

# Anti-NMDAR encephalitis induced in mice by active immunization with peptides of the amino-terminal domain of the GluN1 subunit

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## Short report

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# Abstract

Anti-N-methyl-D-aspartate receptor (NMDA) receptor encephalitis is a recently discovered autoimmune syndrome associated with psychosis, dyskinesias, and seizures. Ectopic expression of NMDA receptors associated with ovarian teratoma is thought to mediate the initial autoimmune response against NMDA receptor encephalitis. Due to the lack of suitable animal models, the underlying mechanism of the disease remains unclear. This study described a new mice model of active immunization against the NMDA receptor with amino-terminal domain (ATD) peptides. After 12 weeks of immunization, mice were showed significant behavioral disorders and memory loss. Antibodies from CSF of immunized mice decreased surface NMDAR cluster density on hippocampus neurons. It also impaired the LTP induced at the Schaffer collateral to CA1 synapse and reduced NMDA receptors-induced calcium influx. The new model may help further research into the pathogenesis of the disease and the development of potential new therapies.

## Introduction

Anti-N-methyl-D-aspartate receptor (NMDA) receptor encephalitis predominantly affects children and young women. Almost 80% of patients showed abnormal in cerebral spinal fluid (CSF), presenting as mildly lymphocytic pleocytosis, normal or moderate increased protein levels, and most importantly, specific oligoclonal bands appear in 60% of patients [25, 29].

Ectopic expression of NMDA receptor is thought to trigger the immune response in anti-NMDA receptor encephalitis [1, 27]. Ovarian teratomas and other tumors have been shown to express the NMDA receptor, potentially explaining the association [5]. Antigens released by tumor cells are taken up by antigen-presenting cells that travel to regional lymph nodes, plasma cells produce antibodies that later cross-react with NMDA receptor in the brain following impaired blood-brain barrier permeability [7, 13, 21]. NMDA receptor mediate glutamatergic synaptic transmission and have a prominent role in synaptic plasticity [4, 15]. Previous studies have suggested that the pathogenicity of autoimmune antibodies (ABs) is one of the key mechanisms of anti-NMDA receptor encephalitis [14, 17, 20]. Human CSF-derived NMDA receptor ABs downregulates the level of NMDA receptor both in in vitro and in vivo studies [22]. Meanwhile, the reduced expression of NMDA receptors can result in the increase in extracellular glutamate and further affect the pons medullary respiratory center [22].

The passive immunized mice develop symptoms of depression, anhedonia, and memory deficits following the intrathecal infusion of CSF from affected humans, it provides evidence of the effect of autoantibodies on the disease, but do not produce the pathogenesis of anti-NMDA receptor encephalitis in human [1, 2]. To better mimic the clinical manifestations and mechanism of anti-NMDA receptor encephalitis, it is still worth developing an animal model of active immunity that mimics disease progression.

Previous investigations highlighted the extracellular amino-terminal domain (ATD) of the GluN1 subunit, especially the N368/G369 region that is essential for immunoreactivity [11, 23, 26]. and ATD peptides were used to immunize and induce fulminant encephalitis in mice within 12 weeks. The mice demonstrated behavioral changes and ABs infiltration that were most prominent in the hippocampus. The presence of NMDA receptor ABs was confirmed by and its effect on the NMDA receptor was also confirmed.

## Materials And Methods

### Study design and mice immunization

The study aimed to investigate the effect of active immunization with NMDA receptor peptides on normal adult mice. C57BL/6 Mice (10 weeks old) were immunized with different GluN1 extracellular peptides and emulsified in an equal volume of complete Freund's adjuvant supplemented with of *Mycobacterium tuberculosis* H37Ra (4 mg/mL) at a final concentration of 1 mg/mL. At the tail base, 200 µg of GluN1 peptides or control peptides were injected subcutaneously and boosted twice at 4 and 8 weeks after the first immunization, respectively. All mice were intraperitoneally injected with 400 ng pertussis toxin (PTX) at the last immunization. Behavioral tests, histological staining analysis were performed 10 weeks after the first immunization.

### Patients' sample collection

We collected CSF from patients with high titer of anti-GluN1 ABs (>1:300) during routine clinical practice. All patients fulfilled the clinical diagnostic criteria for anti-NMDAR encephalitis revised in 2016 [12]. The study protocol was approved by the ethics committee of the Nanfang Hospital, Southern Medical University, and written informed consent was obtained from each participant.

### Antibody purification

CSF and serum from patients or immunized mice were purified with protein G Sepharose columns and used to treat neurons or brain slices. 2 ml of diluted sample was incubated with a chromatography spin column (Thermo Scientific) of protein G Sepharose beads for 30 min. After three washes with PBS, the sample were eluted with elution buffer, dialyzed against PBS, concentrated in stock solutions of 4 mg/mL and stored at -80 °C until used.

### Preparation and staining of GluN1-expressing HEK cells

Human embryonic kidney 293 (HEK) cells were transiently transfected with NMDA receptor subunit genes (NR1/NR2A) (DsRed2 labeled) as described [24]. 24 hours later, cells on coverslips were fixed with acetone and incubated with the CSF from patients or immunized mice (starting at 1:1) in 0.1% bovine serum albumin (BSA) in PBS overnight at 4 °C. After washed with PBS, cells were labeled with FITC-conjugated anti-human IgG and observed under a fluorescence microscope (BX51, Olympus)

## Site-directed mutagenesis

Point mutation were made using the Stratagene QuikChange Mutagenesis kit (210518, Agilent) according to the manufacturer's instruction. Primers designed for N368Q point mutation: Forward: 5'-gggatgacatgggtaccttgtagatgccacttgca-3'; Reverse: 5'-tgcaagtgggcatctaccaagggtacccatgtcatccc-3'.

## Primary neuron culture

Hippocampal neurons were prepared and maintained from embryonic day 18 rat brains. The hippocampi were dissociated with papain at 37 °C for 30 minutes, and then separated with a fire polished Pasteur pipette. After centrifugation at 300 g, the cells were resuspended in neurobasal medium. Cells were counted and plated on poly-D-lysine (PDL) treated 24-well plates. After 6 hours, the supernatant was removed and replaced with 500 µL of fresh culture medium. Cells were cultured for 14 days for subsequent experiments.

## Immunostaining

Immunostaining is used to detect binding to brain slices and autoantibodies. On day 14 after immunization, CSF was obtained from mice, purified and incubated with brain slice. Slices were imaged with confocal microscope (Carl Zeiss LSM 880). To stain surface NMDA receptor, Neurons were treated and incubated with AB derived from CSF of patients or immunized mice, after incubation for 18 h, the surface antibody-bound receptors were labeled with a fluorescent conjugated secondary antibody (Alexa Fluor 488) (1:200, anti-Mouse Alexa Fluor 488, A-11029; 1:200, anti-Human Alexa Fluor 488, A-11013; Invitrogen). Neurons were then fixed, permeabilized, incubation with anti-PSD95 primary AB (1:200, Synaptic Systems) to label postsynaptic densities and visualized after staining with a different fluorescent secondary antibody (Alexa Fluor 647) (1:200, anti-Rabbit Alexa Fluor 647, A-21244; Invitrogen).

## Electrophysiological Recordings

Hippocampal slices (300 µm) of mice were prepared with a vibratome (Leica VT1000S). Slices were kept at 30 °C for at least 60 min before experiments in artificial cerebrospinal fluid (ACSF) (NaCl 124 mM, KCl 2.5 mM, MgSO<sub>4</sub> 2.0 mM, NaH<sub>2</sub>PO<sub>4</sub> 1.25 mM, NaHCO<sub>3</sub> 26 mM, CaCl<sub>2</sub> 2 mM, glucose 10 mM, pH=7.3), bubbled with a mixture of 95 % O<sub>2</sub> and 5 % CO<sub>2</sub>. Field excitatory postsynaptic potentials (fEPSPs) were evoked in CA1 stratum radiatum by stimulation of Schaffer collaterals with a two-concentric bipolar stimulating electrode and recorded with ACSF-filled glass pipettes. LTP was induced by applying theta burst stimulation (TBS). purified ABs were diluted with ACSF (100 µg/mL) and applied by switching the perfusion from control ACSF to ABs-containing ACSF. In each recording, baseline synaptic transmission was monitored for 10 min before ABs perfusion and washed out with ACSF continuously after TBS up to the end of the experiment. The acquired data were analyzed with pClamp 10 software (Molecular Devices).

## Calcium Imaging

Hippocampal neurons were incubated with 20 µg/mL patient or mice ABs at 37 °C for 18 hours. To detect calcium flux, cells were loaded with 1µM Fluo-4 and incubated at 37 °C for 15–30 minutes, followed by 15–30 minutes at room temperature. After washed in Tyrodes solution, cells were transferred into wells containing 10 µM NBQX. NMDA was used to stimulate NMDAR-mediated calcium influx at a concentration of 10 µM. Imaging was performed using inverted fluorescence microscope (Eclipse TE2000-U, Nikon) with a charge-couple device camera, A series of images was acquired at 800-ms intervals for 40 s at an excitation wavelength of 470 nm. The fluorescence intensity for each time point was measured using ImageJ.

## Behavioral assessment

After 12 weeks of immunization, LE30 immunized mice were tested through a series of behavioral experiments. Mice immunized with a control peptide was used as a control. Behavioral testing was conducted at a uniform time, from 09: 00 to 12:00 hours by researchers' blind to the mice's group allocation and behavioral parameters were recorded by a video tracking system (Smart 3.0, Panlab, Spain).

**Novel Object Recognition (NOR):** The test apparatus consisted of a dark open box made of plexiglass, and the objects made of plastic with different shapes. Before the test, the mice were allowed to explore the instrument for 5 minutes in 3 consecutive days, on the day of the test, two 5-minute tests were performed. First two identical objects were placed in opposite corners of the device. A mouse was placed in the middle of the device was left to explore these two identical objects. after 5 minutes, a sample was replaced by A new object. The times spent by each mouse in exploring each object were recorded.

**Three-Chamber Test (TCT):** The opaque plastic device consists of side chambers of equal size (30 × 30 cm), each side chamber being located on both sides of the same central chamber and delimited by removable dividers. After 10 minutes of getting used to a 3-chambered arena, an empty cage and a cage containing an unfamiliar mouse (same sex) were placed in opposite side chambers. The location of cages was systematically alternated between animals. The time spent by each mouse in each chamber was recorded for 10 minutes.

**Opening Field Test (OFT):** Place each mouse in a 40 × 40 cm square plastic room for 15 minutes, total distance traveled by the mice, and the time spent by each mouse in the center of the field (15 × 15 cm) was recorded.

**Elevated Plus-Maze (EPM):** The black plastic device consists of two closed (10 × 45 × 40 cm) and two open (10 × 45 cm) arms that extend from the same central platform (10 × 10 cm). Each mouse spent 5 minutes in the EPM. Each mouse was placed in the center of the maze, facing one of the closed arms, and the time spent in opening and closing the arms and the number of entries per arm were recorded.

## Western blot analyses

Cytoplasmic and total proteins were extracted from the primary neurons using the Minute™ Cytoplasmic Extraction Kit (Invent Biotechnologies) according to the manufacturer's protocol. Proteins in SDS-loading buffer were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane. After blocking with 5% BSA, the membranes were incubated with anti-GluN1 ABs (1:500, 114-011, Synaptic Systems) and a secondary HRP-conjugated antibody. The immunocomplexes were detected using enhanced chemiluminescence.

## Statistical analyses

Data were presented as mean  $\pm$  SEM. Independent sample t-tests or Mann Whitney-U test were used as indicated for each experiment. Kruskal–Wallis tests were used for nonparametric data. All analyses were performed using SPSS 24.0 and the histograms were made by Prism 6.0 or ImageJ.  $p < 0.05$  was considered significant.

## Results

### Detection of antigen specificity and binding properties of the peptide-induced autoantibodies

To induce an active-immune mice model of anti-NMDA receptor encephalitis, mice were immunized by subcutaneously injection with 200  $\mu$ g peptide emulsified in Complete Freund's Adjuvant (CFA) (Fig. 1A). CSF from peptide-immunized mice was collected 14 days after immunization. The CSF AB was purified and detected on HEK293T cells transfected with GluN1 subunit (DsRed2 labeled). We found that only the LE30 peptide could induce autoantibodies in CSF that specific for GluN1 (Fig. 1B). The binding of ABs from peptide-immunized mice were detected by immunofluorescence. ABs from immunized mice were mainly deposited in the hippocampus. It is similar to the binding of ABs extracted from the patients (Fig. 1C). Antibody binding was also found in cortical glial cells and gray matter of the spinal cord (Fig. 1D).

### Levels of LE30-peptide-induced autoantibodies expression in mice Behavioral experiments to assess behavioral changes in the mice model

The NMDA receptor antibody-mediated internalization of receptors is a reversible process [22]. When the antibody disappears, the function of the NMDA receptors may be restored. To induce high concentrations of autoantibodies targeting NMDA receptors, we repeatedly immunized mice with LE30-peptide at 4 and 8 weeks after the first immunization with LE30-peptide. We found that this procedure can maintain titer of autoantibodies within the CSF and serum of mice. At the same time, we drew the brains from LE30-peptide immunized mice for immunofluorescence targeting autoantibodies against NMDA receptors at 12 weeks. We found significant antibody deposits throughout the brain, including the hippocampus, cortex and cerebellum regions (Fig. 2A, B, C).

Symptoms of memory deficits and schizophrenia-like changes are the main presentations occurred in patients with anti-NMDA receptor encephalitis [6]. To verify the effect of ABs on neurological functions

and behavioral phenotypes in mice, we performed a series of experiments targeting memory, anhedonia, depressive-like behavior, and anxiety, aggression and locomotor activity in mice after immunization. 12 weeks after immunization, evident neurological deficits were gradually observed. Most of the peptide-treated mice exhibited abnormal behaviors like hyperactivity and hunched back. Decreased exploration time and lower discrimination index were observed in LE30 immunized mice in the novel object recognition test ( $P = 0.03$ ) (Fig. 2D). Three-chamber experiment showed reduced visits to strangers in LE30-immunized mice compared with mice immunized with CFA ( $P = 0.01$ ) (Fig. 2E). In contrast, no significant differences were noted in tests of anxiety (EPM) or depressive behavior (OFT) (Fig. 2F, G).

Binding properties of peptide-induced ABs to GluN1 protein and its internalization to NMDA receptors

Binding of patients' ABs to GluN1 in patients can be prevented by single-amino acid mutation in the ATD of GluN1 (N368Q) [22]. We generated a GluN1 subunit construct with amino acid 368 mutated (N368Q) and tested the GluN1-specific clones for their reactivity in transfected HEK cells. Indeed, binding to the mutant was eliminated for the LE30 ABs (Fig. 3A).

Binding of LE30 AB to GluN1 led to the question that whether LE30 AB mediates internalization of NMDA receptors in the neurons. Primary murine hippocampal neurons were incubated with the purified human or mice CSF antibody. Antibody-binding resulted in profound downregulation of NMDA receptors-positive synaptic clusters (Fig. 3C, D). Western blots of GluN1 also showed a significantly reduced density in the membrane fractions of LE30 AB-treated neurons, indicating profound loss of synaptic NMDA receptor (Fig. 3B, D).

Inhibitory effect of ABs on LTP in the hippocampal CA1 region and NMDA receptor-mediated calcium influx.

The synaptic plasticity of neurons is closely related to learning and memory. We next tested whether the tetanus-induced LTP at Schaffer collateral-CA1 synapse in hippocampal slices was affected, CSF from patients or immunized mice was applied for 10 min before TBS was applied. Each CSF did not affect baseline transmission. The amplitude of LTP was smaller in the LE30 CSF than in the ACSF control as well as the other control groups (Fig. 4A). The magnitude of LTP at 30 min post-tetanus was significantly smaller in the human CSF ( $104.0 \pm 11.1\%$ ,  $n = 5$ ,  $p < 0.005$ ) and mice CSF ( $110.7 \pm 8.1\%$ ,  $n = 5$ ,  $p < 0.01$ ) than in the ACSF control ( $151.7 \pm 6.2\%$ ,  $n = 5$ ) (Fig. 4B, C). Consistent with the electrophysiological observations, calcium imaging experiments using patients' AB or LE30 AB revealed a profoundly reduced NMDA receptors-induced calcium influx, affecting the total amount of calcium influx (patients' AB,  $P < 0.005$ ; LE30 AB,  $P < 0.005$ ) (Fig. 4D).

## Discussion

NMDA receptor encephalitis is a common cause of autoimmune encephalitis, predominantly affecting young adults [8]. Ectopic expression of NMDA receptors associated with ovarian teratoma and other tumors is thought to mediate the initial autoimmune response against NMDA receptor encephalitis [5].

Active immune-induced animal models mimic the process of antibody production in autoimmune diseases and has played an important role in the study of neurological diseases [16, 18]. Previous studies have shown that peptide fragment was able to induce autoantibodies to the glutamate receptor (AMPA GluN3B) and cause behavioral changes and lower epileptic thresholds in mice [9, 10]. In this study, we demonstrate that active immunization with peptide LE30 targeting the ATD of GluN1 is sufficient to induce high titers of pathogenic anti-GluN1 autoantibodies and to reproduce key symptom characteristics of typical anti-NMDA receptor encephalitis in mice.

We first tested whether peptide induction could produce antibodies against GluN1 in CSF. As shown, antibodies from LE30 immunized mice specifically bind to GluN1-transfected HEK293 cells, LE30 AB showed similar characteristics with patients AB in mouse brain slice. Besides, the site-mutation N368Q in ATD of GluN1 prevented the binding of LE30 AB to GluN1, suggesting that LE30 AB share similar epitope with patient AB.

Current data supports the idea that autoantibodies targeting NMDA receptor are responsible for disease pathogenesis [3, 22]. Antibody titers appear to correlate with clinical symptoms [14, 25]. After 12 weeks of immunization, the mice exhibited significant behavioral disorders, memory loss, and elevated rates of agitation, which is consistent with key symptoms in patients with anti-NMDA receptor encephalitis [25]. Further study showed that the LE30 antibody treatment significantly reduced the GluN1 density in the membrane of primary hippocampal neurons and impaired the function of the NMDA receptor. NMDA receptor is essential in establishing synaptic plasticity and memory formation. The hippocampus targeted by the GluN1 antibody showed severe damage to LTP formation in Schaffer collateral/CA1 synapses [32]. Our electrophysiological studies have shown that GluN1 antibodies from immunized mice have similar effects. LTP records in CA1 synapses of LE30 AB-treated brain slices show severe impairment of hippocampal synaptic plasticity, which may explain memory and behavioral deficits observed in our animal models.

Over the past decade, anti-NMDA receptor encephalitis has been identified as one of the most common types of autoimmune encephalitis [28, 30]. Studies have shown that autoantibodies target NMDA receptors can induce receptor internalization, affecting normal electrophysiology and transmitter metabolism mediated by NMDA receptors. Further research may need to be completed, including immune triggers against NMDA receptor encephalitis, specific indicators related to the disease, and the involvement of T cells and other inflammatory mediators [14, 19, 31].

Our research may provide a promising model of active immunity against NMDA receptor encephalitis, which may help further research into the pathogenesis of the disease and the development of potential new therapies.

## Conclusion

We demonstrate establish a novel anti-NMDA receptor encephalitis model by active immunization with peptide LE30 targeting the ATD of GluN1. Antibodies from LE30 immunized mice showed similar

pathogenic effect with patients' antibodies. Unlike the passive antibody transfer model, this active immune model is better simulates the immunological characteristics of anti-NMDA receptor encephalitis. We will explore the pathological mechanism of anti-NMDA receptor encephalitis using this active immune model.

## Declarations

### Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Nanfang Hospital Southern Medical University. Written informed consent was obtained from individual or guardian participants.

### Consent for publication

Not applicable.

### Availability of data and materia

All data generated or analysed during this study are included in this published article.

### Competing interests

The authors declare that they have no competing interests.

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Not applicable.

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### Authors' contributions

WHH, ZJ and XW designed the study. DYW, ZZY, CJY, PY performed the experiments, analyzed the data, DYW, ZZY prepared the manuscript. WHH, QW and WHT edited the manuscript.

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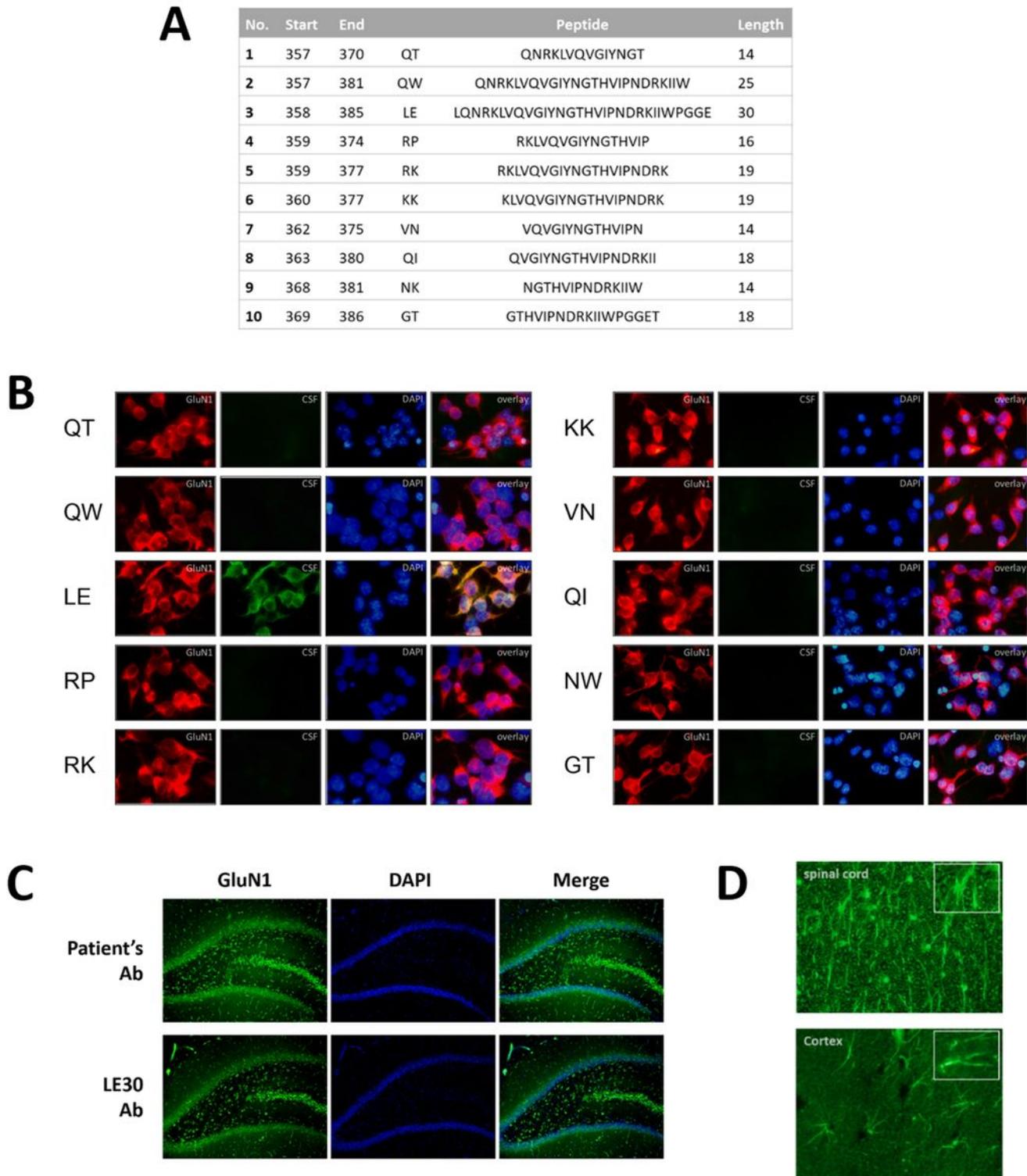
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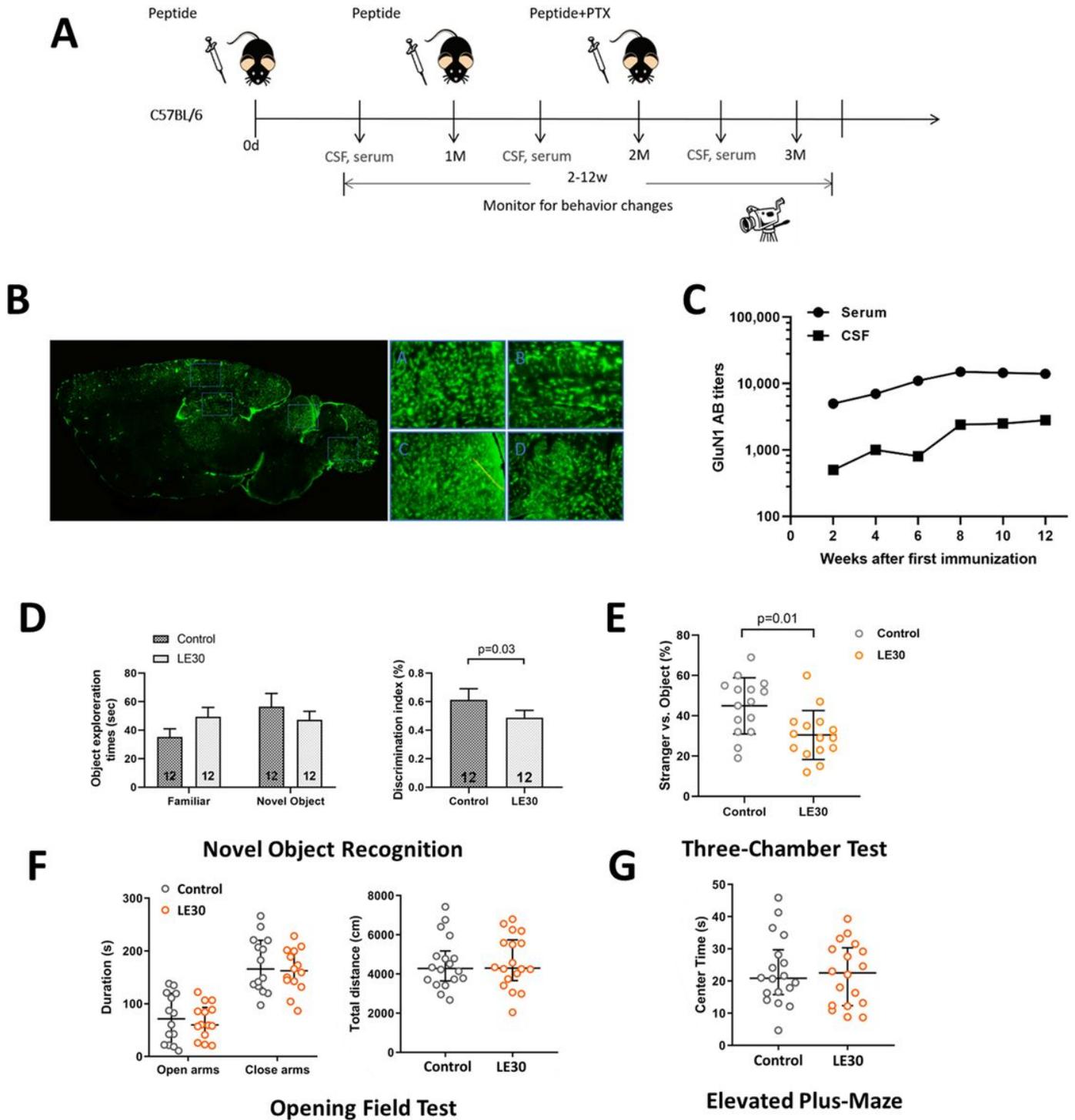
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## Figures



**Figure 1**

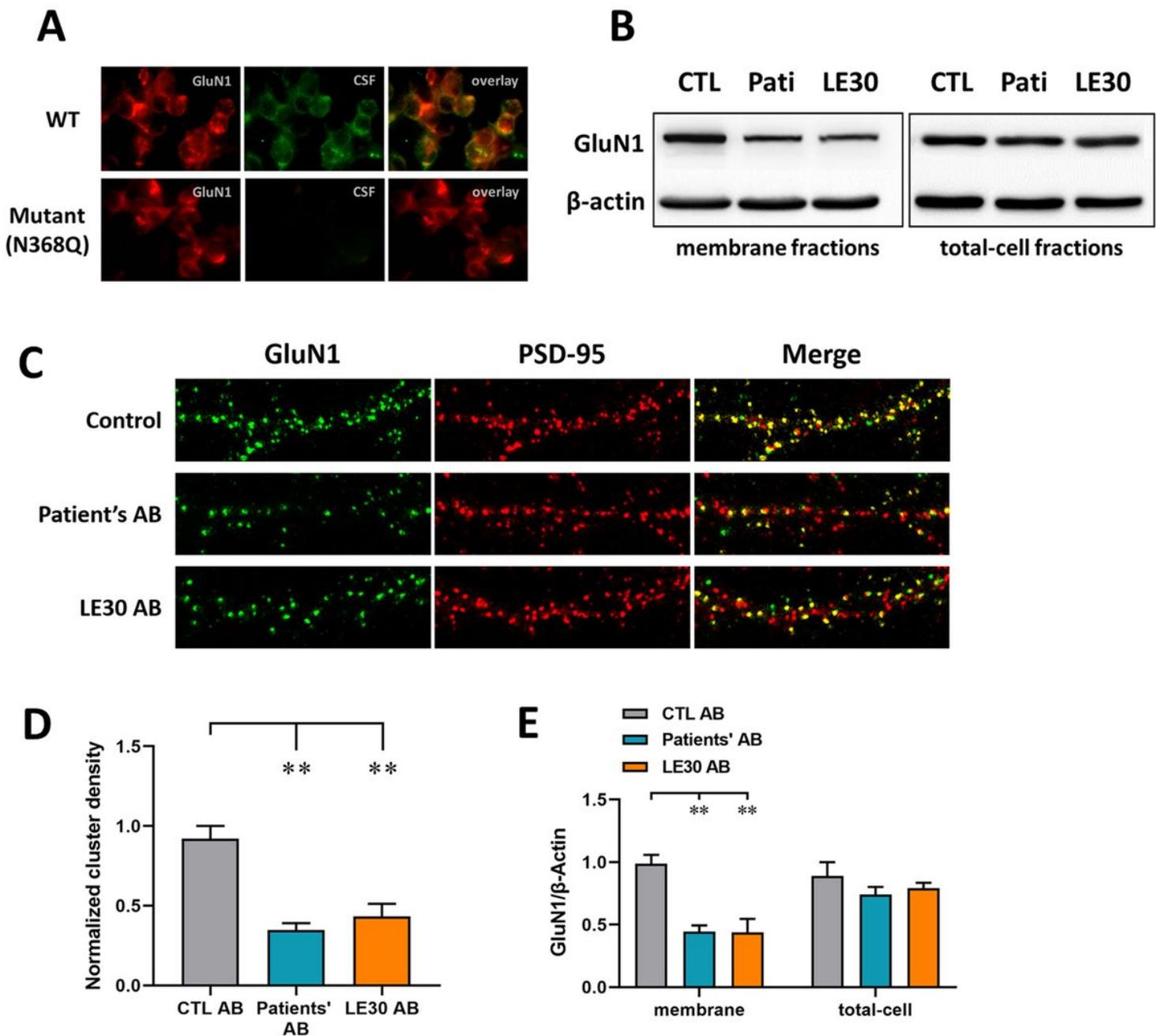
Immunostaining of mouse ABs on HEK293 and mouse brain tissue. Sequence of peptides used in the immunization experiment (A). Detection of antigen specificity of peptide-induced ABs against GluN1 protein using HEK293 cells (B). Immunofluorescence detection of antibody binding properties induced by antigen peptides in the central nervous system (C, D).



**Figure 2**

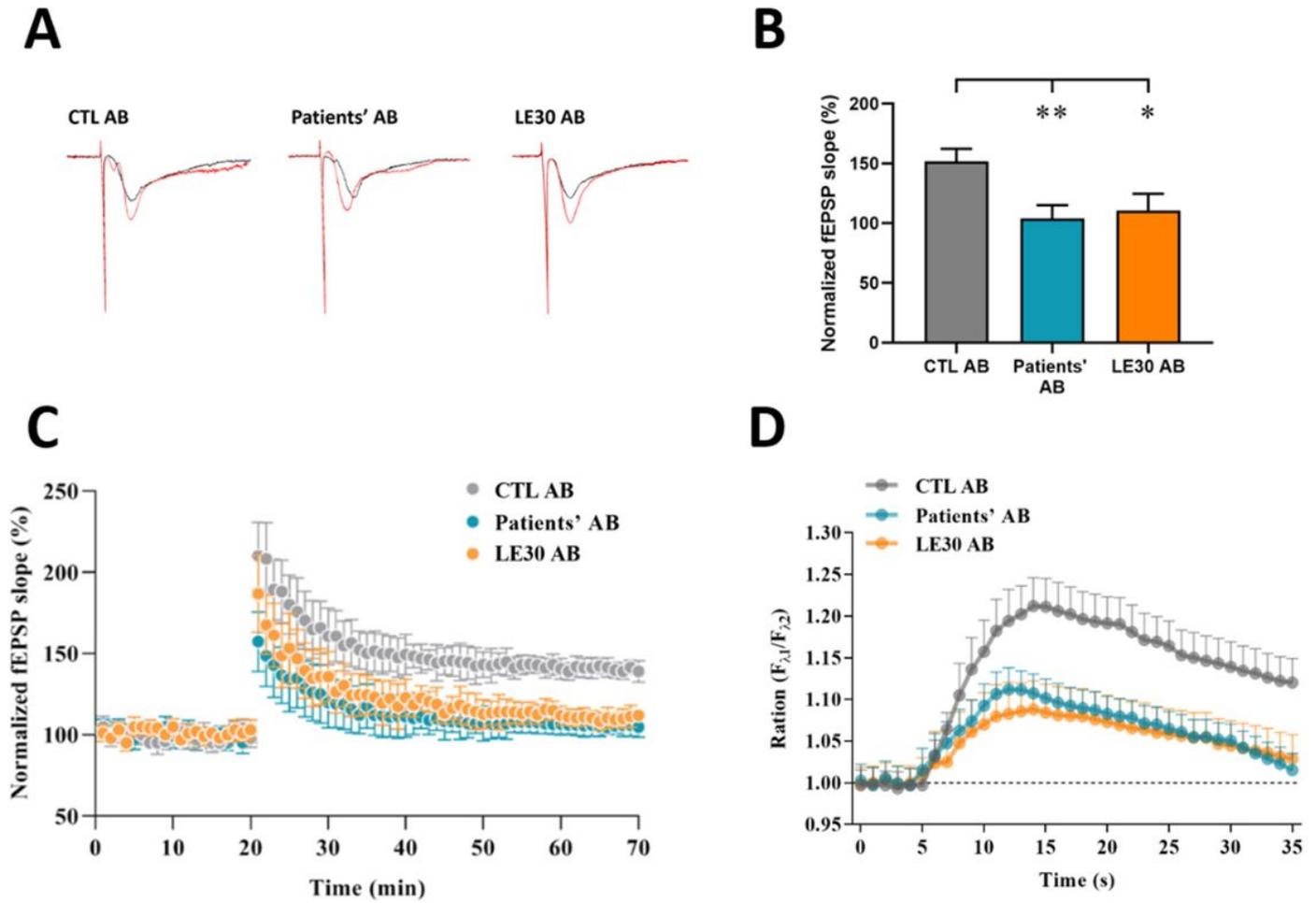
Active antibody-induced mouse antibody expression in vivo. Experimental design of active immunization of mice at different time points (A). Immunofluorescence detection of antibody deposition in mice brain tissue at week 12 (B); CSF and serum antibody titer in mice within 12 weeks (C). Memory and behavioral deficits in mice after 12 weeks of immunization. Novel-object recognition 24 h after the acquisition. object exploration during the 5 min test phase of the object recognition test (n=12) (D). Mice's social

exploration differential was affected significantly after LE30 immunization (n=15) (E). LE30 immunization does not alter the tests of depression and anxiety (F, G).



**Figure 3**

The binding characteristics of LE30 AB to GluN1 after single site mutation and its internalization to NMDA receptors. HEK cells were transfected with wild-type GluN1 or a construct with amino acid 368 mutated (N368Q). LE30 AB strongly recognized GluN1 but staining of the mutant was eliminated (A). LE30 AB reduced the expression of GluN1 on the surface, but did not affect total GluN1 on hippocampal neurons (B, C, D, E).



**Figure 4**

Electrophysiological effects in neurons after exposure to CSF from immunized mice. Effect of patient and mouse CSF-derived ABs on the tetanus-induced LTP (A). Normalized fEPSP slope in hippocampus slice after acute CSF application (B, C). Effect of patients' AB and LE30 AB on NMDA receptor-mediated calcium influx (D).