Anti-NMDAR encephalitis induced in mice by active immunization with a peptide from amino-terminal domain of the GluN1 subunit

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Short report

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Abstract

Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis is a recently discovered autoimmune syndrome associated with psychosis, dyskinesia, and seizures. However, the underlying mechanisms of this disease remain unclear, in part because of a lack of suitable animal models. This study describes a novel mouse model of anti-NMDAR encephalitis that was induced by active immunization against NMDARs using amino-terminal domain peptides. After 12 weeks of immunization, the mice showed significant behavioral disorders and memory loss. Furthermore, antibodies from the cerebrospinal fluid of immunized mice lowered the surface NMDAR cluster density in hippocampal neurons. Immunization also impaired long-term potentiation at Schaffer collateral–CA1 synapses and reduced NMDAR-induced calcium influx. This novel mouse model may allow further research into the pathogenesis of anti-NMDAR encephalitis and aid in the development of new therapies for this disease.

Background

Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis predominantly affects young women. The typical clinical manifestations include a progressive development of neurologic and psychiatric symptoms, including abnormal movements, seizures, impaired memory, and behavior disorders (1). Almost 80% of patients show cerebrospinal fluid (CSF) abnormalities, presenting as mild lymphocytic pleocytosis and normal or moderately increased protein levels and 60% of patients have specific CSF oligoclonal bands (2, 3).

Approximately 50% of women having an underlying ovarian teratoma that contains nervous tissue expressing NMDARs (4). It is believed that the ectopic expression of NMDARs contributes to triggering the immune response (5). Antigens released by tumor cells are taken up by antigen-presenting cells that travel to regional lymph nodes, and plasma cells produce antibodies that later cross-react with NMDARs in the brain following impaired blood–brain barrier permeability (6). NMDARs mediate glutamatergic synaptic transmission and have a prominent role in synaptic plasticity. Previous studies have suggested that the pathogenicity of autoimmune antibodies (ABs) is a key mechanism of anti-NMDAR encephalitis (7, 8). Human CSF-derived NMDAR ABs downregulate NMDAR levels in both *in vitro* and *in vivo* studies. In addition, reduced NMDAR expression can result in increased extracellular glutamate and thus affect the pons/medullary respiratory center (9).

Passive immunization in mice, using intrathecal infusion of CSF from affected humans, leads to behaviors of depression, anhedonia, and memory deficits. However, although these mice provide evidence of the effects of autoantibodies on the disease, passive immunization does not produce anti-NMDAR encephalitis pathogenesis in humans (4, 10). Thus, an animal model of active immunity is needed that more closely mimics disease progression.

Previous investigations have highlighted the extracellular amino-terminal domain (ATD) of the GluN1 subunit, especially the N368/G369 region, as essential for immunoreactivity (11, 12). Therefore, ATD

peptides were used to immunize mice within 12 weeks. The mice demonstrated behavioral changes and AB infiltration that were most prominent in the hippocampus. The presence of NMDAR ABs and its effect on NMDARs was also confirmed.

Methods

Study design and mice immunization

The study aimed to investigate the effects of active immunization with NMDAR peptides in normal adult mice. C57BL/6 mice (10 weeks old) were immunized with different GluN1 extracellular peptides emulsified in an equal volume of Complete Freund's Adjuvant supplemented with *Mycobacterium tuberculosis* H37Ra (4 mg/mL) at a final concentration of 1 mg/mL. Mice were subcutaneously injected with 200 μg of GluN1 peptides or control peptides at the tail base, and received two booster injections with CFA at 4 and 8 weeks after the first immunization. All mice were intraperitoneally injected with 400 ng pertussis toxin at the last immunization. Behavioral tests and histological staining were performed 12 weeks after the first immunization.

Patient sample collection

We collected CSF from patients with high titers of anti-GluN1 ABs (> 1:300) during routine clinical practice. All patients fulfilled the clinical diagnostic criteria for anti-NMDAR encephalitis, revised in 2016 (13). The study protocol was approved by the ethics committee of Nanfang Hospital, Southern Medical University, and written informed consent was obtained from each participant or their guardian.

AB purification

The CSF and serum ABs from patients or immunized mice were purified using protein G Sepharose columns and were then used to treat neurons or brain slices. For the purification process, 2 mL of diluted sample was incubated in a chromatography spin column (Thermo Scientific) of protein G Sepharose beads for 30 min. After three washes with phosphate-buffered saline (PBS), the samples 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 (HEK293) cells were transiently transfected with NMDAR subunit genes (NR1/NR2A; DsRed2-labeled) as previously described (14). Twenty-four hours later, cells were fixed on coverslips with acetone and incubated overnight at 4 °C with the purified ABs from patient or immunized mice CSF (starting at 1:1) in 0.1% bovine serum albumin (BSA) in PBS. After washing with PBS, the cells were labeled with fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (ab7149, Abcam) or FITC-conjugated anti-mice IgG (ab6785, Abcam) 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'-gggatgacatgggtaccttggtagatgcccacttgca-3'; Reverse: 5'-tgcaagtgggcatctaccaaggtacccat gtcatccc-3'.

Primary neuronalcultures

Hippocampal neurons were prepared and maintained from embryonic day 18 rat brains as previously described (15). The hippocampi were dissociated with papain at 37 °C for 30 min, 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 onto poly-D-lysine-treated 24-well plates. After 6 h, the supernatant was removed and replaced with 500 μL of fresh culture medium. Cells were then cultured for 14 days for subsequent experiments.

Immunocytochemistry

Immunocytochemistry was used to detect binding to brain slices and autoantibodies. On day 14 after immunization, CSF was obtained from mice, and was then purified and incubated with brain slices. The slices were imaged using a confocal microscope (LSM 880, Carl Zeiss, Germany). To stain surface NMDAR, neurons were incubated with ABs derived from the CSF of patients or immunized mice. After incubating for 18 h, the AB-bound surface receptors were labeled with a fluorescent-conjugated secondary AB (1:200, anti-Mouse Alexa Fluor 488, A-11029; 1:200, anti-Human Alexa Fluor 488, A-11013; Invitrogen). Neurons were then fixed, permeabilized, incubated with anti-PSD95 primary AB (1:200, Synaptic Systems, 124-003) to label postsynaptic densities, and visualized after staining with Alexa Fluor 647 (1:200, Invitrogen, A-21244).

Electrophysiological recording

Brain slices preparing and electrophysiological recording were performed as previously described (16). Mouse hippocampal slices (300 μm) were prepared using a vibratome (VT1000S, Leica, Germany). The slices were kept at 30 °C for at least 60 min before experiments in artificial CSF (ACSF; NaCl 124 mM, KCl 2.5 mM, $MgSO_4$ 2.0 mM, NaH_2PO_4 1.25 mM, $NaHCO_3$ 26 mM, $CaCl_2$ 2 mM, and glucose 10 mM; pH 7.3), bubbled with a mixture of 95% O_2 and 5% CO_2. Field excitatory postsynaptic potentials were evoked in the CA1 stratum radiatum by stimulating Schaffer collaterals with a two-concentric bipolar stimulating electrode, and were recorded with ACSF-filled glass pipettes. LTP was induced by applying theta burst stimulation. Purified ABs were diluted in ACSF (100 μg/mL) and applied by switching the perfusion from the control ACSF to the AB-containing ACSF. For each recording, the baseline synaptic transmission was monitored for 10 min before AB perfusion, and was washed out with ACSF continuously after theta burst stimulation until the end of the experiment. The acquired data were analyzed with pClamp 10 software (Axon Instruments, USA).

Calcium imaging

Hippocampal neurons were incubated with 20 μg/mL of patient or mice ABs at 37 °C for 18 h. To detect calcium flux, the cells were loaded with Fura-2 (1 μM, Invitrogen, F1221) and incubated at 37 °C for 15–30 min, followed by 15–30 min incubation at room temperature. After washing in Tyrode's solution, the cells were transferred into wells containing NBQX (10 μM, Tocris, 0373). NMDA (10 μM, Sigma, M3262) was used to stimulate NMDAR-mediated calcium influx. Imaging was performed using an inverted fluorescence microscope (Nikon Eclipse TE2000-U, Japan) with a charge-coupled device camera. Series of images were acquired at 800 ms intervals for 40 s at an excitation wavelength of 470 nm. The fluorescence intensity at each timepoint was measured using ImageJ (NIH).

Behavioral assessments

After 12 weeks of immunization, LE30-immunized mice were tested using a series of behavioral experiments. Mice immunized with a control peptide were used as the control. Behavioral testing was conducted at uniform times, from 09:00 to 12:00, by researchers blinded to the group allocations. Behavioral parameters were recorded using a video tracking system (Smart 3.0, Panlab, Spain). Details of behavioral tests are described in the *Supplementary Methods*.

Western blot analyses

Minute Plasma Membrane Protein Isolation Kit (SM-005, Invent Biotechnologies) was used to extract membrane and total cell proteins from the primary neurons after incubated with purified human or mice CSF ABs according to the manufacturer's protocol. Proteins in SDS loading buffer were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking with 5% BSA, the membranes were incubated with anti-GluN1 ABs (1:500, Synaptic Systems, 114-011) and a secondary HRP-conjugated AB. The immunocomplexes were detected using enhanced chemiluminescence.

Statistical analyses

Data were presented as mean ± SEM. Independent sample *t*-tests or Mann–Whitney *U* tests were used as appropriate for each experiment. Kruskal–Wallis tests were used for nonparametric data. All analyses were performed using SPSS V24.0 (IBM, USA) and the histograms were made using GraphPad Prism 6.0 (GraphPad Software, USA) or ImageJ. $P<0.05$ was considered statistically significant.

Results

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

To induce an active immune mouse model of anti-NMDAR encephalitis, mice were immunized by subcutaneous injection with 200 μg peptide emulsified in Complete Freund's Adjuvant (Fig. 1A, B). CSF from peptide-immunized mice was collected 14 days after immunization. The CSF ABs were purified and

detected on HEK293T cells transfected with GluN1 subunits (DsRed2-labeled). We found that only the LE30 peptide could induce CSF autoantibodies that were specific for GluN1 (Fig. 1C). The binding of ABs from peptide-immunized mice were detected using immunofluorescence. ABs from immunized mice were mainly deposited in the hippocampus. This was similar to the binding of ABs extracted from patients (Figure 1D). AB binding was also observed in cortical glial cells and spinal cord gray matter (Fig. 1E).

Levels of LE30-peptide-induced autoantibodies expression in mice

The AB-mediated internalization of NMDAR is a reversible process (9). When the ABs disappear, NMDAR function may be restored. To induce high concentrations of autoantibodies targeting NMDARs, we repeatedly immunized mice with LE30 peptide, with three immunizations at 4-week intervals. This procedure maintained the titer of autoantibodies within the CSF and serum of mice. At 12 weeks after the first immunization, we used the brains of LE30-peptide-immunized mice for immunofluorescence experiments targeting autoantibodies against NMDARs. There were significant AB deposits throughout the brain, including in the hippocampus, cortex, and cerebellum (Fig. 2A, B).

Behavioral changes of mice after immunized with LE30-peptide

Symptoms of memory deficits and schizophrenia-like changes are the main presentations that occur in patients with anti-NMDAR encephalitis (17). To verify the effects of ABs on neurological function and behavioral phenotypes in mice, we performed a series of experiments investigating memory, anhedonia, depressive-like behavior, anxiety, aggression, and locomotor activity in immunized mice. At 12 weeks after immunization, neurological deficits were observed. Most of the peptide-treated mice exhibited abnormal behaviors, including hyperactivity and hunched backs. We also observed decreased exploration time and a lower discrimination index in LE30-immunized mice in the novel object recognition test (n=12, P=0.03) (Fig. 2C). The three-chamber experiment revealed that LE30-immunized mice made reduced visits to strangers compared with CFA-immunized mice (n=15, P=0.01) (Fig. 2D). In contrast, there were no differences between the mice in tests of anxiety or depressive behavior (open field test and elevated plus-maze) (Fig. 2E, F).

Binding properties of peptide-induced ABs to GluN1 protein and the internalization of NMDARs

The binding of ABs to GluN1 in human patients can be prevented by a single amino acid mutation in the ATD of GluN1 (N368Q) (11). We therefore generated a GluN1 subunit construct with mutated amino acid 368 (N368Q) and tested the GluN1-specific clones for their reactivity in transfected HEK293 cells. Binding to the mutant was eliminated for the LE30 ABs (Fig. 3A).

The binding of the LE30 ABs to GluN1 led to question whether LE30 AB mediates the internalization of NMDARs in neurons. We therefore incubated primary murine hippocampal neurons with purified human or mice CSF ABs. AB binding resulted in a marked downregulation of NMDAR-positive synaptic clusters (Fig. 3B, C). Western blots of GluN1 also revealed significantly reduced density in the membrane fractions of LE30-AB-treated neurons, indicating a profound loss of synaptic NMDARs (Fig. 3D, E).

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

The synaptic plasticity of neurons is closely related to learning and memory. We next tested whether tetanus-induced LTP was affected at Schaffer collateral–CA1 synapses in hippocampal slices. To do this, CSF from patients or immunized mice was applied for 10 min before theta burst stimulation was applied. Neither of the CSF types affected baseline transmission. The LTP amplitude was smaller in the LE30 CSF group compared with the ACSF control and other control groups (Fig. 4A). The magnitude of LTP at 30 min post-tetanus was significantly smaller with human CSF (104.0±11.1%, n=5, P<0.005) and mice CSF (110.7±8.1%, n=5, P<0.01) compared with the ACSF control (151.7±6.2%, n=5) (Fig. 4B, C). Consistent with these electrophysiological observations, calcium imaging experiments using patient ABs or LE30 ABs revealed a marked reduction in NMDAR-induced calcium influx, affecting the total amount of calcium influx (patients' ABs, n=5, P<0.05; LE30 ABs, n=5, P<0.05) (Fig. 4D).

Discussion

Anti-NMDAR encephalitis is a common cause of autoimmune encephalitis, predominantly affecting young adults (18). The ectopic expression of NMDARs that is associated with ovarian teratoma and other tumors is thought to mediate the initial autoimmune response in anti-NMDAR encephalitis (19). Active immune-induced animal models mimic the process of AB production in autoimmune diseases and have played an important role in the study of neurological diseases (20, 21). Previous investigations have revealed that peptide fragments are able to induce autoantibodies to glutamate receptors (AMPA GluN3B), lower epileptic thresholds, and cause behavioral changes in mice (22). In the present study, we demonstrated that active immunization with peptide LE30 targeting the ATD of GluN1 is sufficient to induce high titers of pathogenic anti-GluN1 autoantibodies. This immunization also reproduced many typical anti-NMDAR encephalitis symptoms in mice.

We first tested whether peptide induction was able to produce ABs against GluN1 in the CSF. The ABs from LE30-immunized mice specifically bound to GluN1-transfected HEK293 cells, and LE30 ABs showed similar characteristics to patient ABs in mouse brain slices. In addition, the site mutation at N368Q in the ATD of GluN1 prevented the binding of LE30 ABs to GluN1, suggesting that LE30 ABs share similar epitopes with patient ABs.

Current data support the idea that autoantibodies targeting NMDARs are responsible for disease pathogenesis (23, 24), and AB titers appear to correlate with clinical symptoms (25). After 12 weeks of immunization, the mice in our study exhibited behavioral disorders, memory loss, which is consistent with the key symptoms of patients with anti-NMDAR encephalitis (1). Further investigation revealed that LE30 AB treatment significantly reduced GluN1 density in the membrane of primary hippocampal neurons, and impaired NMDAR function. NMDARs are essential for establishing synaptic plasticity and memory formation. When the hippocampus is targeted by GluN1 ABs, there is severe damage to LTP formation in Schaffer collateral–CA1 synapses (16). Our electrophysiological results revealed that GluN1 ABs from immunized mice have similar effects. The LTP recordings from CA1 synapses of LE30-AB-treated brain

slices showed severe impairment of hippocampal synaptic plasticity, which may explain the memory and behavioral deficits observed in our animal models.

Over the past decade, anti-NMDAR encephalitis has been identified as one of the most common types of autoimmune encephalitis (26, 27). Studies have shown that autoantibodies targeting NMDARs can induce receptor internalization, thereby affecting NMDAR-mediated normal electrophysiology and transmitter metabolism (28, 29).

At present, the triggering factors for the autoimmune response to NMDA receptor is still unclear. Previous research using holoprotein immunogens of tetrameric Xenopus laevis GluN1/GluN2B or rat GluN1/GluN2A receptor to produce NMDA receptor antibodies has showed clinical symptoms similar to patients (21). Active immunization of $ApoE^{-/-}$ mice against peptides fragments leads to high circulating levels of NMDAR AB and was able to induce psychosis-like symptoms upon MK-801 challenge, characterized by an open blood-brain barrier (23, 30). Intranasal infection with herpes simplex virus has also been shown to induce circulating NMDAR antibodies (31), which may be used to explain the pathogenesis of secondary anti-NMDA receptor encephalitis in patients with herpes simplex virus encephalitis. The peptide from ATD of the GluN1 subunit was used as immunogens in our study and is showed sufficient to induce high titers of pathogenic anti-GluN1 autoantibodies. Active immunization also reproduced typical anti-NMDAR encephalitis symptoms in mice. it may be a convenient alternate endogenous model of anti-NMDAR encephalitis, which may be useful for further research into the pathogenesis of this disease and aid in the development of potential new therapies.

Conclusion

We established a novel anti-NMDAR encephalitis model using active immunization with peptide LE30 targeting the ATD of GluN1. ABs from LE30-immunized mice had a similar pathogenic effect to ABs from patients. Compared with the passive AB transfer model, this active immune model better simulates the immunological characteristics of anti-NMDAR encephalitis. In future studies, we will explore the pathological mechanisms of anti-NMDAR 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 Declaration of Helsinki and was approved by the Human Ethics Committee of Nanfang Hospital Southern Medical University. Written informed consent was obtained from individual participants or their guardians.

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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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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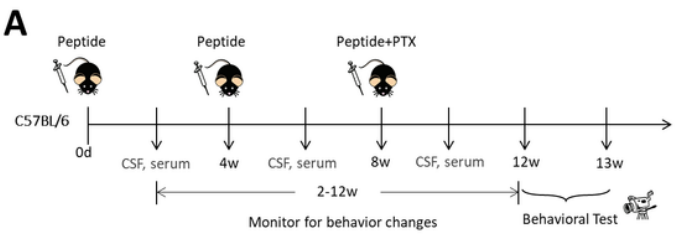
Figures



B

No.	Start	End		Peptide	Length
1	357	370	QT	QNRKLVQVGIYNGT	14
2	357	381	QW	QNRKLVQVGIYNGTHVIPNDRKIIW	25
3	356	385	LE	LQNRKLVQVGIYNGTHVIPNDRKIIWPGGE	30
4	359	374	RP	RKLVQVGIYNGTHVIP	16
5	359	377	RK	RKLVQVGIYNGTHVIPNDRK	19
6	360	377	KK	KLVQVGIYNGTHVIPNDRK	19
7	362	375	VN	VQVGIYNGTHVIPN	14
8	363	380	QI	QVGIYNGTHVIPNDRKII	18
9	368	381	NW	NGTHVIPNDRKIIW	14
10	369	386	GT	GTHVIPNDRKIIWPGGET	18

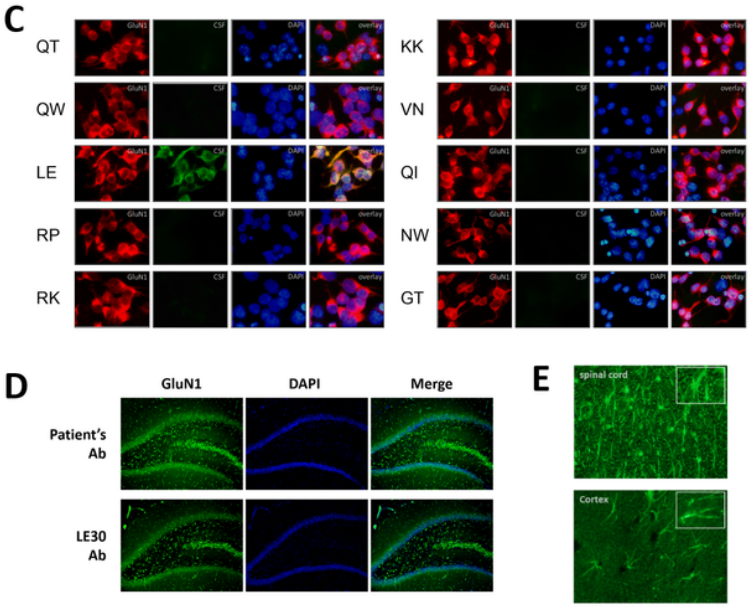


Figure 1

Active immunized mouse AB expression in vivo. The experimental design for active immunization of mice at different time points (A). The peptide sequence used in the immunization experiment (A). Immunofluorescence detection of peptide-induced ABs against GluN1 protein using HEK293 cells (B). Immunofluorescence detection of the AB binding properties induced by antigen peptides in the central nervous system (C, D).

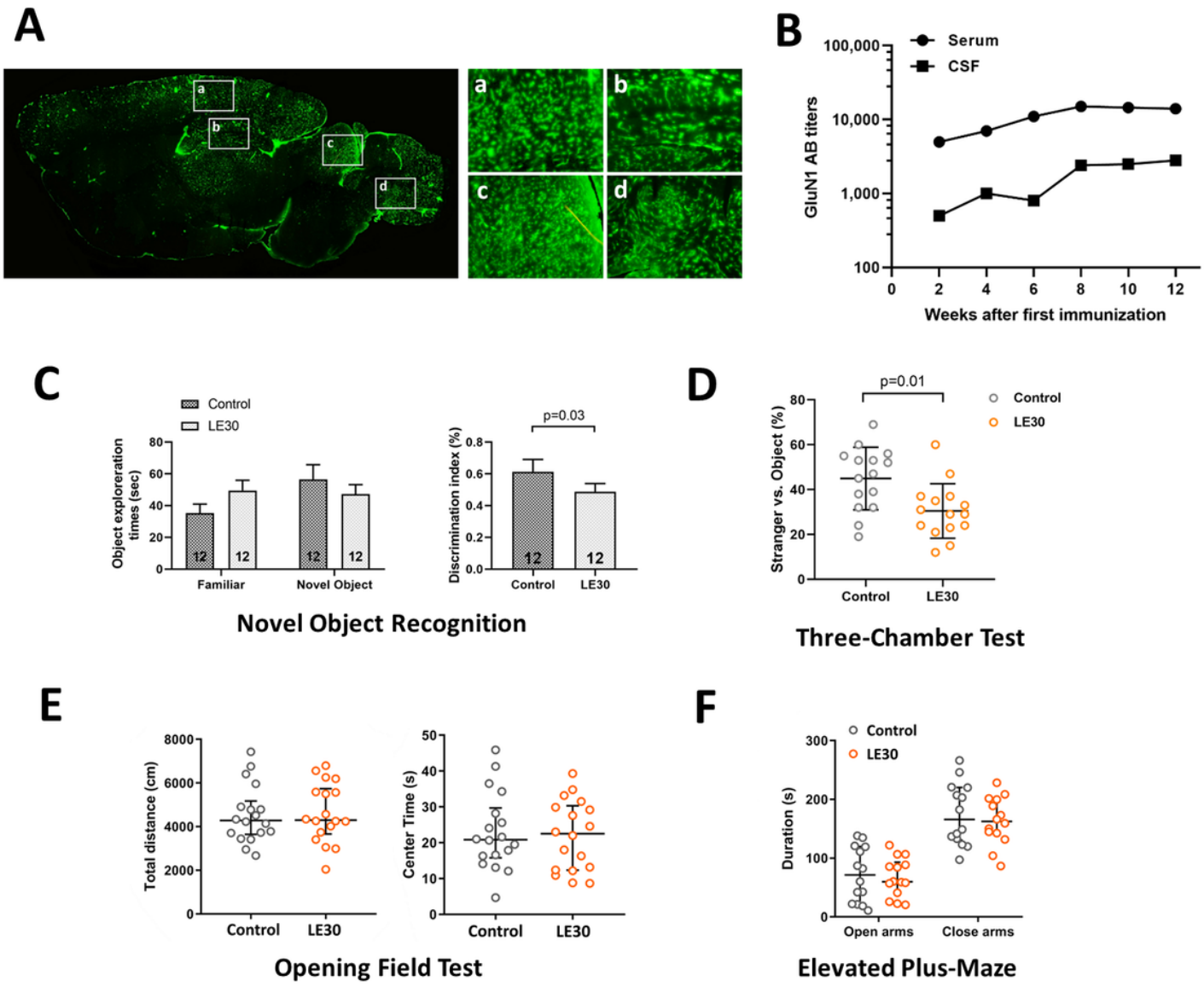


Figure 2

ABs produce and Behavioral changes of mice after 12-week immunization with LE30-peptide. Immunostaining of endogenous ABs on mouse brain tissue (A); CSF and serum AB titer in mice after 12 weeks of immunization (B). Memory and behavioral deficits in mice after 12 weeks of immunization. Novel object recognition 24 h after acquisition. Object exploration during the 5-min test phase of the object recognition test (n=12) (C). The social exploration differential was significantly affected in mice after LE30 immunization (n=15) (D). LE30 immunization did not alter depression and anxiety behaviors (E, F).

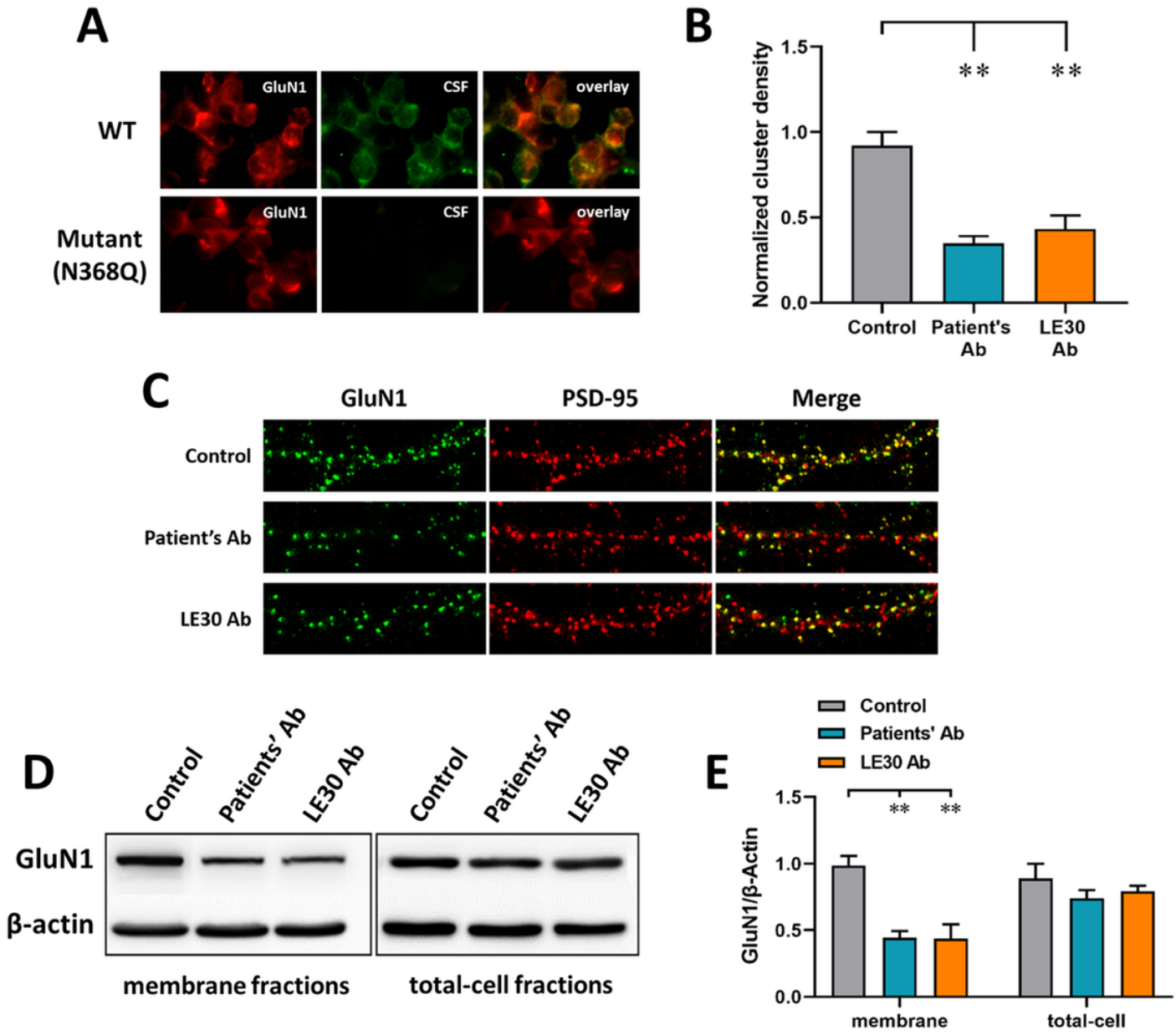


Figure 3

Binding characteristics of LE30 ABs to GluN1 after single-site mutation, and NMDAR internalization. HEK293 cells were transfected with wild-type GluN1 or a construct with mutated amino acid 368 (N368Q). LE30 ABs strongly recognized GluN1, but staining was eliminated with the mutant (A). LE30 ABs reduced the expression of GluN1 on the cell surface, but did not affect the total GluN1 in hippocampal neurons (B, C, D, E). *p<0.05; **p<0.01.

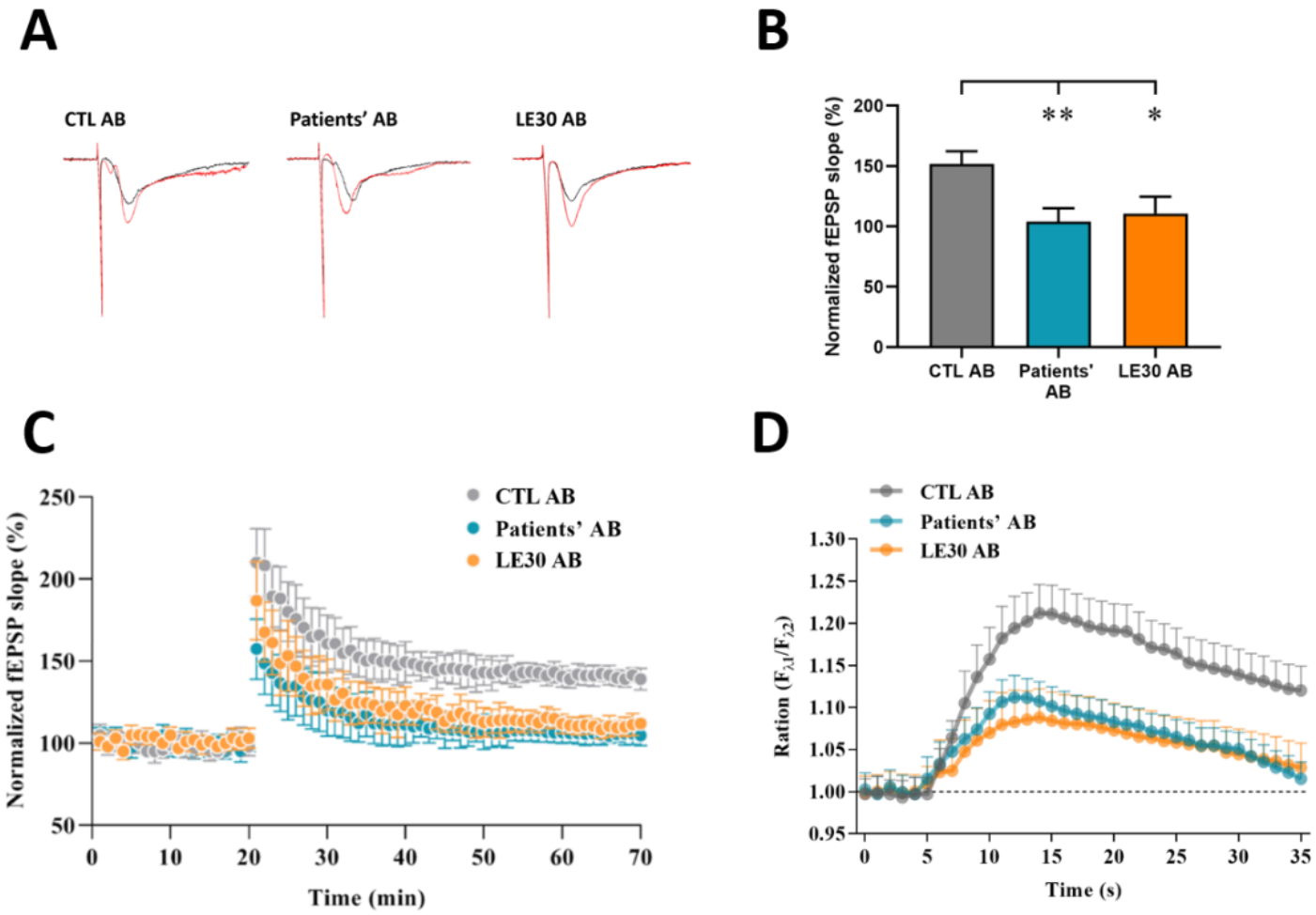


Figure 4

Electrophysiological effects on neurons of exposure to CSF from immunized mice and patients. The effects of patient- and mouse-CSF-derived ABs on tetanus-induced LTP (A). Normalized slope of field excitatory postsynaptic potentials in a hippocampal slice after acute CSF application (B, C). Effects of patient ABs and LE30 ABs on NMDAR-mediated calcium influx (D). *p<0.05; **p<0.01.

Supplementary Files

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- SupplementaryMethods.docx