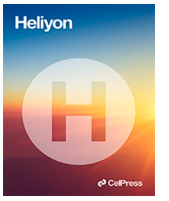

Research article

A peptide from the Japanese encephalitis virus failed to induce the production of anti-N-methyl-D-aspartate receptor antibodies via molecular mimicry in mice

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ABSTRACT

Background: The development of anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis following viral encephalitis, such as Japanese encephalitis, has received increasing attention in recent years. However, the mechanism of anti-NMDAR antibody production following Japanese encephalitis has not been explored.

Methods: A peptide from the Japanese encephalitis virus (JEV), which shares a similar amino acid sequence with GluN1, was identified by sequence comparison. We then explored whether active subcutaneous immunization with the JEV peptide could induce the production of anti-NMDAR antibodies and related pathophysiological and behavioral changes in mice. In addition, a published active immune model of anti-NMDAR encephalitis using a GluN1 peptide was used as the positive control.

Results: A 6-amino-acid sequence with 83 % similarity between the envelope protein of the JEV (HGTVVI) and GluN1 (NGTHVI) was identified, and the sequence included the N368/G369 region. Active immunization with the JEV peptide induced a substantial and specific immune response in mice. However, anti-NMDAR antibodies were not detected in the serum of mice immunized with the JEV peptide by ELISA, CBA, and TBA. Moreover, mice immunized with the JEV peptide presented no abnormities related to anti-NMDAR antibodies according to western blotting, patch clamp, and a series of behavioral tests. In addition, active immunization with a recently reported GluN1 peptide failed to induce anti-NMDAR antibody production in mice.

Conclusions: In this study, the attempt of active immunization with the JEV peptide to induce the production of anti-NMDAR antibodies via molecular mimicry failed. The pathogenesis of anti-NMDAR encephalitis following Japanese encephalitis remains to be elucidated.

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1. Introduction

The most common form of autoimmune encephalitis is anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis, which is characterized by psychiatric or behavioral disorders, movement disorders, seizures, speech dysfunction, and autonomic dysfunction [1]. Although the effect of anti-NMDAR autoantibodies on the GluN1 subunit is well studied [2], the mechanisms by which this specific autoantibody is produced still need to be clarified. Currently, studies have shown that tumors, particularly ovarian teratomas, are the dominant trigger for anti-NMDAR encephalitis [3,4]. However, the development of tumors in children with anti-NMDAR encephalitis is extremely rare [3,4].

Recently, many studies have revealed the underlying relationship between infection and autoimmune encephalitis [5]. Previous studies revealed that patients with viral encephalitis could suffer from late-stage symptoms after recovery from the acute stage, which was finally diagnosed as autoimmune encephalitis, which includes anti-NMDAR encephalitis [6]. In 2017, we originally reported that children with Japanese encephalitis (JE) might develop anti-NMDAR encephalitis during convalescence [7]. We then reported the incidence and clinical features of children with JE-induced anti-NMDAR encephalitis [8,9]. Several other researchers also reported similar cases [10,11]. However, the mechanism of anti-NMDAR antibody genesis after JE has not yet been explored.

The current hypotheses of anti-NMDAR antibody production following central nervous system infection mainly include molecular mimicry, antigen exposition, and genetic predisposition [12]. The molecular mimicry mechanism has been widely proven in different autoimmune diseases, including Guillain-Barre syndrome [13]. He et al. reported that Japanese encephalitis virus (JEV) shares a seven amino acid sequence with the acetylcholine receptor (AChR). Based on this finding, immunization with a live-attenuated Japanese encephalitis vaccine successfully induced antibodies against AChR in mice via molecular mimicry [14]. Thus, we assumed that JEV could also induce an autoimmune response against the NMDA receptor through molecular mimicry. Moreover, the N368/G369 region of the GluN1 subunit extracellular amino-terminal domain (ATD) is critical for anti-NMDAR antibody immunoreactivity [15]. Wagnon et al. successfully induced anti-NMDAR antibodies in mice by active immunization using a single peptide containing this crucial region [16]. Inspired by the above results, we first tried to identify whether the JEV and GluN1 subunit share similar amino acid sequences that could serve as the fundamental molecular mimics. We then explored whether this peptide could induce the production of anti-NMDAR antibodies in mice through active immunization.

2. Material and methods

2.1. Animals

Fifty-four six-to eight-week-old male C57BL/6J mice (20–24 g) were obtained from the Experimental Animal Center of Chongqing Medical University. Sample sizes were determined using the Power Analysis and Sample Size software (PASS, version 15.0) with full consideration of previous experience, the Reduction, Replacement, Refinement principle, and technical limitations [17,18]. The mice were housed in standard laboratory conditions with an automated light/dark cycle of 12/12 h, a humidity level of 50–60 %, and a room temperature (RT) of 22–24 °C. Food and water were provided *ad libitum*. The protocol was approved by the Ethics Committees of the Children's Hospital of Chongqing Medical University (CHCMU-IACUC20220429012).

2.2. Peptide determination and synthesis

The amino acid sequences of the GluN1 subunit and JEV (SA-14-14-2) were obtained from the Protein database of the PubMed

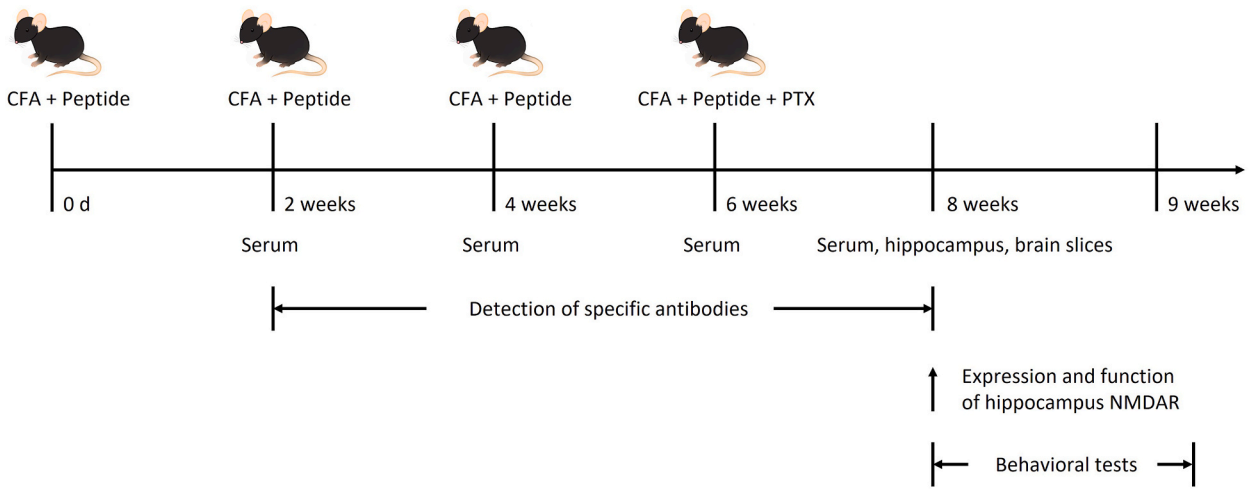

Fig. 1. The experimental procedure. CFA: complete Freund's adjuvant, PTX: pertussis toxin.

website. The Basic Local Alignment Search Tool (BLAST) was used to explore potential similar sequences between the GluN1 subunit and JEV. Three-dimensional models of the crystal structure of the GluN1subunit and each protein of JEV were obtained from the structure database of the PubMed website and visualized using PyMOL software.

To select an appropriate peptide with enough immunogenicity to induce an immune response, we screened the immunogenicity of different peptides in regions containing similar sequences by using the BepiPred linear epitope prediction method (www.iedb.org).

Peptides without any C- or N-terminal modification were synthesized and purified to 95 %, which was verified by high-performance liquid chromatography (GL Biochem LTD, China).

2.3. Study design and active immunization

Action immunization was conducted as previously reported [16]. The peptides were dissolved in saline (2 mg/ml), and then fully emulsified in an equal volume of complete Freund's adjuvant containing 1 mg/ml heat-killed *Mycobacterium tuberculosis* H37Ra [(CFA), #F5881, Sigma Aldrich, USA]. Mice were randomly assigned to experimental, negative control, or positive control groups. Each mouse received a subcutaneous injection of emulsion (200 μl containing 200 μg of peptide) at four sites on the back, which was repeated three times at 2, 4, and 6 weeks (Fig. 1). The $GluN1_{359\text{-}378}$ peptide emulsion was injected as the positive control. For the sham groups, an equal volume of saline emulsified in CFA was used in place of the peptide. Two hundred nanograms of pertussis toxin (1 ng/μl, #181, List Biological Laboratories, USA) was intraperitoneally administered to each mouse at the time of the last immunization and 48 h later.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Retro-orbital bleeds were collected at 2, 4, 6, and 8 weeks after immunization. To evaluate specific immune responses induced by peptides, ELISA plates (#3509, Costar) were coated with 0.1 μg of JEV peptide or GluN1 peptide in 100 μl carbonate buffer solution (0.01 M, pH 9.6)/well overnight at 4 °C and blocked with 5 % BSA at 37 °C for 1 h. Then, 50 μl of mouse serum (1:100) was added for 1 h at 37 °C. Horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000, #511103, Zenbio, China) and 3,3′,5,5′-tetramethylbenzidine substrate (#IT0001, Leagene, China) were subsequently used to detect peptide-specific IgG. A microplate reader (BioTek Epoch, USA) was used to measure the absorbance at 450 nm.

Anti- NMDAR antibody levels in the serum were measured using a mouse NMDAR1-Ab ELISA kit (#JL20420, Jianglai Biological, China).

2.5. Cell-based assay

The full-length NR1 and NR2B subunits of NMDAR (GFP labeled) were transfected into human embryonic kidney 293 cells. After 24 h, the cells were fixed with 4 % paraformaldehyde for 30 min and then treated with 0.3 % Triton™ X-100 (Sigma Aldrich, USA). Following a 1-h blocking step at RT with 5 % bovine serum albumin, the cells were incubated with mouse serum (1:10–1:1000) at 4 °C overnight. Moreover, a commercial rabbit monoclonal NR1 antibody (1:50, #ab109182, Abcam) was used as the positive control. The following day, the cells were washed with PBS and incubated with the corresponding TRITC-conjugated secondary antibodies (1:1000, #511102 and #511202, Zenbio, China) at RT for 1 h. Coverslips were mounted with an antifade medium and the slides were observed under a fluorescence microscope (Nikon 90i, Japan).

2.6. Immunohistochemistry

Frozen coronal brain sections (7 μm thick) of adult rat brains were prepared for immunohistochemistry, in which mouse serum (1:10–1:1000) was applied overnight at 4 °C. A commercial mouse monoclonal NR1 antibody (1:500, #67717-1-Ig, Proteintech) was used as the positive control. Following the manufacturer's protocol, immunoperoxidase staining was performed using a commercial HRP/DAB Detection IHC Kit (#ab64264, Abcam).

2.7. Western blotting

Total or membrane proteins were extracted from the hippocampus (#BB-3101-100T, BestBio; #KGP350, KeyGEN BioTECH, China) and the concentrations were quantitated using the BCA assay (#23225, Thermo Scientific, USA). Loading buffer (P0015L, Beyotime, China) was added to the protein samples, and the mixture was boiled at 100 °C for 5 min. After electrophoresis on a 10 % SDS-polyacrylamide gel, the protein (30 μg) was transferred to polyvinylidene difluoride (PVDF) membranes (#1620177, Bio-Rad, USA). The PVDF membranes were incubated with skim milk (5 %) at RT for 1 h to block nonspecific sites, followed by incubation with rabbit monoclonal anti-NMDAR1 (1:4000, ab109182, Abcam), mouse monoclonal anti-β-actin (1:10000, #66009-1, Proteintech) or mouse monoclonal anti-GAPDH (1:10000, #60004-1, Proteintech) at 4 °C overnight. The membranes were then incubated with the corresponding HRP-conjugated secondary antibodies (1:5000, #ab205718, Abcam; 1:5000, #511103, Zenbio, China) at RT for 1 h and then visualized by electrochemiluminescence (Bio-Rad, USA) on a ChemiDocTM Touch Imaging System (Bio-Rad, USA). Normalized protein expression was analyzed using Image Lab 6.0 software (Bio-Rad, USA).

2.8. Electrophysiology

Brain sections preparation. The mice were sacrificed after being anesthetized by isoflurane. The brain was quickly immersed in an ice-cold solution containing (in mM) 220 sucrose, 26 $NaHCO_3$, 10 D-glucose, 4 $MgSO_4$, 3 KCl, 2 lactate-Na, 2 $MgCl_2$, 2 pyruvate-Na, 1.25 NaH_2PO_4, 0.4 vitamin C, and 0.1 $CaCl_2$, and there was continuous ventilation with 95 % O_2 and 5 % CO_2. Hippocampal sections (400 μm) were prepared using a vibratome (VT1200s, Leica, Germany) and transferred to artificial CSF containing (in mM) 124 NaCl, 26 $NaHCO_3$, 10 D-glucose, 3 KCl, 2 lactate-Na, 2 $CaCl_2$, 2 pyruvate-Na, 1.2 $MgSO_4$, 1.25 NaH_2PO_4 and 0.4 vitamin C at 32 °C for 1 h and then allowed to recover at 22–24 °C for at least 1 h, with continuous aeration with the 95 % O_2 and 5 % CO_2 mixture.

Long-term potentiation (LTP) recording. Hippocampal sections were kept in a recording chamber with continuous perfusion of artificial CSF (1 mL/min) aerated with a mixture of 95 % O_2 and 5 % CO_2. Extracellular field excitatory postsynaptic potentials (fEPSPs) were evoked from the CA1 stratum radiatum and were recorded using a glass pipette filled with artificial CSF (2–4 MΩ). After recording the baseline fEPSPs for 30 min, high-frequency stimulation (HFS, 100 Hz, 1 s duration) was applied to induce LTP. Potentiated fEPSPs were recorded at 28 s intervals for 60 min. LTP was quantified by calculating the percentage change of the potentiated fEPSP slope compared to the average baseline fEPSP slope in the last 20 min. The recordings were analyzed using Clampfit 10.7 software.

2.9. Behavioral tests

Two weeks after the last immunization, behavioral experiments were performed to evaluate anxiety-like symptoms [open field test (OF) and elevated plus maze test (EPM)], depressive-like behaviors [forced swim test (FS)], and recognition function [Morris's water maze test (MWM)]. All tests were conducted at uniform times by researchers who were blinded to the group assignment. All behavioral tests were conducted using ANY-maze software (Stoelting, USA). Detailed protocols for the behavioral tests are presented in the supplementary materials.

A

Protein	Amino acid region	Amino acid sequence	Identities	Positive
JEV	613-618	HGTVVI		
		+GT VI	67%	83%
GluN1	368-373	NGTHVI		

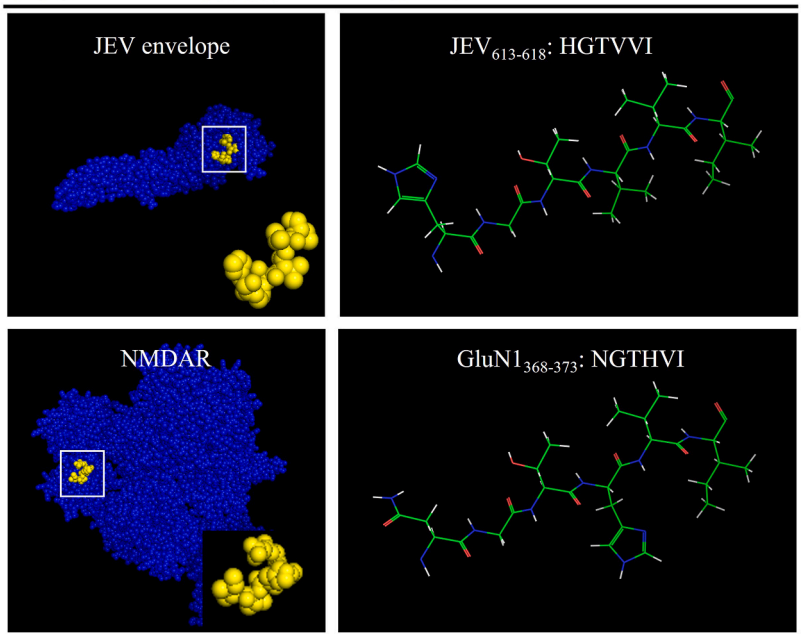

Fig. 2. Results of sequence alignment of JEV and GluN1. (A) An 83 % similarity was identified in a 6-amino-acid sequence shared by both the JEV envelope protein (HGTVVI) and NMDAR (NGTHVI), which included the N368/G369 amino acids, by comparing the amino sequence of the JEV protein and $GluN1_{359-378}$. (B, C) Structural analysis of these 6-amino-acid sequences. Three-dimensional models showed that these 6-amino-acid sequences give rise to similar surface structures. The $JEV_{613-618}$ and $GluN1_{368-373}$ peptides were highlighted in yellow dots. JEV: Japanese encephalitis virus. NMDAR: N-methyl-D-aspartate receptor. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.10. Statistical analysis

Data are shown as the mean ± standard deviation (SD). Data normality was tested on all samples using the Shapiro-Wilk and Kolmogorov–Smirnov tests (α-error of 0.05). According to the distribution of data, either the Student's t-test or the Mann-Whitney *U* test was used to measure differences between the two groups. One-way or two-way ANOVA was used to test differences between multiple groups, and Tukey's test was chosen for post hoc analysis. The Kruskal-Wallis test was used if data were abnormally distributed. Statistical analyses were performed using GraphPad Prism version 7 (GraphPad Software, USA). Statistically significant was considered when $P < 0.05$.

3. Results

3.1. Identification of the JEV peptide

To screen potential sequences for molecular mimicry, we first compared the whole amino acid sequence of JEV (SA-14-14-2) with that of NMDAR using BLAST. However, no appropriate similarity was found. We then compared the JEV sequence with a previously reported sequence ($GluN1_{359\text{-}378}$: RKLVQVGIYNGTHVIPNDRK) that successfully induced an anti-NMDAR response in mice. A 6-amino-acid sequence, which included the N368/G369 amino acids, shared by both the JEV envelope protein (HGTVVI) and NMDAR (NGTHVI) was found to be 83 % identical between the two proteins (Fig. 2A). Three-dimensional models showed that these two sequences construct a similar surface structure (Fig. 2B and C). These data suggested that the $JEV_{613\text{-}618}$ peptide could theoretically serve as a molecular mimic of NMDAR. To produce a peptide with enough immunogenicity, we used the BepiPred linear epitope prediction method to evaluate several peptides encompassing $JEV_{613\text{-}618}$. The $JEV_{606\text{-}621}$ peptide (KNPVDTGHGTVVIELS), which had the highest theoretical immunogenicity, was used in further experiments (Supplementary Table 1).

3.2. Evaluation of specific peptide-induced immune response

The efficacy of immunization was determined by ELISA. As shown in Fig. 3, a significant increase in anti-peptide antibody levels was found in the serum of mice immunized with JEV peptide when compared with the control mice. Moreover, this level of anti-$JEV_{606\text{-}621}$ peptide antibodies increased with the number of active immunizations. These data suggest that the $JEV_{606\text{-}621}$ peptide successfully induces a specific immune response in mice.

3.3. Evaluation of anti-NMDAR antibody levels

Three different methods were used to evaluate anti-NMDAR antibody levels in mice. There was no significant difference in anti-NMDAR antibody titers between serum from mice immunized with $JEV_{606\text{-}621}$ peptide and control mice at 2 or 8 weeks, according to the ELISA results (Fig. 4A and B). No specific antibodies binding to the HEK293 cells transfected with GluN1 subunits were observed in the serum from mice immunized with $JEV_{606\text{-}621}$ peptide or control mice in CBA at 2, 4, 6, and 8 weeks (Fig. 4C). Moreover, compared with commercial anti-NMDAR antibodies, the serum of mice immunized with $JEV_{606\text{-}621}$ peptide or control exhibited no specific binding pattern to the rodent's hippocampus slice (Fig. 4D). These results suggest that the anti-$JEV_{606\text{-}621}$ peptide antibodies may have no immunoreaction with NMDAR.

3.4. Pathophysiological effects of anti-$JEV_{606\text{-}621}$ peptide antibodies

While anti-NMDAR antibodies were not detected in serum, we still further tested whether the anti-$JEV_{606\text{-}621}$ peptide antibodies

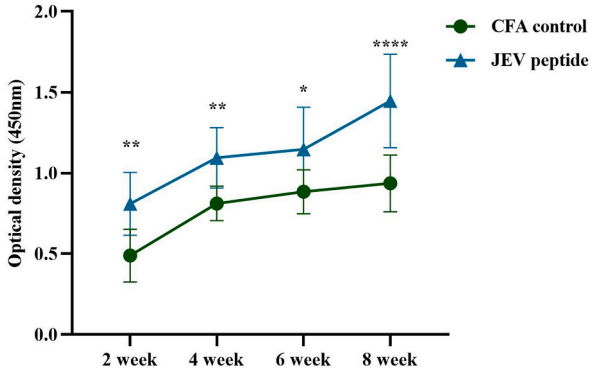

Fig. 3. Measurement of serum $JEV_{606\text{-}621}$ peptide antibody levels using ELISA. Two-way ANOVA was used. *P < 0.05, **P < 0.01, ****P < 0.001. CFA: complete Freund's adjuvant, JEV: Japanese encephalitis virus.

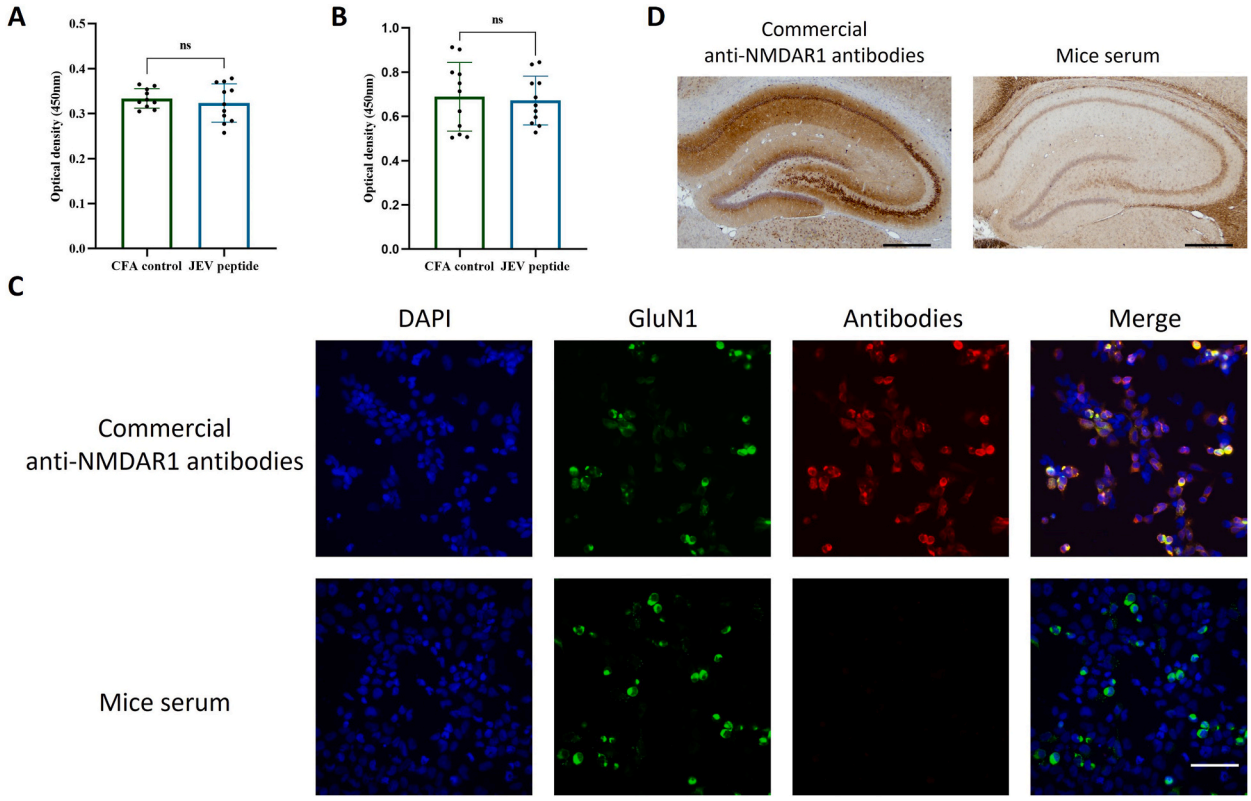

Fig. 4. Measurement of serum anti-NMDAR antibody levels in mice immunized with the $JEV_{606\text{-}621}$ peptide. ELISA at 2 weeks (A) and 8 weeks (B) after the first injection. (C) Representative immunofluorescence images using cell-based assays. Scale bar = 100 μm. (D) Representative immunohistochemical images using tissue-based assays. Scale bar = 500 μm. Student's t-test was used. ns: no significance. CFA: complete Freund's adjuvant, JEV: Japanese encephalitis virus.

had pathological effects on mice. Two weeks after the last immunization, western blotting indicated that $JEV_{606\text{-}621}$ peptide immunization did not lead to a significant decrease in hippocampal GluN1 protein levels on the cell membrane (Fig. 5A and B) or in the total cells (Fig. 5C and D). Moreover, the electrophysiological studies (Fig. 5E and F) showed that the magnitude of LTP was not impaired in mice immunized with $JEV_{606\text{-}621}$ peptide compared with the control mice (186.3 % vs. 168.9 %, P = 0.0981).

3.5. *Behavioral changes in mice immunized with the $JEV_{606\text{-}621}$ peptide*

A series of behavioral tests were performed to evaluate the anti-NMDAR encephalitis-related symptoms in mice immunized with the $JEV_{606\text{-}621}$ peptide (Fig. 6A–G). Two weeks after the last immunization, we observed similar escape latency (P = 0.4647) and numbers of island crossings (2.5 vs. 1.5, P = 0.3022) or time in the target quadrant (20.6 vs. 16.8 P = 0.6069) in the MWM between mice immunized with $JEV_{606\text{-}621}$ peptide or control mice. Moreover, there were no significant differences between these two groups in center time (OF, 28.0 vs. 29.1, P = 0.9858), open arms time (EPM, 22.4 vs. 24.1, P = 0.9468), or immobile time (FS, 71.5 vs. 68.7, P = 0.9864).

3.6. *Immunization with a previously reported peptide failed to induce anti-NMDAR antibodies in mice*

A previous study reported that the $GluN1_{359\text{-}378}$ peptide, which contains the NMDAR1-N368/G369 region, was used as a positive control. We first immunized mice strictly following the protocols. However, two weeks after immunization, we did not detect any signals of anti-NMDAR antibodies in mouse serum by ELISA, CBA and TBA (Fig. 7A–C). We then conducted three booster injections with the $GluN1_{359\text{-}378}$ peptide emulsion every two weeks after the first immunization in another independent cohort of mice to enhance the immune response. To confirm the immune response, the levels of specific anti-$GluN1_{359\text{-}378}$ peptide antibodies were measured by ELISA. Although anti-$GluN1_{359\text{-}378}$ peptide antibodies were successfully detected in mouse serum (Fig. 7D), we found no immune response to anti-NMDAR antibodies according to the ELISA (Fig. 7E), TBA, and CBA tests (data not shown). Moreover, for mice immunized with the $GluN1_{359\text{-}378}$ peptide, no significant decrease in surface or total hippocampal NMDAR protein levels was observed by western blotting (Fig. 7F–I). Electrophysiological tests showed unimpaired LTP function (Fig. 7J and K). Behavioral testing did not show any cognitive or schizophrenia-like change (Fig. 6).

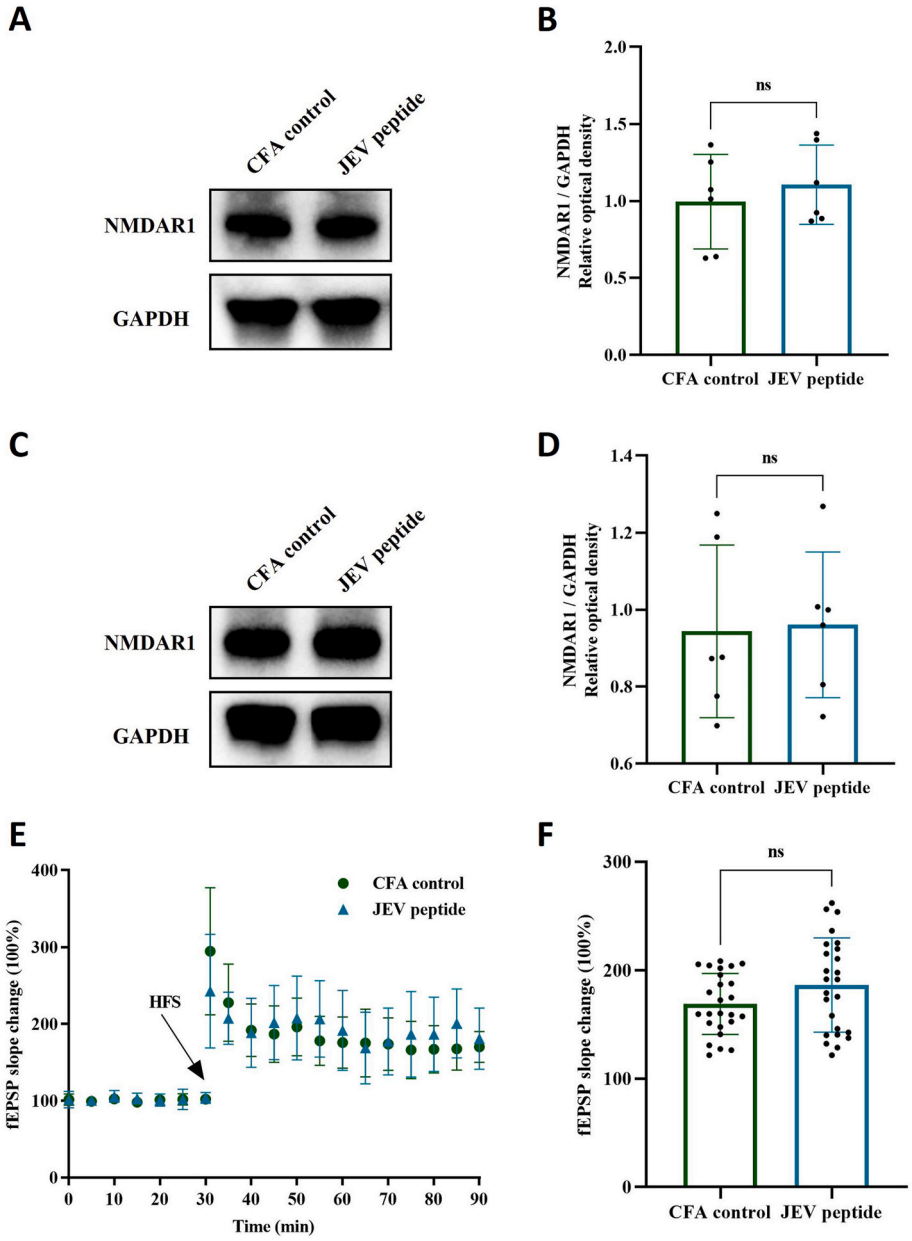

Fig. 5. Analysis of the effect of the anti-$JEV_{606\text{-}621}$ antibodies. Western blotting of the hippocampal NMDAR levels on the membrane (A, B n = 6) and in the total cells (C, D n = 6). Original images of Fig. 5A and C are presented in the supplementary materials. (E) Traces of fEPSPs recording in the mice immunized with $JEV_{606\text{-}621}$ peptide (n = 5, recordings from 4 mice) and control mice (n = 5, recordings from 3 mice). (F) Quantification of fEPSP changes during the last 20 min. Student's t-test was used. ns: no significance, HFS, high-frequency stimulation, CFA: complete Freund's adjuvant, JEV: Japanese encephalitis virus.

4. Discussion

In the current study, we explored the possibility that JEV could induce the production of anti-NMDAR antibodies via molecular mimicry. We also tested the reproducibility of a published active immune model of anti-NMDAR encephalitis. Although all of the results were negative, this study could provide an important reference for further research.

JE is a viral infection caused by mosquitos carrying the JEV. It is characterized by high fever, convulsion, disturbance of consciousness, and thalamus involvement according to MRI data [19]. Although patients with JE usually have an acute reaction, a few patients may develop a biphasic stage, mainly characterized by movement disorders, psychiatric disorders, and cognitive dysfunction [20]. Our previous studies confirmed that the reason for relapse in some children with biphasic JE was the development of anti-NMDAR encephalitis, which has an incidence of 5 % [7,8]. Further investigation showed that the median time between the onset

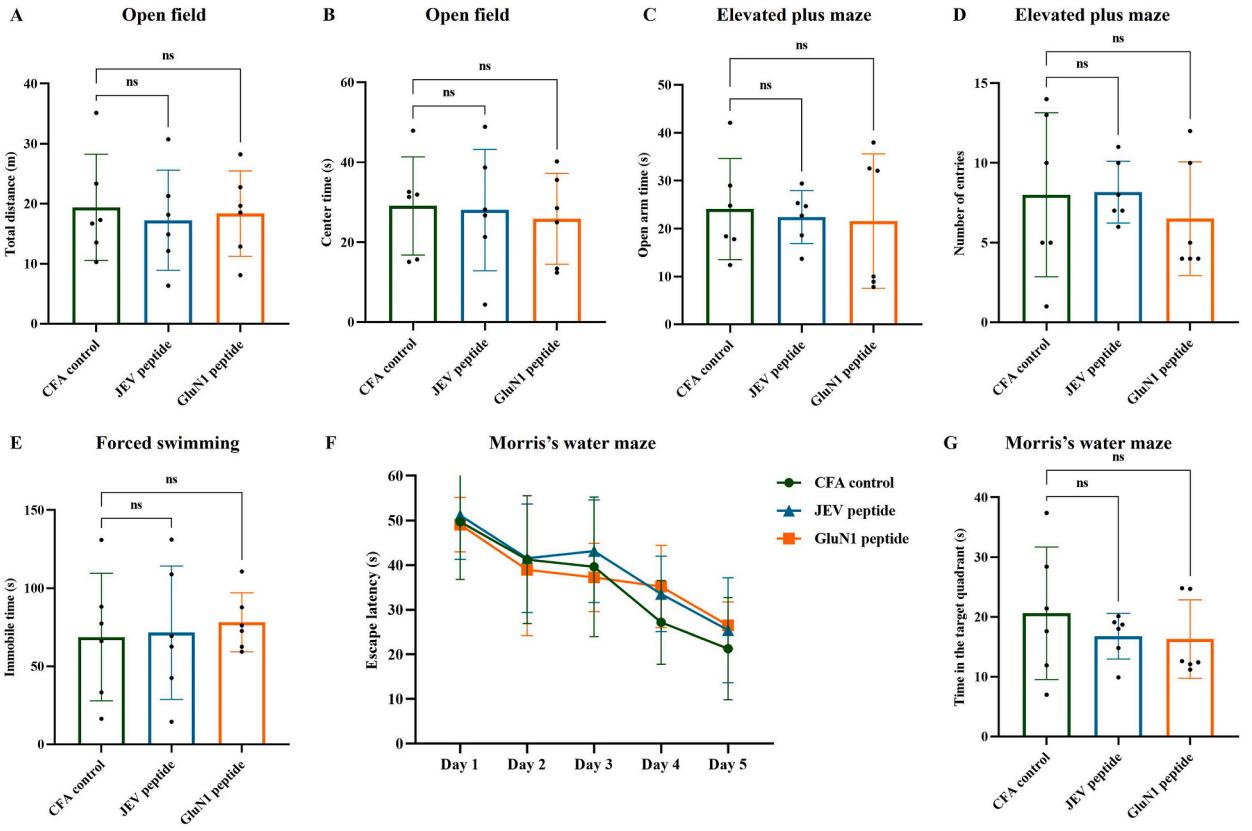

Fig. 6. Behavioral tests were conducted on mice two weeks after the last immunization. Total distance (A) and time in the central zone (B) were assessed in the open field test. The time spent in the open arms (C) and the number of entries (D) were assessed in the elevated plus maze test. Immobility time (E) was assessed in the forced swimming test. Escape latency (F) and time in the target quadrant (G) were assessed in the Morris's water maze. n = 6 in every group. There were no significant differences between mice immunized with the $JEV_{606\text{-}621}$ peptide and $GluN1_{359\text{-}378}$ peptide and control mice in the behavioral tests. One-way ANOVA was used for open field and forced swimming tests. The Kruskal-Wallis test was used for the elevated plus maze test. Two-way ANOVA and the Kruskal-Wallis test were used for the Morris's water maze. ns: no significance, CFA: complete Freund's adjuvant, JEV: Japanese encephalitis virus.

of JE and anti-NMDAR encephalitis was 29 days, and these patients were prone to poor outcomes [9]. However, no study has been performed on the mechanism underlying this phenomenon.

The mechanisms between infection and autoimmune diseases of the nervous system have yet to be fully understood. Current hypotheses include molecular mimicry, antigen release, bystander activation, and genetic susceptibility [12]. Generally, molecular mimicry refers to pathogens having similar protein structures/amino acid sequences/epitopes to neuronal antigens, leading to specific autoimmunoreaction. (e.g., rheumatic chorea after *Streptococcus* infection and Guillain-Barre syndrome after *Campylobacter jejuni* infection) [13]. In this study, we found that the envelope protein of JEV, which is the main antigen that stimulates humans to produce protective neutralizing antibodies, shares a 6-amino-acid sequence with the GluN1 protein by sequence alignment. We speculated that this sequence could act as a potential basis of molecular mimic. However, the results of mice immunized with this $JEV_{606\text{-}621}$ peptide failed to induce any anti-NMDAR antibodies and their related effects. This exploratory experiment suggested that molecular mimicry seems unlikely to be involved in the process of JE-induced anti-NMDAR encephalitis.

Previous studies have revealed that the N368/G369 region in the extracellular ATD of the GluN1 subunit is essential for anti-NMDAR antibody immunoreactivity, which is one of the premises of the current study [15]. Some issues remain to be explained. Various phenotypes, some even paradoxical, were observed in the murine model of anti-NMDAR antibody passive transfer [21–25]. This observation could partially be explained by heterogeneity among studies. However, anti-NMDAR antibodies are also present in other disorders. Anti-NMDAR antibodies in patients with schizophrenia disrupt the interaction between NMDARs and EphrinB2 receptors, resulting in decreasing synaptic NMDAR currents, which is consistent with the characteristics of anti-NNMDAR encephalitis [26,27]. However, the anti-NMDAR antibodies in patients with schizophrenia and anti-NMDAR encephalitis did not compete for binding to live cultured neurons, and mutation of the N368 region could only partially interfere anti-NMDAR antibody binding [26, 28]. Moreover, anti-NMDAR antibodies from healthy controls or patients with autism spectrum disorder did not affect surface NMDAR expression [26,29]. These results highly suggested the presence of other epitopes outside the N368/G369 region and intrinsic differences in activity or affinity among anti-NMDAR antibodies. Moreover, anti-NMDAR antibodies targeting the extracellular peptide located in the S2 domain of GluN1 (673–678: RNPSDK) and targeting the cleft of the ATD clamshells of GluN2A, or GluN2B were

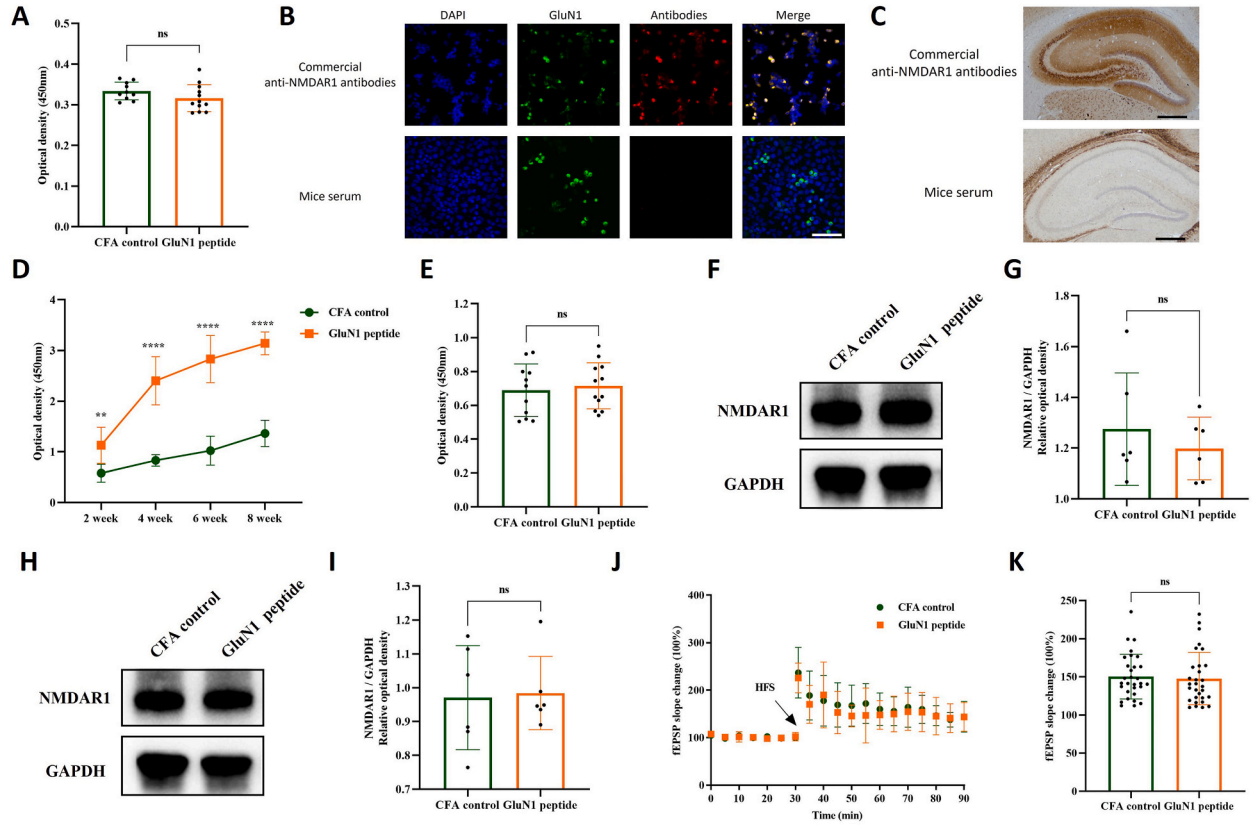

Fig. 7. Immunization with the GluN1 peptide failed to induce anti-NMDAR antibodies and related changes in mice. (A) Measurement of anti-NMDAR antibody levels at 2 weeks after immunization using ELISA. (B) Representative immunofluorescence images using cell-based assays. Scale bar = 100 μm. (C) Representative immunohistochemical images using tissue-based assays. Scale bar = 500 μm. (D) Measurement of serum $GluN1_{359\text{-}378}$ peptide antibody levels using ELISA. (E) Measurement of anti-NMDAR antibody levels at 8 weeks after first immunization using ELISA. Western blotting of hippocampal NMDAR levels on the membrane (F, G, n = 6) and in the total cells (H, I, n = 6). Original images of Fig. 7F and H are presented in the supplementary materials. (J) Traces of fEPSP recording in mice immunized with the $GluN1_{359\text{-}378}$ peptide (n = 6, recordings from 5 mice) and controls (n = 6, recordings from 4 mice). (K) Quantification of fEPSP changes during the last 20 min. Student's t-test and two-way ANOVA were used. $^{**}P < 0.01$, $^{***}P < 0.001$, ns: no significance. HFS: high-frequency stimulation, CFA: complete Freund's adjuvant, JEV: Japanese encephalitis virus.

reported in previous studies [30,31]. Thus, sequence alignment between JEV and different domains of NMDAR is still worthy of further study.

The association between herpes simplex virus encephalitis (HSE) and anti-NMDAR encephalitis has been well-studied. In 2018, an observational study indicated that 27 % of patients presented autoimmune encephalitis after HSE, of whom 64 % had anti-NMDAR encephalitis, with a median interval of 32 days [6]. Linnoila et al. developed an HSE-induced anti-NMDAR encephalitis mouse model by intranasal inoculation of herpes simplex virus, which further established the relationship between anti-NMDAR encephalitis and herpes simplex virus [32]. However, the mechanism of anti-NMDAR encephalitis genesis after HSE also remains obscure. Despite the predominance of anti-NMDAR antibodies, other antibodies, including anti-$GABA_BR$, anti-$GABA_AR$, anti-Dopamine-2R, anti--CASPR2, and anti-AMPAR antibodies, or antibodies targeting unknown neuronal antigens were reported in patients with autoimmune encephalitis after HSE [12,33]. In line with this report, our previous study showed that anti-$GABA_BR$ and antibodies against currently unknown neuronal antigens were detected in children with autoimmune encephalitis after JE [34]. Given that anti-NMDAR antibodies could be secondary to various viral encephalitis and that a single virus could induce different autoantibodies, antigen release after direct neuron damage is a more reasonable hypothesis for the development of anti-NMDAR encephalitis after viral encephalitis, including JE. However, although no sequence similarity between HSV and NMDAR has been identified, a previous study revealed that patients with anti-NMDAR encephalitis without previous HSE have more frequent anti-HSV antibodies than healthy controls, indicating a specific association between HSV and anti-NMDAR antibodies [35]. Moreover, in addition to the similarity of similar amino acid sequences or epitopes, spatial structural similarity between the microbe and its host could also lead to cross-reaction that could not be established by simple sequence comparisons [13]. Thus, the current studies could not rule out the potential involvement of molecular mimicry in anti-NMDAR encephalitis after JE.

In multiple studies, the pathogenicity of anti-NMDAR antibodies has been well proven in vitro and in vivo through the passive transfer of patients' antibodies into rodents' central nervous systems [19,36–39]. However, the exact mechanism and

pathophysiological process of anti-NMDAR encephalitis are still unclear, and an established active immunization model is needed. A previous study reported that the $GluN1_{359-378}$ peptide successfully induced the production of anti-NMDAR antibodies in mice by a widely used active immune procedure. Mice immunized with this peptide exhibited a remarkable B-cell response and anti-NMDAR encephalitis-related behavioral changes [16]. However, the induction of autoimmune reactions against NMDAR using either the same or enhanced procedure completely failed in this study. Immunized mice presented no pathological, electrophysiological, or behavioral changes related to anti-NMDAR encephalitis, although we did not conduct every experiment due to technical issues. This considerable discrepancy may be due to the heterogeneity among mice or the peptide. We did not know whether the $GluN1_{359-378}$ peptide was purified or whether any modification of the peptide existed in the original study. Nevertheless, a recent study also tried to reproduce this model by meticulously following the original protocol. Unfortunately, although a specific anti-$GluN1_{359-378}$ peptide immune response was observed, which was in line with our finding, replication in two large independent cohorts of wild-type mice failed [18]. Moreover, several different peptides containing the NMDAR1-N368/G369 region or not were used to develop an active model of anti-NMDAR encephalitis in two studies, which also resulted in variable results [40,41]. Altogether, these findings demonstrated the urgent need for a stable, highly repeatable active immune model.

This study has several limitations. Firstly, the lack of positive controls due to the failure to replicate a published GluN1 peptide weakened the reliability of our results. Thus, we still cannot exclude the possibility that JEV could induce the production of anti-NMDAR antibodies via molecular mimicry. As mentioned above, only the similarity of amino acid sequences, the most reported and easiest type of molecular mimicry to investigate, was explored in this study. Further studies could test whether structural similarities exist between GluN1 and JEV or other viruses. In addition, we did not replicate all of the original experiments in the previously reported model. Although experimental fluctuations commonly exist, validation from multiple studies is necessary to establish a representative animal model, which is very important to researchers in this field.

5. Conclusion

The failure of this study suggested that molecular mimicry is less likely to be involved in the development of anti-NMDAR encephalitis after JE. Further studies are also required to develop more stable and repeatable active immune models for anti-NMDAR encephalitis.

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Data availability statement

The data associated with this study has not been deposited into a publicly available repository. Data will be made available on request.

CRediT authorship contribution statement

Hanyu Luo: Data curation, Formal analysis, Methodology, Project administration, Software, Visualization, Writing – original draft, Writing – review & editing. **Jiaxin Yang:** Data curation, Methodology, Project administration, Validation, Writing – review & editing. **Xiaoyue Yang:** Investigation, Methodology, Writing – review & editing. **Ziyao Han:** Investigation, Methodology, Writing – review & editing. **Zhixu Fang:** Investigation, Methodology, Writing – review & editing. **Dishu Huang:** Investigation, Methodology, Writing – review & editing. **Jianxiong Gui:** Investigation, Methodology, Writing – review & editing. **Ran Ding:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Hengsheng Chen:** Resources, Software, Supervision. **Li Cheng:** Methodology, Resources, Software, Supervision. **Jiannan Ma:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Li Jiang:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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