

Identification and Characterization of GABA_A Receptor Autoantibodies in Autoimmune Encephalitis

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Autoimmune forms of encephalitis have been associated with autoantibodies against synaptic cell surface antigens such as NMDA- and AMPA-type glutamate receptors, GABA_B receptor, and LGI1. However, it remains unclear how many synaptic autoantigens are yet to be defined. Using immunoproteomics, we identified autoantibodies against the GABA_A receptor in human sera from two patients diagnosed with encephalitis who presented with cognitive impairment and multifocal brain MRI abnormalities. Both patients had antibodies directed against the extracellular epitope of the β 3 subunit of the GABA_A receptor. The β 3-subunit-containing GABA_A receptor was a major target of the patients' serum antibodies in rat hippocampal neurons because the serum reactivity to the neuronal surface was greatly decreased by 80% when the β 3 subunit was knocked down. Our developed multiplex ELISA testing showed that both patients had similar levels of GABA_A receptor antibodies, one patient also had a low level of LGI1 antibodies, and the other also had CASPR2 antibodies. Application of the patients' serum at the time of symptom presentation of encephalitis to rat hippocampal neuron cultures specifically decreased both synaptic and surface GABA_A receptors. Furthermore, treatment of neurons with the patients' serum selectively reduced miniature IPSC amplitude and frequency without affecting miniature EPSCs. These results strongly suggest that the patients' GABA_A receptor antibodies play a central role in the patients' symptoms. Therefore, this study establishes anti-GABA_A receptor encephalitis and expands the pathogenic roles of GABA_A receptor autoantibodies.

Key words: autoantibody; autoimmune encephalitis; cognitive impairment; GABA_A receptor; seizure; thymoma

Introduction

Autoimmune neurological disorders are induced through the production of autoantibodies. Identifying the target antigens and elucidating the pathogenic mechanisms of autoantibodies play extremely important roles in the diagnosis and treatment of autoimmune disorders (Vincent et al., 2006; Moscato et al., 2010;

Lancaster and Dalmau, 2012). In particular, autoantibodies to synaptic cell surface antigens have attracted considerable attention because such antibodies may be directly pathogenic by interfering with synaptic functional proteins.

In the CNS, antibodies to the metabotropic glutamate receptor 1, which cause cerebellar ataxia, were found in two patients with Hodgkin's disease (Sillevis Smitt et al., 2000). Antibodies to the ionotropic NMDA-type glutamate receptor were then identified in many patients with ovarian tumors, psychiatric symptoms, amnesia, seizures, and impaired consciousness (Dalmau et al., 2007). This disease has since been established as "anti-NMDA receptor encephalitis" (Dalmau et al., 2008). Since 2009, immunoprecipitation coupled with mass spectrometry analysis using patient serum antibodies has accelerated the identification of target antigens associated with limbic encephalitis characterized by subacute onset of amnesia and seizures. Another major ionotropic glutamate receptor, the AMPA receptor (Lai et al., 2009), the inhibitory metabotropic GABA_B receptor (Lancaster et al., 2010), and CASPR2 and LGI1, which were previously recognized as the voltage-gated potassium channel (VGKC) (Irani et al., 2010; Lai et al., 2010), were identified as cell surface autoantigens in patients with limbic encephalitis. In addition, antibodies to inhibitory ionotropic glycine receptor were reported in a spec-

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trum of brainstem and spinal hyperexcitability disorders (stiff-person syndrome phenotype) (Hutchinson et al., 2008; McKeon et al., 2013).

The ionotropic GABA_A receptor mediates most of the fast inhibitory synaptic transmission in the brain and is composed of heteropentameric assemblies of different subunit subtypes [α (1–6), β (1–3), γ (1–3), δ , ϵ , θ , π , and ρ (1–3)] to form chloride ion channels (Macdonald and Olsen, 1994; Jacob et al., 2008; Rudolph and Knoflach, 2011). The majority of GABA_A receptors contain two α subunits, two β subunits, and one γ or δ subunit. The GABA_A receptor plays a central role in the regulation of brain excitability and is targeted by many antiepileptic, sedative, and anxiolytic drugs, including benzodiazepines and barbiturates. In addition, mutations in human GABA_A receptor subunits, including α 1, β 3, γ 2, and δ , cause genetic epilepsy syndromes (Macdonald et al., 2010) and genetic loss of the β 3 subunit in mice causes seizures and learning and memory deficits (DeLorey et al., 1998). Therefore, although the GABA_A receptor can be a strong candidate affected in autoimmune CNS disorders, GABA_A receptor antibodies have not yet been reported.

Here, using a nonbiased proteomic method, we identified autoantibodies against the GABA_A receptor in two patients with encephalitis. The patients' GABA_A receptor antibodies specifically caused downregulation of GABA_A receptors. The present study establishes a pathogenic role of GABA_A receptor antibodies in certain cases of encephalitis.

Materials and Methods

Experiments. The experiments using human sera were reviewed and approved by ethic committees at the National Institute for Physiological Sciences (NIPS), Nagoya University, and Kagoshima University, and written informed consent was obtained from all patients or their family members. All animal studies were reviewed and approved by the ethic committees at NIPS and were performed according to the institutional guidelines concerning the care and handling of experimental animals.

Study population and serum samples. We collected ~1200 serum samples from patients who were diagnosed with or suspected of immune-mediated disorders of the CNS or PNS. These patients were seen by us or by clinicians at other institutions in Japan. To screen for synaptic cell surface autoantigens, we selected serum samples from 116 patients diagnosed with or suspected of immune-mediated encephalitis (59 males and 57 females), which included 76 limbic encephalitis, 24 encephalitis, nine encephalopathy, four anti-NMDA receptor encephalitis, and three Hashimoto encephalopathy cases. These patients presented with subacute onset of some CNS symptoms, including cognitive impairment, confusion/disorientation, and/or seizures. For the first screening, these 116 samples with encephalitis (and also 49 samples with other immune-mediated neurological disorders described in the following sentence) were tested for binding to the cell surface of cultured rat hippocampal neurons. For the second round of screening for binding to the cell surface of COS7 cells expressing the GABA_A receptor, we tested serum samples from all of the 116 patients with encephalitis and an additional 94 control subjects (54 males and 40 females): 49 patients (32 males and 17 females) with or suspected of other immune-mediated neurological disorders, including 35 neuromyotonia, six cramp-fasciculation syndrome, three myasthenia gravis, two Morvan syndrome, one stiff-person syndrome, one Guillain-Barre syndrome, and one chronic inflammatory demyelinating polyneuropathy case; 22 patients (10 males and 12 females) with neurodegenerative diseases, including seven amyotrophic lateral sclerosis, six spinocerebellar degeneration, five multiple system atrophy, two Parkinson's disease, one corticobasal degeneration, and one frontotemporal lobar degeneration case; and 23 healthy individuals (12 males and 11 females). The study population and control subjects contained 19 patients with the complications of thymoma (including 12 invasive thymoma cases). The serum samples of Patient 1 and Patient 2 used for the

present screening were both from their initial episodes of encephalitis (Table 1). Due to the current unavailability of patients' CSF samples, only the serum samples were used in the present study.

Antibodies. The antibodies used in this study included the following: rabbit polyclonal antibodies to GABA_A receptor α 2 (catalog #600-401-D45 RRID:AB_11182018; Rockland Immunochemicals), α 5 (catalog #AB9678 RRID:AB_570435; Millipore), β 3 (catalog #ab4046 RRID:AB_2109564; Abcam), γ 2 (extracellular epitope, catalog #224 003 RRID:AB_2263066; Synaptic Systems), and AMPA receptor GluA1 (catalog #AB1504 RRID:AB_2113602; Millipore; and extracellular epitope, catalog #PC246-100UG RRID:AB_564636; Millipore); guinea pig polyclonal antibodies to vGAT (catalog #131 005 RRID:AB_1106810; Synaptic Systems) and vGluT1 (catalog #AB5905 RRID:AB_2301751; Millipore); and mouse monoclonal antibodies to GABA_A receptor α 1 (catalog #75-136 RRID:AB_2108811; NeuroMab), β 1 (catalog #75-137 RRID:AB_2109406; NeuroMab), β 2/ β 3 (extracellular epitope, catalog #MAB341 RRID:AB_2109419; Millipore), β -catenin (catalog #610153 RRID:AB_397554; BD Biosciences), gephyrin (catalog #147 021 RRID:AB_1279448; Synaptic Systems), PSD-95 (catalog #MA1-046 RRID:AB_2092361; Thermo Scientific), and N-cadherin (catalog #610921 RRID:AB_398236; BD Biosciences).

Plasmid construction. The cDNAs of rat GABA_A receptor α 1 (NM_183326), α 2 (NM_001135779), α 5 (NM_017295), β 1 (NM_012956), and β 3 (NM_017065) were cloned from rat brain total RNA by RT-PCR. These cDNAs were subcloned into pCAGGS vector. Dr. Bernhard Lüscher (Pennsylvania State University) kindly provided pRK5:Myc-mouse GABA_A receptor γ 2 (Fang et al., 2006).

Rat GABA_A receptor β 3 subunit was knocked down by the miR-RNAi system (Life Technologies). BLOCK-iT RNAi Designer was used to select the targeting sequences and the following targeting sequences were used: miR- β 3-211, 5'-AGCATCGACATGGTTTCTGAA-3' (an alternative sequence: miR- β 3-347, 5'-TCTGGGTGCTGACACATATT-3'; both sequences yielded the same results) and miR-LacZ (β -galactosidase), 5'-GACTACACAAATCAGCGATTT-3' as a negative control. After subcloning these oligonucleotides into pcDNA6.2-EmGFP-miR, the pre-miRNA expression cassette of pcDNA6.2-EmGFP-miR was transferred to pCAGGS vector with a β -actin promoter. The resultant miR constructs were validated for the knock down of cotransfected rat GABA_A receptor β 3 expression in HEK293T cells by Western blotting (see Fig. 3A). Rat GABA_A receptor β 3 subunit rescue construct [miR- β 3-211-resistant β 3 (res β 3) in pCAGGS], which has two different nucleotides in the target sequences, was generated using site-directed mutagenesis (5'-AACATCGACATCGCCAGCATTGATATGGTTTCTGAAGTCAACAT-3'; changed nucleotides are shown in italic font). All PCR products were analyzed by DNA sequencing (Functional Genomics Facility, National Institute for Basic Biology).

Immunofluorescence analysis of cultured hippocampal neurons. Cultured rat hippocampal neurons (5×10^4 cells) were obtained from embryonic day 18–19 embryos and seeded onto poly-L-lysine-coated 12 mm coverslips in 24 well dishes. For selecting the serum samples that bind to neuronal cell surface, live neurons (21–28 DIV) were incubated with the serum from 116 patients diagnosed with or suspected of immune-mediated encephalitis for 1 h at 37°C (diluted 1:100). The neurons were subsequently fixed with 4% paraformaldehyde/120 mM sucrose/100 mM HEPES, pH 7.4, at room temperature for 10 min and blocked with PBS containing 10 mg/ml BSA for 15 min. The bound human Ig (IgG) was visualized using Cy3-conjugated secondary antibody. For Figure 1D, live neurons were incubated with the patient serum (diluted 1:200) together with anti-GABA_A receptor γ 2 antibody (against the extracellular epitope) for 30 min at 37°C. The neurons were fixed and labeled by Cy3-conjugated human IgG and Alexa Fluor 488-conjugated rabbit IgG antibodies. The neurons were then permeabilized and incubated with anti-gephyrin antibody, followed by staining with Alexa Fluor 647-conjugated mouse IgG antibody.

For Figure 3, knock down of the GABA_A receptor β 3 subunit was performed using the miR-RNAi system as described previously (Fukata et al., 2013). Briefly, hippocampal neurons (10 DIV) were transfected with the knock-down vector (miR-LacZ or miR- β 3) with or without rescue β 3 construct by Lipofectamine 2000. At 5 d after transfection, live

neurons were incubated with anti-GABA_A receptor $\beta 2/\beta 3$ subunit antibody (against the extracellular epitope) or the patient serum (diluted 1:200) together with anti- $\gamma 2$ subunit antibody (against the extracellular epitope) for 30 min at 37°C. The neurons were fixed and blocked with PBS containing 10 mg/ml BSA for 30 min on ice. The $\beta 3$ subunit or the bound human IgG and $\gamma 2$ were visualized using Cy3-conjugated and Alexa Fluor 647-conjugated secondary antibodies, respectively. Neurons transfected with the knock-down vector were reported by co-cistronic expression of EmGFP. To quantify the effect of the knock down (Fig. 3D), we randomly chose neurons from two separate cultures and made the intensity profile along the lines (total $\sim 100 \mu\text{m}$ in length) in somatodendritic regions (LAS AF software; Leica Microsystems). The number of clusters labeled by $\beta 2/\beta 3$ antibody or human IgG (threshold was set at 1000 arbitrary units of mean fluorescent intensity) was counted. Because the expression of rescue $\beta 3$ construct enhanced fluorescence intensity of the clusters, the images of miR- $\beta 3$ -211 + res $\beta 3$ -transfected neurons were acquired with 50% of laser power used for miR-LacZ + GST- or miR- $\beta 3$ -211 + GST-transfected neurons (GST was used as a mock vector).

For Figures 1D and 3, fluorescent images were captured with a confocal laser scanning microscopy system (TCS SP5 II; Leica) equipped with an HCX Plan Apochromat 63 \times /1.40 numerical aperture (NA) oil-immersion objective lens combining with the Leica HyD detectors. For Figures 2, 4, and 5, images were captured with a system (LSM5 Exciter; Carl Zeiss) equipped with a Plan Apochromat 63 \times /1.40 NA oil-immersion objective lens.

Immunoprecipitation and mass spectrometry. Rat hippocampal neurons (5×10^5 cells/well) were seeded in six well plates. The neurons were incubated with the patient serum (diluted 1:50) for 1 h at 37°C. The neurons were washed and subsequently lysed with buffer A containing the following: 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 1.3% Triton X-100, and 50 $\mu\text{g}/\text{ml}$ PMSF. The lysates were cleared by centrifugation at 10,000 \times g for 5 min at 4°C. The immune complexes were precipitated with Protein A Sepharose (GE Healthcare). The immunoprecipitates were separated by SDS-PAGE and the gels were subsequently analyzed by silver staining and Western blotting. All of the specific protein bands were excised from a silver-stained gel and analyzed with liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described previously (Fukata et al., 2010). The gel pieces with the corresponding molecular weights in the control serum sample were also analyzed to rule out nonspecific binding to human serum antibodies.

Cell-based binding assay. COS7 cells were transfected with the indicated GABA_A receptor subunits. Twenty-four hours after transfection, the cells were fixed with 4% paraformaldehyde/120 mM sucrose/100 mM HEPES, pH 7.4, at room temperature for 10 min and blocked with PBS containing 10 mg/ml BSA for 15 min. The fixed cells were incubated with the patient serum (diluted 1:10), followed by staining with the Cy3-conjugated secondary antibody. The cells were then permeabilized with 0.1% Triton X-100 for 10 min, blocked with PBS containing 10 mg/ml BSA, and incubated with antibodies to individual GABA_A receptor subunits, followed by staining with the Alexa Fluor 488-conjugated secondary antibody. For the second round of screening, serum samples from all of the 116 patients with encephalitis and 94 control subjects were tested for binding to COS7 cells expressing the GABA_A receptor $\alpha 1/\beta 3/\gamma 2$ subunits. We confirmed that any serum samples did not bind to untransfected cells that did not express the GABA_A receptor subunits through distinguishing untransfected cells with Hoechst dye (33342; Invitrogen) nucleic acid staining (Figs. 2A, 4A) and that neither the patient sera nor the control sera bound to COS7 cells that had not been treated with Lipofectamine transfection reagent (data not shown). To quantify the intensity of bound human IgG (Fig. 2B), we randomly chose 10 cells and measured the mean intensities in green and red channels. The ratio of the human IgG intensity to the GABA_A receptor subunit intensity was graphed.

The identities of human and rat GABA_A receptor $\alpha 1$, $\beta 3$, and $\gamma 2$ subunits in their amino acid sequence are 100%, 97%, and 99%, respectively; and that of the human and mouse $\gamma 2$ subunit is 99%, suggesting that the results obtained by using rat or mouse GABA_A receptor constructs and rat neurons do not affect the interpretation of our results. Cell-based binding assay for LGI1, CASPR2, and DCC (Fig. 4A) was described previously (Ohkawa et al., 2013).

Cell-based ELISA. Cell-based ELISA testing for LGI1, CASPR2, and DCC was described previously (Ohkawa et al., 2013) and the ELISA testing for the GABA_A receptor $\beta 3$ subunit basically followed this procedure. Briefly, plasmids (0.1 $\mu\text{g}/\text{well}$ for LGI1, CASPR2, and $\beta 3$; 0.06 $\mu\text{g}/\text{well}$ for DCC) were lipofected into HEK293T cells grown in polyethylenimine-coated 96-well plates (3×10^4 cells/well; Nunclon TC; Nunc). After a 24 h incubation, the cells were treated with the serially diluted sera (1:50, 150, 450, and 1350) from patients and control subjects together in the same plate for 15 min at 37°C. The cells were washed and fixed with 4% paraformaldehyde for 15 min. After washing and blocking, the cells were incubated with an HRP-conjugated anti-human IgG antibody in a 1:2000 dilution for 15 min at 37°C. After washing, the cell-bound human IgG was detected using Ultra-TMB substrate solution (Thermo Scientific). The colorimetric reaction was stopped upon the addition of 2 M sulfuric acid and the resulting plates were measured at 450 nm absorption with MULTISKAN FC microplate reader (Thermo Scientific). Wells containing nontransfected cells were used to subtract the background signals. The absorbance at a dilution of 1:50 was used as the antibody value for an individual patient. We previously determined the best cutoff point to predict the patient with limbic encephalitis from LGI1-ELISA values using a receiver operating characteristic curve composed of sensitivity and false-positive rate (Ohkawa et al., 2013). We showed that the ELISA test for LGI1 is useful for the diagnosis of limbic encephalitis with high specificity (94.2%), sensitivity (49.2%), and positive predictive value (85.3%) at the 0.8 cutoff point and that the ELISA for CASPR2 is useful for the diagnosis of neuromyotonia with high specificity (96.5%), sensitivity (26.7%), and positive predictive value (66.7%) at the 0.3 cutoff point (Ohkawa et al., 2013).

Tests of effects of GABA_A receptor autoantibodies. To determine the effect of the patients' serum antibodies on the surface or synaptic GABA_A receptor density, cultured rat hippocampal neurons (~ 30 DIV) were treated with the indicated serum samples for 2 d. 4.5 μl of the serum was added daily to 300 μl of each culture medium (final 3% concentration). After washing, live neurons were then incubated with an antibody to an extracellular epitope of GABA_A receptor $\gamma 2$ for 15 min at 37°C. After fixation, the surface-expressed $\gamma 2$ subunit was visualized with the Cy3-conjugated antibody. After permeabilizing neurons, the neurons were incubated with anti-gephyrin and vGAT antibodies, followed by staining with Alexa Fluor 488- and Alexa Fluor 647-conjugated secondary antibodies, respectively. Serum-treated sister cultures were also independently stained with anti-GABA_A receptor $\beta 3$ subunit antibody (the intracellular epitope) after cell permeabilization to visualize the GABA_A receptors containing the $\beta 3$ subunit. To quantify the synaptic GABA_A receptors, we randomly chose dendrites and analyzed the number of GABA_A receptor $\gamma 2$ and $\beta 3$ clusters along dendrites (20 μm length). Three to 10 neurons were examined from each separate culture. Synaptic $\gamma 2$ and $\beta 3$ clusters that were adjacent to both vGAT and gephyrin and bigger than $1/\pi \mu\text{m}$ in diameter (threshold was set at 70 arbitrary units of mean fluorescent intensity) were counted. The quantification of gephyrin clusters apposed to vGAT was analyzed by the same criteria. The quantification of synaptic AMPA receptor was described previously (Ohkawa et al., 2013).

Biotinylation of cell surface proteins was performed as described previously (Hughes et al., 2010). Briefly, neurons were incubated with 2.3 mM Sulfo-NHS-Biotin (Thermo Scientific) for 30 min at 4°C. Neurons were then incubated with quenching buffer containing 100 mM glycine for 30 min and lysed in buffer B containing the following: 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 1% SDS, and 50 $\mu\text{g}/\text{ml}$ PMSF. After a 20 min extraction, the lysates were diluted with 10 volumes of buffer B containing 1% Triton X-100 instead of SDS. After centrifugation at 20,000 \times g for 20 min, the supernatant was incubated with NeutrAvidin agarose beads (Thermo Scientific) for 12 h at 4°C. The isolated surface proteins were separated by SDS-PAGE and analyzed by Western blotting with indicated antibodies. For the quantification, ImageJ software was used.

Electrophysiology. Cultured rat hippocampal neurons (1.5×10^4 cells) were seeded onto poly-L-lysine-coated 12 mm coverslips in 24 well dishes. Neurons (33–46 DIV) were treated with control serum or patients' serum samples containing GABA_A receptor antibodies for 24 h.

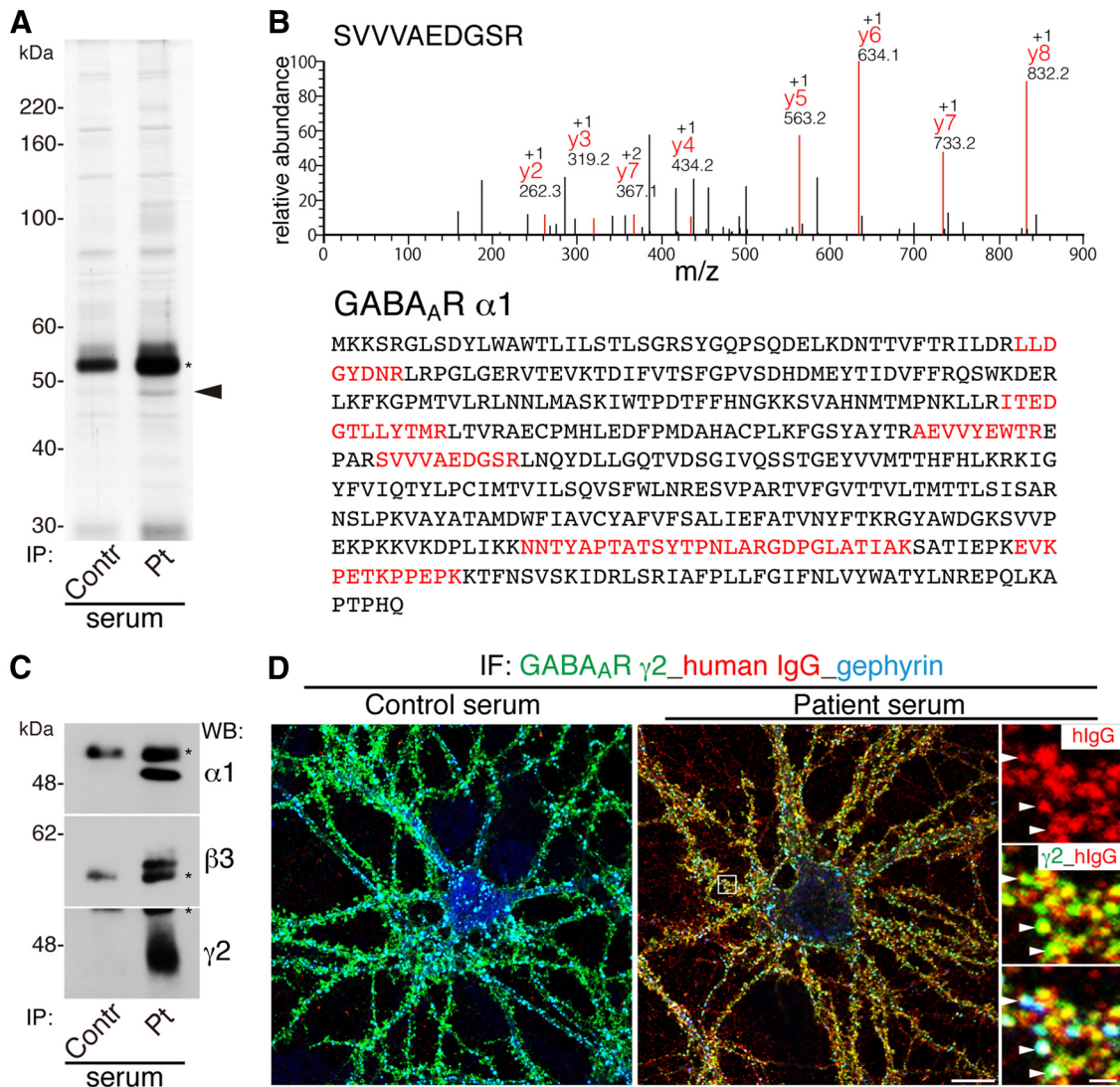


Figure 1. Identification of GABA_A receptor autoantibodies in a patient with autoimmune encephalitis. **A**, Immunoprecipitation of cell surface target proteins with patient's serum antibodies. The immunoprecipitates of serum antibodies bound to rat hippocampal neurons were analyzed by SDS-PAGE with silver staining. The specific band at 48 kDa (arrowhead) was analyzed by the LC-MS/MS. **B**, MS/MS spectra of a peptide unique for the GABA_A receptor α1 subunit (*m/z* value of the parent ion, 509.74) obtained from the trypsinized protein band shown in **A** (arrowhead). The matched fragment *y*⁺ ion series is indicated in red. Identified peptides in the amino acid sequence of GABA_A receptor α1 are indicated in red. The accession number is P62813. The patient serum used came from the initial episode of encephalitis of Patient 1. **C**, Western blotting with the subunit specific antibodies showed that the α1, β3, and γ2 subunits of the GABA_A receptor were present in the immunoprecipitate by the patient serum antibodies. Asterisks indicate the position of the human IgG heavy chain (**A**, **C**). **D**, Patient serum antibodies bind to the inhibitory GABA_A receptors at the cell surface of rat hippocampal neurons. The serum reactivity (red; human IgG) was well overlapped with surface-expressed γ2 subunits of GABA_A receptor (green), which were apposed to gephyrin scaffold (blue; marked by arrowheads). Magnified view of the region indicated by a white square. Scale bars, 10 μm (1 μm, magnified). IP, immunoprecipitation; Contr, control; Pt, patient; GABA_AR, GABA_A receptor; WB, Western blotting; IF, immunofluorescence; hlgG, human IgG.

Nine microliters of the serum was added to 300 μl of the culture medium (final 3% concentration). The culture slips were transferred to a recording chamber mounted on the microscope stage (BX51WI; Olympus) and continuously superfused with an artificial CSF (ACSF) containing the following (in mM): 138.6 NaCl, 3.35 KCl, 2.5 CaCl₂, 1.0 MgCl₂, 21.0 NaHCO₃, 0.6 NaH₂PO₄, and 10.0 glucose equilibrated with 95% O₂ and 5% CO₂, pH 7.4, at room temperature. Flow rate was 1.0 ml/min and all experiments were performed at room temperature. Synaptic currents were recorded from the hippocampal neurons by whole-cell voltage clamping (Satake and Imoto, 2014) under Nomarski optics with a water-immersion objective (63×/0.90 NA; Olympus). Patch-clamp electrodes (resistance, 3–6 MΩ) were filled with an internal solution containing the following (in mM): 150.0 Cs-methanesulfonate, 5.0 KCl, 0.1 EGTA, 5.0 HEPES, 3.0 Mg-ATP, and 0.4 Na₃-GTP, pH 7.4. Membrane potential was held with a voltage-clamp amplifier (EPC-10; HEKA Elektronik) controlled by PatchMaster software (HEKA Elektronik). Currents were filtered at 3 kHz and digitized at 20 kHz. To

record miniature IPSCs (mIPSCs), neurons were held at −20 mV in the presence of 1 μM tetrodotoxin (TTX), 20 μM CNQX, and 50 μM APV (Satake et al., 2004). To record AMPA-receptor-mediated miniature EPSCs (mEPSCs), neurons were held at −80 mV in the presence of 1 μM TTX, 10 μM bicuculline, and 50 μM APV. The Mini Analysis program (Synaptosoft Systems) was used to detect and measure mIPSCs and mEPSCs; the threshold for detection of events was threefold more than variance (σ^2) of basal noise. All data were obtained on age-matched sister cultures by an experimenter who was blinded with regard to serum treatment.

Statistical analysis. Statistical comparisons between two groups were performed by the Student's *t* test. Statistical comparisons between multiple groups were performed by one-way ANOVA with Tukey's *post hoc* analysis. When the sample sizes were unequal, Scheffe's *post hoc* analysis was used. For Figure 6C, statistical analysis of cumulative distribution of the mIPSC amplitude was performed using two-way ANOVA. Error bars indicate SEM in all figures.

Table 1. Clinical features of patients with encephalitis and GABA_A receptor antibodies

| Pt# | Age | Sex | At onset of encephalitis ^a | | History | | Autoantibodies at onset of encephalitis | | | Reference | |
|-----|-----|-----|--|--|---|--|--|---|--|---|-----------------------|
| | | | Symptoms/signs | Brain MRI (T2) | Cancer and other remarks (age) | Autoimmune diseases (age); autoantibodies ^b | In original case report ^b | Determined in the present study | Treatment | | Outcome |
| 1 | 46 | M | Subacute onset of aphasia, visual hallucination, and generalized seizures with delirium; residual thymoma | Multifocal signal abnormalities | Invasive thymoma (42); thymectomy, radiation, and chemotherapy (42) | MG (42); Anti-AchR, 500 nm; anti-VGKC, 63.7 μM (serum) | Anti-AchR, 130 nm; anti-VGKC, 649 μM (serum) | Anti-GABA _A R, anti-LG11, and anti-DCC (serum) | Corticosteroids and IVIg; AED | Epileptic seizures disappeared; severe cognitive impairment and psychological symptoms remained | Miyazaki et al., 2012 |
| 2 | 59 | F | Subacute onset of amnesia (short-term memory impairment), disorientation, and no evidence of recurrent thymoma; 10 months later, progressive memory impairment and recurrence of thymoma | Multifocal signal abnormalities in the medial temporal lobe, insular cortex, frontobasal cortex, and cingulate gyrus | Invasive thymoma (56); thymectomy and radiation (56); carbamazepine (200 mg/d) for postherpetic neuralgia (57–63) | NA | Anti-AchR, anti-Hu, and anti-Yo, negative; anti-VGKC, 403 μM (serum) | Anti-GABA _A R, anti-CASPR2, and anti-DCC (serum) | Chemotherapy for recurrent thymoma (carboplatin and etoposide) | Mental state partially improved | Ohshita et al., 2006 |

^aThe serum samples at the onset of encephalitis were used for the present screening.

^bAnti-VGKC, normal <100 μM; anti-AchR, normal <0.2 nm.

MG, Myasthenia gravis; AchR, acetylcholine receptor; IVIg, intravenous immunoglobulin; AED, antiepileptic drug; NA, not applicable.

Results

Identification of GABA_A receptor autoantibodies in patients with encephalitis

To identify novel cell surface autoantigens involved in autoimmune encephalitis, we analyzed serum samples from 116 patients with or suspected of immune-mediated encephalitis (see Study Population section in Materials and Methods). We first screened serum antibodies for binding to the cell surface of cultured rat hippocampal neurons. The serum antibodies from 37 patients bound to the neuronal cell surface (data not shown). Target proteins were immunoprecipitated from cultured neurons with the bound serum antibodies and identified by mass spectrometry. We found the previously reported autoantibodies to LGI1, AMPA receptor, CASPR2, DPP10, and DCC in patients with encephalitis (data not shown) (Lai et al., 2009; Irani et al., 2010; Lai et al., 2010; Ohkawa et al., 2013). In addition, a protein with a molecular mass of 48 kDa (p48) was specifically detected in the immunoprecipitate by serum antibodies from one patient (Fig. 1A), who was originally diagnosed as having invasive thymoma with the complications of myasthenia gravis and then developed encephalitis associated with VGKC-complex antibodies (Patient 1, Table 1) (Miyazaki et al., 2012). The molecular identity of p48 was determined by LC-MS/MS (Fig. 1B). Molecular weights of seven peptide fragments derived from p48 coincided with those from the rat GABA_A receptor α1 subunit: LLDGYDNR, ITEDGTLTYTMR, AEVVYEWTR, SVVVAEDGSR, NNTYAPTATSYTPNLAR, GDPGLATIAK, and EVKPETKPPEPK. The estimated molecular weight of rat GABA_A receptor α1 (51.7 kDa) was close to that of p48. In addition, peptide fragments coinciding with those from the GABA_A receptor β3 subunit (NVVFATGAYPR and IKIPDLTDVNAIDR) were present in the same immunoprecipitate (the corresponding band at 54.2 kDa was masked by the human IgG heavy chain). Western blotting with antibodies specific to the α1, β3, and γ2 subunits of GABA_A receptor confirmed the immunoprecipitation of heteromeric GABA_A receptors (Fig. 1C). Consistently, the serum antibodies showed overlapped signals with inhibitory synapses marked by GABA_A receptor γ2 and gephyrin antibodies in rat hippocampal neurons (Fig. 1D).

GABA_A receptor autoantibodies are directed to extracellular epitope of the β3 subunit

To determine whether the patient serum antibodies bind directly to the GABA_A receptor and, if so, which of GABA_A receptor subunits the antibodies recognize, the cell-based binding assay was performed. Because the native GABA_A receptor is a heteropentamer composed of two α, two β, and one γ subunits and the patient serum immunoprecipitated α1, β3, and γ2 subunits from hippocampal neurons (Fig. 1C), α1, β3, and γ2 subunits were coexpressed to display heteropentameric GABA_A receptors at the cell surface of COS7 cells. Transfected cells were then fixed and incubated with the patient serum without cell permeabilization. We found that the serum antibodies from Patient 1 robustly reacted to the surface-expressed GABA_A receptors (α1/β3/γ2; Fig. 2A, left). Among 19 individual GABA_A receptor subunits, we then examined the binding to individual GABA_A receptor subunits (α1, α2, α5, β1, β3, and γ2), which are known to be included in GABA_A receptor heteromers expressed in hippocampus (Pirker et al., 2000). The serum antibodies from Patient 1 strongly reacted to the cells expressing the β3 subunit alone and weakly reacted to those expressing the γ2 subunit alone, but did not to those expressing the α1, α2, α5, or β1 subunit alone (Fig. 2A, B). Then, as the second round of screening, we tested serum samples from all of the 116 patients with encephalitis for binding to the cell surface of COS7 cells expressing the GABA_A receptor (α1/β3/γ2). We additionally tested serum samples from 94 control subjects (see Materials and Methods). We found that another patient (Patient 2) diagnosed with encephalitis had antibodies against the GABA_A receptor α1/β3/γ2 and that the antibodies also strongly recognized the β3 subunit, but neither the α1 nor the γ2 subunit (Fig. 2A, B). Patient 2 had invasive thymoma and limbic encephalitis associated with VGKC-complex antibodies (Ohshita et al., 2006) (Table 1). Therefore, the common clinical features between two patients with GABA_A receptor antibodies are cognitive impairment, multifocal abnormal brain MRI signals, and invasive thymoma. Patient 1 had seizures/status epilepticus, but Patient 2 had no seizure episodes (see Discussion). We did not find any control subjects that bound to the GABA_A re-

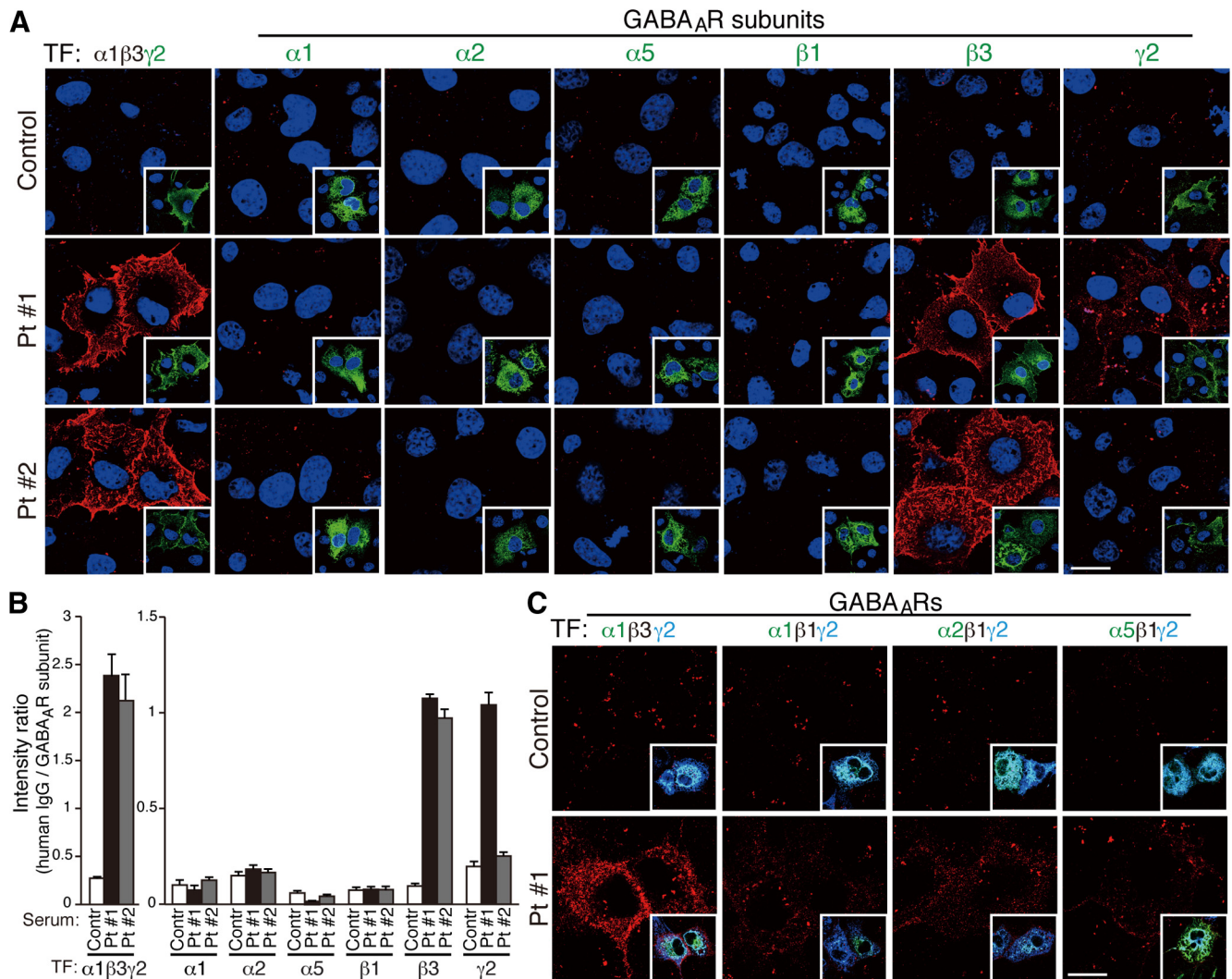


Figure 2. Patients' GABA_A receptor antibodies are directed to extracellular epitope of $\beta 3$ subunit. **A, B**, COS7 cells were transfected (TF) to surface express the indicated GABA_A receptor subunits. Transfected cells were fixed and doubly stained with the patient sera (Patient 1 or Patient 2; red, human IgG) together with the antibodies specific to the individual expressed subunits (green, insets). Nuclear DNA was stained by Hoechst 33342 (blue) to distinguish untransfected cells. To clearly show the weak binding of the serum from Patient 1 to the $\gamma 2$ subunit, the detector gain of the red channel is enhanced upon image acquisition (right, middle). The ratio of the human IgG intensity to the GABA_A receptor subunit intensity was graphed (**B**). Error bars indicate SEM; $n = 10$ transfected cells. **C**, COS7 cells were transfected to surface express the indicated heteromeric GABA_A receptors and tested for binding of serum antibodies (red). Transfected cells were detected by staining with the individual α subunit (green) and $\gamma 2$ subunit (blue) antibodies. Merged images are shown in insets. Scale bars, 20 μm .

ceptor ($\alpha 1/\beta 3/\gamma 2$; a representative is shown in Fig. 2A), although this screening might have missed some serum samples that contained antibodies to other GABA_A receptor subunits than $\alpha 1/\beta 3/\gamma 2$. Because neither serum antibody from the two patients bound to COS7 cells expressing the α subunit alone, one may wonder whether the α subunit might not have been efficiently expressed at the cell surface without other subunits. To further examine the possible involvement of α subunit antibodies in the patient serum, COS7 cells were transfected with various combinations of three subunit genes of the GABA_A receptor, $\alpha 1/\beta 3/\gamma 2$, $\alpha 1/\beta 1/\gamma 2$, $\alpha 2/\beta 1/\gamma 2$, or $\alpha 5/\beta 1/\gamma 2$ (Fig. 2C). There were no apparent differences in the weak binding of serum antibodies from Patient 1 to three different GABA_A receptors, $\alpha 1/\beta 1/\gamma 2$, $\alpha 2/\beta 1/\gamma 2$, and $\alpha 5/\beta 1/\gamma 2$, indicating that the binding of serum antibodies was attributed to the $\gamma 2$ subunit, but not to the $\alpha 1$, $\alpha 2$, or $\alpha 5$ subunits. Serum from Patient 2 did not show any apparent binding to $\alpha 1/\beta 1/\gamma 2$, $\alpha 2/\beta 1/\gamma 2$, or $\alpha 5/\beta 1/\gamma 2$ (data not shown). Together, these results indicate that the two patients with immune-mediated encephalitis had autoantibodies directed against the

GABA_A receptor and that the extracellular part of the $\beta 3$ subunit was the antigenic epitope recognized by the patients' GABA_A receptor antibodies. One of the two patients also had a low level of $\gamma 2$ autoantibodies (Patient 1), but neither patient had any autoantibodies to the $\alpha 1$, $\alpha 2$, $\alpha 5$, or $\beta 1$ subunit.

GABA_A receptor containing $\beta 3$ subunit is the main target of the patient serum antibodies

We next investigated whether the GABA_A receptor is the main target of the patient serum antibodies in neurons. We took advantage of knock-down approach in cultured rat hippocampal neurons. MicroRNAs (miRNA- $\beta 3$ -211 and miRNA- $\beta 3$ -347) for the GABA_A receptor $\beta 3$ subunit were first validated by the reduced expression of exogenously expressed rat GABA_A receptor $\beta 3$ in HEK293T cells (Fig. 3A). Then, by cell surface staining with anti- $\beta 2/\beta 3$ antibody, we quantified the knock-down effect on the $\beta 3$ subunit expression in neurons. When miRNA- $\beta 3$ -211 for the GABA_A receptor $\beta 3$ subunit was expressed in neurons, the number of $\beta 3$ subunit clusters in soma and dendrites that were stained

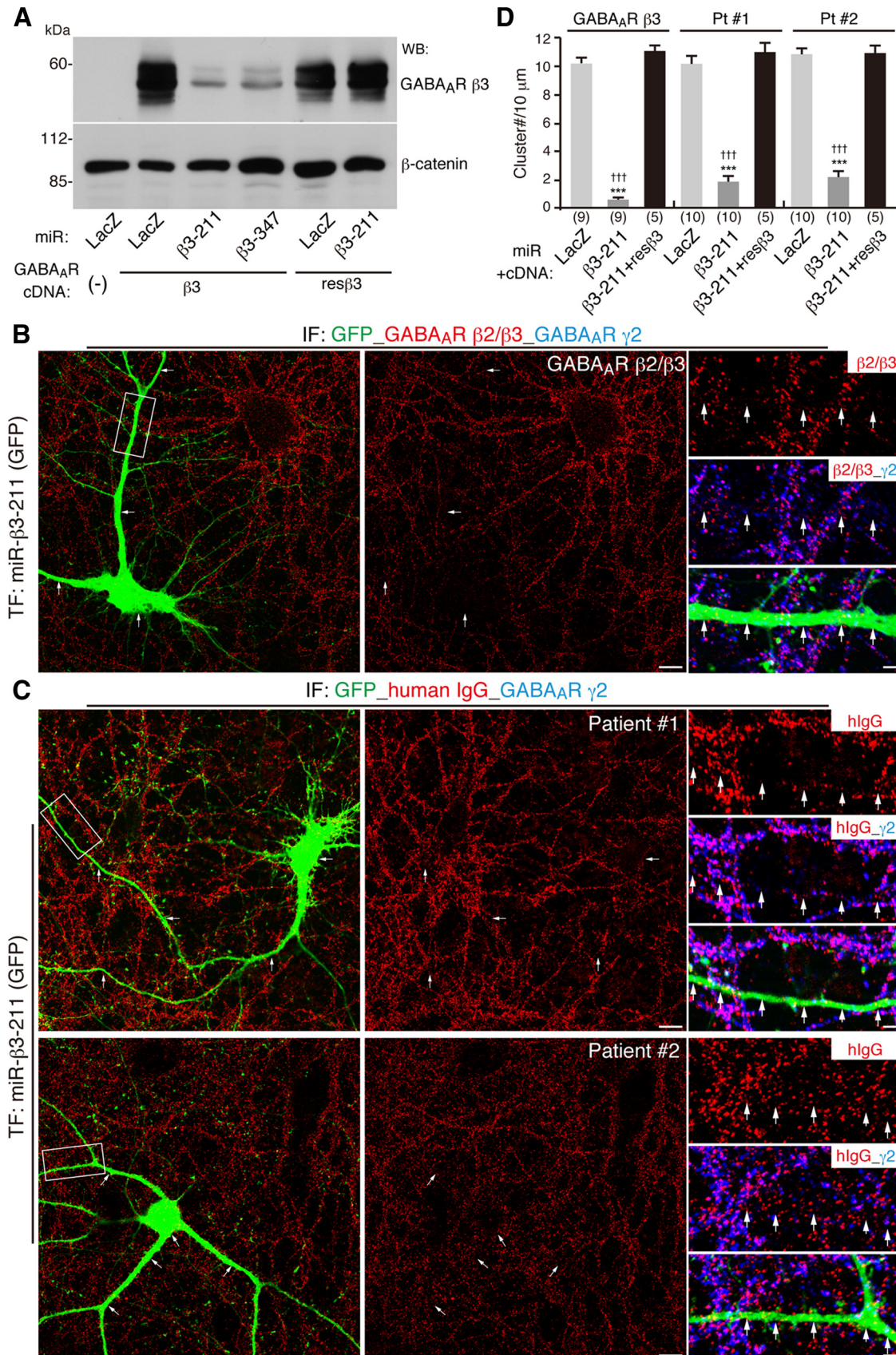


Figure 3. GABA_A-receptor-containing $\beta 3$ subunit is a major target of the patients' antibodies in hippocampal neurons. **A**, Validation of miRNA constructs for the GABA_A receptor $\beta 3$ subunit. HEK293T cells were cotransfected with the indicated knock-down (miR) and $\beta 3$ expression vectors. Three days after the transfection, the cell lysates were analyzed by Western blotting with GABA_A receptor $\beta 3$ and β -catenin antibodies. miR-LacZ, Control miRNA targeting to LacZ; res $\beta 3$, miR-211-resistant $\beta 3$. **B**, Effective knock down of the endogenous $\beta 3$ subunit. Cultured rat hippocampal neurons were transfected with the miR- $\beta 3$ expression vector at 10 DIV. Cell surface GABA_A receptor $\beta 2/\beta 3$ (red) and $\gamma 2$ (blue) subunits were stained at 15 DIV. (Figure legend continues.)

by anti- $\beta 2/\beta 3$ antibody was robustly reduced, showing that $\beta 3$ subunit expression was decreased to $5.47 \pm 3.60\%$ (Fig. 3*B,D*). This is consistent with the previous report showing that $\beta 2$ expression is very low in hippocampal neurons (Pirker et al., 2000). This reduction was not due to off-target effects of miRNA expression because it was completely rescued by coexpression of the knock-down-resistant $\beta 3$ construct (res $\beta 3$) with miRNA- $\beta 3$. We noted that $\gamma 2$ subunit clusters were also decreased in neurons in which the $\beta 3$ subunit was knocked down, confirming an essential role of the $\beta 3$ subunit in the GABA_A receptor function in hippocampal neurons (DeLorey et al., 1998). Under these conditions, the overall immunoreactivity of the sera from Patient 1 and Patient 2 to the neurons was greatly reduced by the expression of miRNA- $\beta 3$ -211 and rescued by coexpression of the knock-down-resistant $\beta 3$ construct. Importantly, the residual immunoreactivity upon $\beta 3$ knock down was $18.2 \pm 10.8\%$ for Patient 1 and $19.8 \pm 11.7\%$ for Patient 2 (Fig. 3*C,D*). These results indicate that the binding of the patients' antibodies to the neuronal surface was mostly ($\sim 80\%$) attributed to the GABA_A receptor containing the $\beta 3$ subunit and that the patients had other autoantibodies in addition to GABA_A receptor antibodies.

Coexisting antibodies with GABA_A receptor antibodies in the patient serum

We therefore performed the cell-based binding assay (Fig. 4*A*) and the cell-based ELISA test, which quantifies the frequent serum antibodies against LGI1, CASPR2, DCC (Ohkawa et al., 2013), and GABA_A receptor $\beta 3$ (Fig. 4*B*). We found that the serum samples of Patient 1 and Patient 2, but no other tested serum samples, bound to the GABA_A receptor $\beta 3$ (Fig. 4*A*) and showed similar positive values for GABA_A receptor antibodies (ELISA absorbance = 0.57 for Patient 1; 0.52 for Patient 2; Fig. 4*B*). We also found that Patient 1 had low levels of LGI1 antibodies (absorbance = 0.37) and DCC antibodies (absorbance = 0.26) in addition to GABA_A receptor antibodies, but not CASPR2 antibodies. In contrast, serum from Patient 2 contained CASPR2 antibodies (absorbance = 0.51) and a low level of DCC antibodies (absorbance = 0.21) in addition to GABA_A receptor antibodies, but not LGI1 antibodies. However, the low level of LGI1 antibodies of Patient 1 is unlikely to cause the patient's CNS symptoms, because the value for LGI1 antibodies of Patient 1 was much lower than the cutoff value (absorbance = 0.8) determined for diagnosis of limbic encephalitis (Ohkawa et al., 2013; see Materials and Methods). In fact, in the present study population, patients with limbic encephalitis and monospecific LGI1 antibodies had much higher levels of LGI1 antibodies (average of ELISA absorbance = 1.41 ± 0.36 , $n = 34$ patients; Patient A as a representative) than patients with neuromyotonia (no CNS symptoms) and LGI1 autoantibodies (0.65 ± 0.16 , $n = 10$ patients; Patient C as a representative) (Fig. 4). CASPR2 and DCC antibodies are also unlikely to be causes of the patient's CNS

symptoms because CASPR2 and DCC autoantibodies are specifically associated with PNS symptoms of neuromyotonia, but are not associated with CNS symptoms observed in encephalitis (Ohkawa et al., 2013). Together, these quantitative analyses (Figs. 3, 4) strongly suggest that the GABA_A receptor containing the $\beta 3$ subunit is a primary target of the patients' serum antibodies and is the main contributor to the patients' symptoms.

Patients' GABA_A receptor antibodies reduce the number of both synaptic and surface GABA_A receptor clusters

Next, we explored a mode of action of patients' GABA_A receptor antibodies. Previous studies showed that autoantibodies against NMDA and AMPA receptors induce the internalization of the corresponding receptors and reduce the number of synaptic receptors (Lai et al., 2009; Hughes et al., 2010). These previous findings inspired us to investigate whether patients' GABA_A receptor antibodies reduce the number of synaptic GABA_A receptors. When hippocampal neurons were treated with the serum from Patient 1 and Patient 2 for 2 d, the number of synaptic GABA_A receptor clusters, represented by $\gamma 2$ or $\beta 3$ subunit clusters adjacent to both gephyrin and vGAT, was significantly reduced (Fig. 5*A*). The effect was specifically attributed to the patients' GABA_A receptor antibodies because treatment of neurons with a control serum without detectable autoantibodies or with the serum from the patient (Patient C) with invasive thymoma and neuromyotonia, who had LGI1 and CASPR2 antibodies but not GABA_A receptor antibodies (Fig. 4), did not affect the synaptic GABA_A receptor clusters. The number of surface $\gamma 2$ subunit clusters, including both synaptic and extrasynaptic GABA_A receptors, was also heavily reduced by treatment with the serum from Patient 1 and Patient 2. The effect of the patients' serum on GABA_A receptor clusters was not complement mediated because the heat-inactivated patient serum reduced the number of both synaptic and surface GABA_A receptor to a similar extent to the non-heat-inactivated patient serum; therefore, we pooled these data. Under these conditions, the number of gephyrin clusters apposed to vGAT was not altered (Fig. 5*A*). The effect of the patients' sera on GABA_A receptor clusters was selective because the same treatment did not affect synaptic or surface AMPA receptor subunit GluA1.

This cell biological results were confirmed by the biochemical experiment: hippocampal neurons were treated with the patient or control serum for 3 d and then the surface-expressed proteins were labeled with biotin and purified by the avidin-conjugated beads (Fig. 5*B*). In the patient serum-treated neurons, the amount of cell surface GABA_A receptor $\beta 3$ subunits was significantly reduced and the total amount of the $\beta 3$ subunit tended to be reduced (but not significantly). This effect was specific to the GABA_A receptor because the amount of the surface GluA1 and N-cadherin was not affected. Together, these results indicate that GABA_A receptor autoantibodies cause a selective decrease in GABA_A receptor surface density and synaptic localization, probably by enhancing the receptor internalization.

To determine the relationship between GABA_A receptor antibodies and patient' symptoms, we compared serum samples of Patient 1 at two different time points, from the episode of invasive thymoma and myasthenia gravis (without encephalitis) and from the episode of encephalitis. The sample of Patient 1 before the episode of encephalitis had acetylcholine receptor (AChR) antibodies, but no detectable GABA_A receptor antibodies (Fig. 5*C*, Table 1) and showed no effects on synaptic GABA_A receptor density (Fig. 5*D*). In contrast, the sample of the same patient at the time of symptom presentation of encephalitis had elevated GABA_A receptor antibodies instead of AChR antibodies and decreased synaptic GABA_A receptor density (Fig. 5*C,D*). Therefore, the clinical course of Patient 1

←

(Figure legend continued.) MicroRNA-transfected neurons were reported by the GFP expression (green). *C*, Binding of serum antibodies (Patient 1 and Patient 2; red) was examined in neurons in which the $\beta 3$ subunit was knocked down (green). Magnified view of the region indicated by a white square (*B*, *C*). Arrows indicate the soma and dendrites of the neuron in which $\beta 3$ was knocked down (*B*, *C*). Scale bars, 10 μm (2 μm , magnified). *D*, Neurons were cotransfected with the indicated miR and the knock-down-resistant construct (res $\beta 3$) or GST (for mock). The number of clusters labeled by $\beta 3$ antibody or human IgG of patients' serum (Patient 1 and Patient 2) was counted and graphed. One-way ANOVA with Scheffe's *post hoc* analysis, $***p < 0.001$ compared with miR-LacZ; $\dagger\dagger p < 0.001$ compared with miR- $\beta 3$ -211 + res $\beta 3$. Error bars indicate SEM. The number of neurons examined is indicated in parentheses.

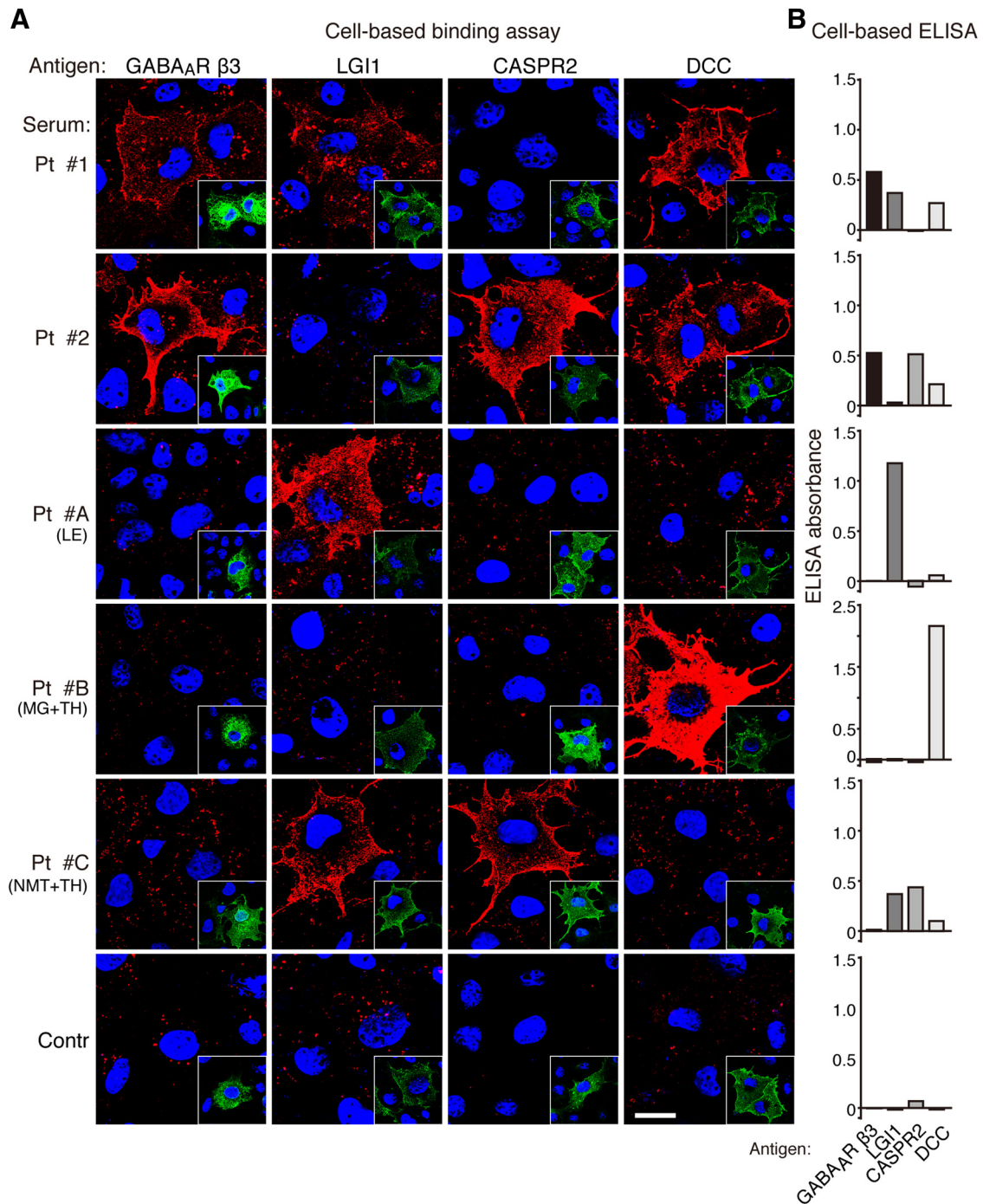


Figure 4. Identification of coexisting antibodies with GABA_A receptor antibodies in the patient serum. **A, B.** Patient 1 and Patient 2 sera from their initial episodes of encephalitis were tested by cell-based binding assay (**A**) and cell-based ELISA tests (**B**) against the GABA_A receptor β3 subunit, LGI1, CASPR2, and DCC. Additional sera were tested: from Patient A with limbic encephalitis (LE), Patient B with myasthenia gravis (MG) and thymoma (TH), and Patient C with neuromyotonia (NMT) and thymoma. Contr, Serum sample from a control patient with a neurodegenerative disease. Scale bar, 20 μm in **A**. Average values from triplicate measurements of the individual serum are shown in **B**.

correlates with the levels and effects of the patient's GABA_A receptor antibodies. Although LGI1 antibodies were also detected only at the time of symptom presentation of encephalitis, the low level of LGI1 antibodies is unlikely to cause the patient's CNS symptoms, as described for Figure 4 (also see Discussion).

Patients' GABA_A receptor antibodies selectively reduce mIPSC amplitude and frequency

Finally, we assessed the effects of two patient sera (Patient 1 and Patient 2) on inhibitory synaptic transmission by whole-cell

patch-clamp recording of mIPSCs in rat hippocampal neurons. We found a significant decrease in the mean amplitude of mIPSCs in patients' serum-treated neurons compared with that of control serum-treated neurons (Fig. 6A, B). This result is consistent with our immunocytochemical data (Fig. 5A) showing that the treatment of neurons with patients' serum reduced the number of synaptic clusters of GABA_A receptors. The frequency of mIPSCs was also decreased in patients' serum-treated neurons (Fig. 6A, B), probably due to the increase in small-amplitude mIPSCs that fell below the threshold of de-

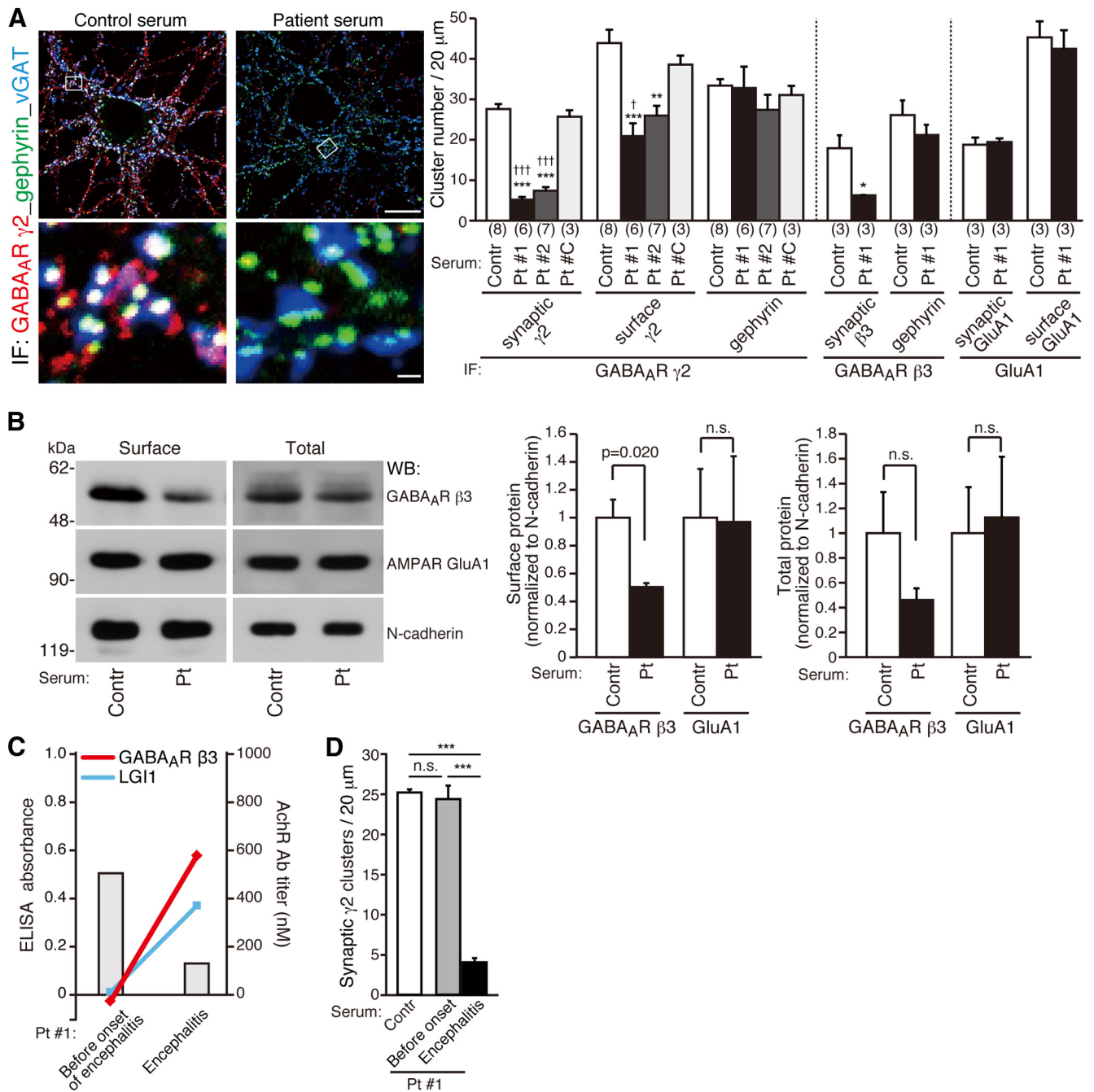


Figure 5. Patients' GABA_A receptor antibodies specifically reduce synaptic and cell surface GABA_A receptor density. **A**, Cultured rat hippocampal neurons were incubated with serum from Patient 1 and Patient 2, serum from Patient C with thymoma that contained both LGI1 and CASPR2 antibodies (Fig. 4), or a control serum for 2 d. Representative images of surface GABA_A receptor clusters in neurons treated with the control or the serum from Patient 1 are shown (left). Bottom, Magnified view of the region indicated by a white square. Synaptic GABA_A receptors, which were γ2 (red) or β3 (data not shown) subunit-positive clusters adjacent to both gephyrin (green) and vGAT (blue), were counted. Surface-expressed GABA_A receptor clusters labeled by the γ2 subunit antibody and gephyrin clusters were also independently counted. In addition, synaptic GluA1 clusters adjacent to both PSD-95 and vGluT1 and surface-expressed GluA1 clusters were counted. Scale bars, 20 μm (1 μm, magnified). Statistical analyses were performed by one-way ANOVA with Scheffé's *post hoc* analysis (γ2 clusters); or by Student's *t* test (β3 and GluA1 clusters). **p* < 0.05; ***p* < 0.01; ****p* < 0.001 compared with control; †*p* < 0.05, ††*p* < 0.001 compared with Patient C. Error bars indicate SEM. The number of separate cultures is indicated in parentheses. **B**, Surface biotinylated and total proteins of the serum from Patient 1 treated hippocampal neurons were analyzed by Western blotting with the indicated antibodies. Student's *t* test; n.s., not significant. Error bars indicate SEM; *n* = 3 separate cultures. **C, D**, Serum samples of Patient 1 at two different time points, from the episode of thymoma and MG (before onset of encephalitis) and from the episode of encephalitis, were analyzed by cell-based ELISA (**C**), and their effects on synaptic GABA_A receptors were investigated (**D**). Average values for GABA_A receptor β3 and LGI1 ELISA from triplicate measurements of the individual serum samples are shown (**C**). Titer of serum antibodies against AchR is shown (bar graph in **C**; Miyazaki et al., 2012). Synaptic GABA_A receptors were counted as in **A**. One-way ANOVA with Tukey's *post hoc* analysis; ****p* < 0.001; n.s., not significant. Error bars indicate SEM; *n* = 3 separate cultures.

tection. In support of this, cumulative distribution of the mIPSC amplitude of the patients' serum-treated neurons showed the significant leftward shift (Fig. 6C). In contrast, patients' antibodies did not affect AMPA-receptor-mediated

mEPSCs (Fig. 6A,D), which is also consistent with no effects of the patients' antibodies on synaptic AMPA receptor clusters (Fig. 5A). Therefore, patients' antibodies specifically reduce the synaptic GABA_A receptor function.

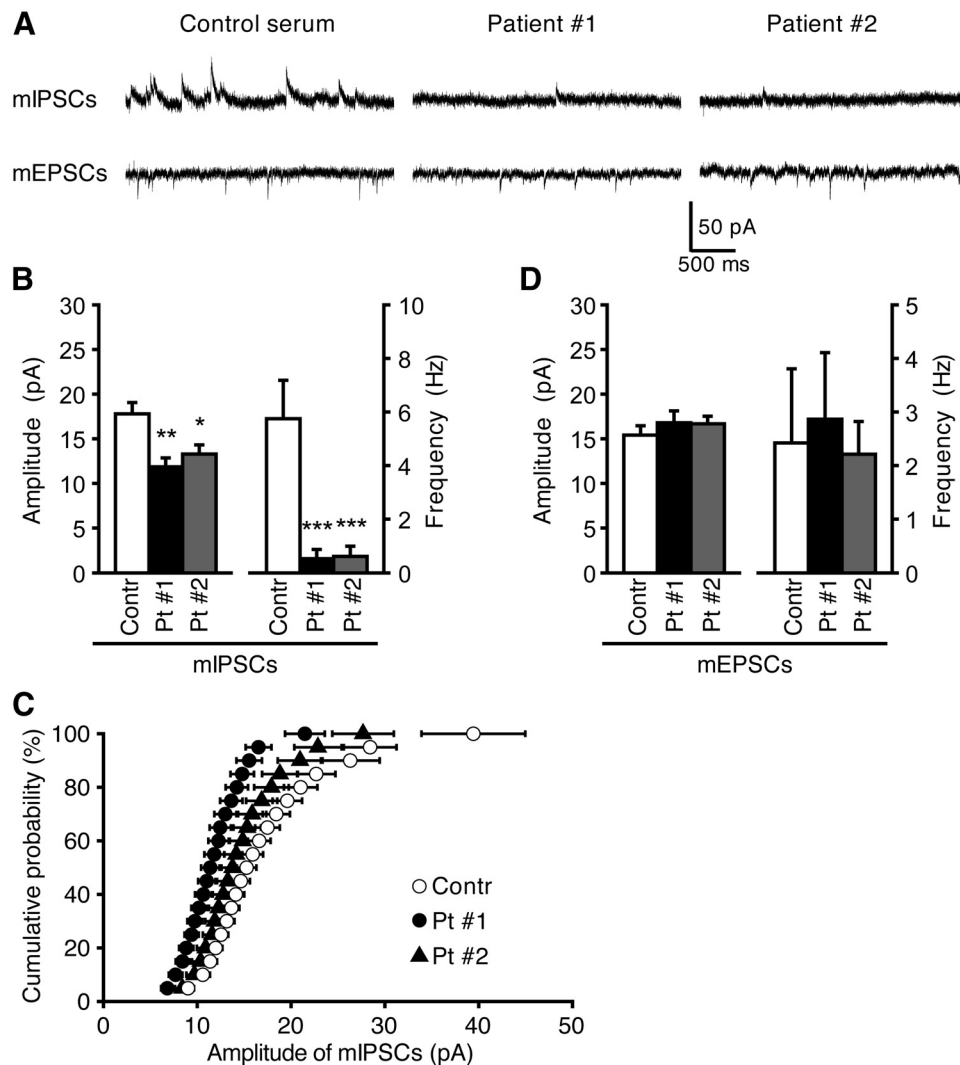


Figure 6. Patients' GABA_A receptor antibodies selectively decrease mIPSCs. **A**, Representative traces of mIPSCs ($V_H = -20$ mV, top) and mEPSCs ($V_H = -80$ mV, bottom) recorded from cultured rat hippocampal neurons, which were incubated with the serum from Patient 1 and Patient 2 or a control individual for 1 d. **B, D**, Treatment of neurons with the patient serum significantly decreased the amplitude and frequency in mIPSCs (**B**), but did not affect those in mEPSCs (**D**). Statistical analyses were performed by one-way ANOVA with Scheffe's *post hoc* analysis. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with control. **C**, Cumulative distribution of the mIPSC amplitude. Note the significant leftward shift in the amplitude distribution in the patients' serum-treated neurons ($F_{(2, 540)} = 62.9, p < 0.001$; two-way ANOVA). Error bars indicate SEM; Control, $n = 9$; Patient 1, $n = 10$; Patient 2, $n = 11$ (**B, C**); Control, $n = 9$; Patient 1, $n = 9$; Patient 2, $n = 12$ (**D**). n values indicate the number of neurons examined from two separate cultures.

Discussion

Identification of GABA_A receptor autoantibodies in autoimmune encephalitis

GABA is a major inhibitory neurotransmitter and exerts its functions through ionotropic GABA_A receptors and metabotropic GABA_B receptors. GABA_B receptor was recently identified as an autoantigen associated with limbic encephalitis (Lancaster et al., 2010). However, antibodies to the ionotropic GABA_A receptor have not been yet reported in any neuroimmunological disorders. Here, we found the autoantibodies to GABA_A receptors in patients with autoimmune encephalitis and revealed a mode of action of the antibodies. One may wonder why GABA_A receptor autoantibodies have not been found for a long time. One possibility is that the immunoprecipitated band of human IgG (heavy chains) almost completely overlaps with bands of co-isolated GABA_A receptor subunits (all are ~50 kDa) in the SDS-PAGE gel, thereby hindering the detection of GABA_A receptor subunits. Very recently, while this manuscript was under review, a related paper was published reporting the identification of GABA_A re-

ceptor autoantibodies in patients with encephalitis showing refractory seizures and/or status epilepticus (Petit-Pedrol et al., 2014). Their history of autoimmunity or cancer seems different from that of our cases. In cases they reported, autoantibodies to GABA_A receptors were sometimes concurrently detected with autoantibodies to GAD65 or GABA_B receptor and were not frequently associated with underlying tumors. In contrast, our cases represent a paraneoplastic subtype of encephalitis with invasive thymoma (Table 1) in which GABA_A receptor autoantibodies coexist with LGI1, CASPR2, or DCC antibodies. It will be worthwhile to test patients presenting with thymoma and encephalitis for GABA_A receptor autoantibodies.

Link between GABA_A receptor autoantibodies and patient symptoms

We found that the GABA_A receptor antibodies of both Patient 1 and Patient 2 targeted the $\beta 3$ subunit of the GABA_A receptor directly. Based on the previous genetic studies showing that mutations in the human GABA_A receptor $\beta 3$ subunit cause genetic

epilepsy syndromes (Macdonald et al., 2010) and that the genetic loss of the $\beta 3$ subunit causes seizures and learning and memory deficits in mice (DeLorey et al., 1998), it is strongly suggested that the patients' GABA_A receptor antibodies are the direct cause of some CNS disorders such as cognitive impairment and/or seizures often observed in encephalitis. Consistently, both the patients' antibodies similarly showed a selective effect on inhibitory synapses (Figs. 5, 6). In addition, only the serum sample from the episode of encephalitis (Patient 1) had decreased synaptic GABA_A receptor density, whereas the sample of the same patient before the onset of encephalitis had no detectable GABA_A receptor antibodies and no effects on synaptic GABA_A receptor density (Fig. 5C,D). To further strengthen the link between GABA_A receptor antibodies and patients' symptoms, we considered two additional factors of two patients, "thymoma" and "VGKC-complex antibodies," as follows.

The Patient 1 and Patient 2 both had invasive thymoma (Table 1) (Ohshita et al., 2006; Miyazaki et al., 2012). Because patients with (invasive) thymoma often develop multiple autoantibodies due to disturbed self-tolerance, we included patients with thymoma as controls. Our subjects for the screening contained 19 patients with thymoma, but only two patients had GABA_A receptor antibodies, indicating that not all patients with thymoma develop GABA_A receptor antibodies. For example, the Patient B, with invasive thymoma and myasthenia gravis, had monospecifically DCC antibodies without GABA_A receptor antibodies, whereas Patient C, with invasive thymoma and neuromyotonia, had LGI1 and CASPR2 antibodies without GABA_A receptor antibodies (Fig. 4). Treatment of hippocampal neurons with these patients' serum did not affect synaptic GABA_A receptor clusters (Fig. 5A for Patient C; cluster number for Patient B serum, $25.0 \pm 4.5/20 \mu\text{m}$ dendrite and control serum, 25.4 ± 4.2 , $p = 0.85$, Student's *t* test; $n = 11$ neurons).

In addition, we included patients with VGKC-complex antibodies as controls because Patient 1 and Patient 2 both had VGKC-complex antibodies (649 pM for Patient 1; 403 pM for Patient 2; Table 1), which are now attributed to LGI1 and/or CASPR2 antibodies, and previous case reports for these patients showed a correlation between patients' symptoms and the follow-up of VGKC-complex antibodies (Ohshita et al., 2006; Miyazaki et al., 2012). Here, we investigated the serum from Patient C as a control because the patient had VGKC-complex antibodies (809 pM; now revealed as LGI1 and CASPR2 antibodies; Fig. 4) but no GABA_A receptor antibodies. Treatment with this patient serum did not affect the synaptic GABA_A receptor clusters (Fig. 5A). In addition, we tested another patient serum with VGKC-complex antibodies (2121 pM) and limbic encephalitis. The patient had high level of monospecific LGI1 antibodies (absorbance = 1.86) without GABA_A receptor antibodies. This serum treatment did not affect the synaptic GABA_A receptor clusters (cluster number for control serum, 27.6 ± 1.2 ; for the patient serum, 24.6 ± 2.3 , $p = 0.39$, Student's *t* test; $n = 3$ separate cultures). These overall results exclude the possibility that coexisting antibodies other than GABA_A receptor antibodies mediate the effects and support the specific role of GABA_A receptor antibodies in the patients' symptoms.

Two patients with GABA_A receptor antibodies shared some clinical features: cognitive impairment, multifocal abnormal brain MRI signals, and invasive thymoma (Table 1). Importantly, Patient 1 had seizures/status epilepticus, but Patient 2 had no seizure episodes. Given that loss of the $\beta 3$ subunit in mice causes seizures and learning and memory deficits (DeLorey et al., 1998), it is reasonable to expect that loss of function of the GABA_A receptor mediated by GABA_A receptor $\beta 3$ antibodies may cause

seizures in human patients. However, at present, it seems too early to conclude that GABA_A receptor antibodies should always cause seizures in human patients. It is conceivable that the brain regions where the antibodies act and the amount of the antibodies at different regions can be highly variable between patients. In addition, other factors such as medication and coexisting antibodies may modify the clinical features. In fact, Patient 2 had suffered from postherpetic neuralgia and had been under treatment with carbamazepine, an antiepileptic and anti-nerve-pain drug, for 5 years, including the periods of the initial episode and the relapse of encephalitis (Ohshita et al., 2006) (Table 1). This medication might have prevented the patient's seizure onset. The exact relationship between GABA_A receptor antibodies and specific CNS symptoms will be clarified in the future as the number of cases increases.

Anti-GABA_A receptor encephalitis as a new class of autoimmune encephalitis

The present study indicates that encephalitis associated with GABA_A receptor antibodies shows different clinical features and mechanisms, at least from limbic encephalitis associated with monospecific LGI1 antibodies. Both cases with the GABA_A receptor antibodies showed the similar brain MRI finding, extensive multifocal lesions involving bilateral temporal lobes (Ohshita et al., 2006; Miyazaki et al., 2012). In contrast, limbic encephalitis with LGI1 autoantibodies is featured by the typical MRI finding with the focal lesion of medial temporal lobes (Cash et al., 2011; Lancaster et al., 2011). We previously found that the monospecific serum against LGI1 (ELISA absorbance = 1.86) from a patient with limbic encephalitis significantly reduce synaptic AMPA receptor density of hippocampal neurons (Ohkawa et al., 2013), but the serum did not affect synaptic GABA_A receptor density. Conversely, serum from Patient 1 showed a selective effect on GABA_A receptor function, but did not affect synaptic AMPA receptor density nor mEPSCs regardless of coexisting LGI1 antibodies (absorbance = 0.37; Figs. 5A, 6). Unlike NMDA, AMPA, and GABA_A receptor antibodies directly targeting ionotropic receptors to induce the receptor internalization (Lai et al., 2009; Hughes et al., 2010; Fig. 5), LGI1 antibodies need to titrate out endogenous LGI1 to prevent LGI1 from binding to its receptor ADAM22 and then to reduce synaptic AMPA receptors. This indirect mode of action of LGI1 antibodies should require a higher concentration of LGI1 antibodies to be effective. Therefore, the loss of effect of serum from Patient 1 on synaptic AMPA receptors is probably due to the low LGI1 antibody level (Fig. 4B) and in turn highlights a predominant role of the GABA_A receptor antibodies in the symptoms experienced by Patient 1. Therefore, it is conceivable that encephalitis with GABA_A receptor antibodies might be distinguished as a new class of autoimmune encephalitis. In addition, we propose that clinical phenotypes of autoimmune anti-GABA_A receptor encephalitis may be further modified by the combination of coexisting autoantibodies such as LGI1, CASPR2, or DCC antibodies, especially if the patient has thymoma. The multiplex ELISA testing to determine the involved autoantibodies will be essential for the accurate diagnosis of a spectrum of autoimmune encephalitis.

In conclusion, we discovered GABA_A receptor autoantibodies associated with autoimmune encephalitis and revealed their pathogenic role, downregulation of the GABA_A receptor function. Given that many agonistic and antagonistic ligands bind to specific sites on the GABA_A receptor, the fine epitope mapping of autoantibodies on the GABA_A receptor $\beta 3$ subunit may contribute to further understanding the pathogenic mechanism causing

abnormal neuronal excitation in the brain and developing therapeutic interventions.

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