

**Figure 1.** Cardiac pathology and CT findings of the paraspinal muscles and gastrocnemius. Hematoxylin and Eosin staining revealed mild myocardial interstitial fibrosis ( $\times 800$ ) (A). A CT revealed low-density areas and atrophic changes (arrows) in the cervical and lumbar paraspinal muscles (B, C) and the left gastrocnemius (D).

## Case Report

A 22-year-old male with normal growth and development was referred to our hospital with a complaint of chest discomfort at the age of 19 years. He had a complete atrioventricular conduction block (heart rate, 38/min), and his serum creatine kinase (CK) was elevated to 3,000 IU/mL (normal range 45-163 IU/mL). A chest X-ray and echocardiography (ECG) revealed no cardiomegaly. His symptoms gradually worsened, and symptoms of dizziness appeared due to bradycardia. The R-R interval of the ECG was extended to 9.2 s. Six months after the initial diagnosis, the patient was implanted with a permanent pacemaker. A sample of the left ventricular myocardium was obtained during cardiac catheterization, and histochemical staining revealed mild myocardial interstitial fibrosis without fiber disarray, fibril deposition, or myocardial injury (Fig. 1A). No abnormalities were detected in the respiratory function tests. Although he had suffered from diplopia and strabismus several years earlier, a neurological examination on admission showed normal ocular movement. His cognition, coordination, and facial muscle strength were also normal. The patient's neck flexion was slightly weak and was limited due to mild neck contracture. There was no scoliosis or contracture of any other joints in the extremities. His extremities showed no significant muscle weakness, atrophy, or sensory disturbances. Serum CK fluctuated between 1,600 IU/mL and 3,000 IU/mL.

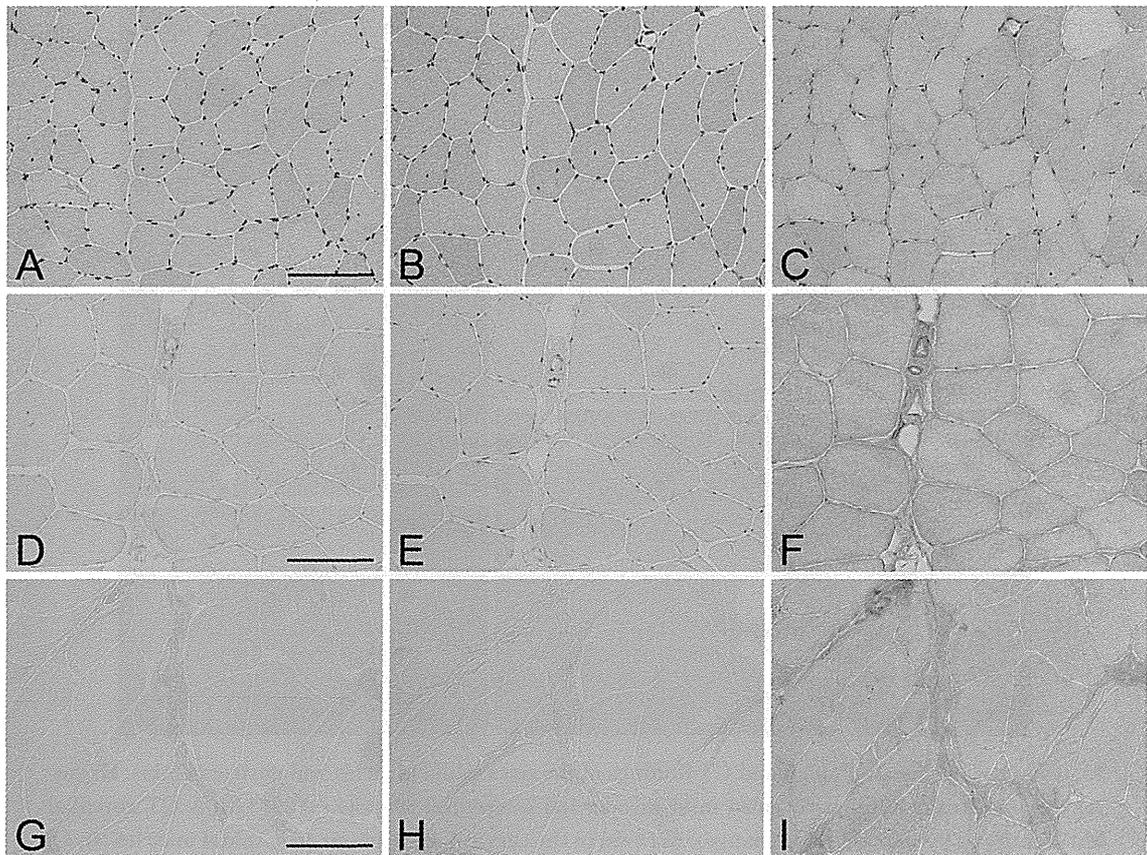
Other routine serologic evaluations and tests for serum autoantibodies revealed no abnormalities. Using electromyography, short-duration and some unstable polyphasic motor unit potentials were detected in the right biceps brachii, but no abnormality was found in the muscles of the lower extremities. A whole-body computed tomography (CT) scan revealed low-density areas and atrophic changes in the cervical and lumbar paraspinal muscles and the left gastrocnemius (Fig. 1B, C, D).

His 48-year-old mother suffered from a first-degree atrioventricular conduction block along with paroxysmal atrial fibrillation, and was treated with warfarin. In addition, unspecified abnormal ECGs were observed when his 24-year-old twin elder sisters were screened, but no abnormalities could be detected in the workup. Periodic health examinations were advised for all three of his family members. Muscle CT examination was not performed in these probable female carriers.

The study protocol was reviewed and approved by the Institutional Review Board of Kagoshima University. The patient provided his written, informed consent to participate in this study.

## Pathological studies

A skeletal muscle sample was obtained from the biceps brachii, but no skin sample was collected. Histochemical staining revealed mild variations in fiber diameter, a few degenerating or regenerating fibers, a slight increase in the

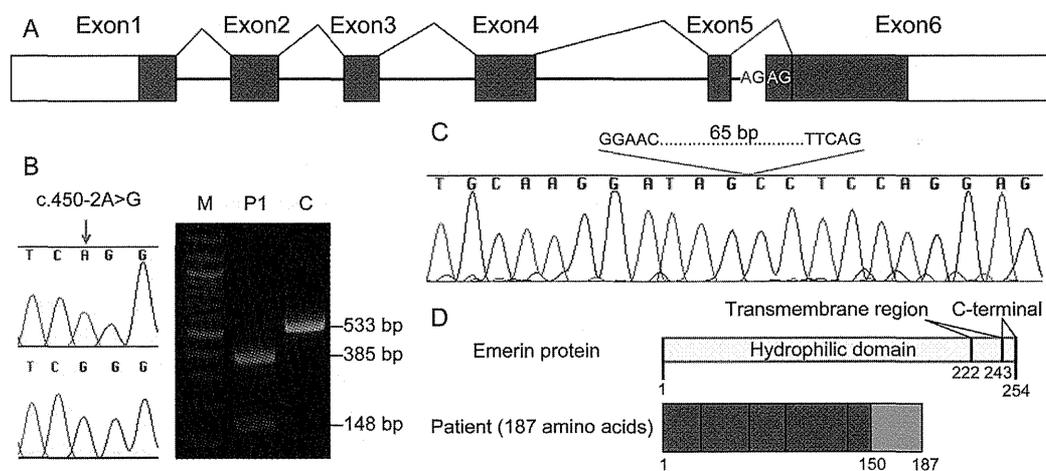


**Figure 2.** Serial immunohistochemical staining with NCL-Emerin (A, D, G), H-12 (B, E, H), and C-20 (C, F, I) antibodies. A-C: Emerin was strongly expressed on the nuclei of skeletal muscle fibers in the healthy control subject. D-F: In the patient, emerin expression was significantly decreased as detected by NCL-Emerin and H-12 labeling, and it was completely absent as assessed by C-20 labeling. G-I: In the disease control, staining experiments revealed a complete absence of emerin with all three antibodies. No counterstain was used. Bar = 100  $\mu$ m

number of internal nuclei, and slight connective tissue proliferation. The number of hypertrophic fibers was increased. Immunohistochemical stains were performed manually with a 1:50 dilution of NCL-Emerin (Novocastra, Leica Microsystems, Newcastle Upon Tyne, UK), H-12 (Santa Cruz Biotechnology, Santa Cruz, USA) monoclonal antibodies for the N-terminus of emerin, and a 1:20 dilution of C-20 affinity purified polyclonal antibody for the C-terminus of emerin (Santa Cruz Biotechnology). A skeletal muscle specimen from an unrelated 39-year-old male with typical X-linked EDMD was used as a disease control. The three antibodies detected emerin expression on the nuclei of the skeletal muscle in the healthy control subject. In our patient, emerin expression was significantly decreased as detected by NCL-Emerin and H-12 labeling, and it was completely absent with C-20 labeling. In the disease control specimen, nuclear staining was absent with all three antibodies against emerin (Fig. 2). In western blot analyses using the three antibodies, we could not detect any clear bands in the patient's samples, and this was probably due to the low expression of protein or detection sensitivity of western blot (data not shown).

### Genetic studies

Genomic DNA was extracted from the patient's blood lymphocytes. We were not able to obtain consent for genetic analysis from his three family members or the disease control patient. Using the Primer3 (v. 0.4.0) online program (<http://frodo.wi.mit.edu/>), we designed oligonucleotide primers flanking the six exons and intron-exon junctions in the *EMD* gene. After hot-start polymerase chain reaction (PCR) amplification, the products were sequenced by dye-terminator chemistry using an ABI3010 sequencer (Applied Biosystems, Foster City, USA). A splice acceptor site mutation located at the second nucleotide before exon six (c.450-2A>G) of *EMD* was identified. This created a new restriction site for *Ava*I. Using the forward (5'-CTCGCCCTGACTCTCTTCTG-3') and reverse (5'-CTAAGGCAGTCAGCCAGAC-3') primers, a 533-bp PCR product covering this mutation was amplified and was divided into 385 bp and 148 bp fragments (Fig. 3A, B). This mutation was not detected in 100 Japanese control chromosomes, and we did not find the c.450-2A>G mutation in the 1,000 Genomes database that catalogs human genetic variations in 2,500 samples, including 500 East Asian (100 Japanese) samples ([-120-](http://</a></p>
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**Figure 3. Genetic studies and an ideogram of the aberrant splicing. A:** The splice acceptor site mutation located at the second nucleotide before exon 6 (red: c.450-2A) of the *EMD* gene. **B:** Sequencing chromatogram of the c.450-2A>G mutation in *EMD* (red arrow) and the amplicon (533 bp) covering exon 6, which was divided into 385-bp and 148-bp fragments by *Ava*I. **C:** This aberrant splicing eliminated 65 nucleotides in the transcript of exon 6. **D:** The mutation shifted the reading frame (gray column) and produced a truncated transcript of 187 amino acids without the transmembrane region and C-terminal tail.

brower.1000genomes.org).

Total RNA was isolated and purified from the biopsied skeletal muscle specimen using a RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion, Austin, USA). Complementary DNA (cDNA) was generated by reverse transcription PCR (RT-PCR) of the isolated RNA (1 µg) using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The cDNA was subsequently amplified with the forward (5'-ACCAGAGCAAGGGCTACAATGACG-3') and reverse primers (5'-GGTGGCCTTTGGTTAATCCCCTC-3') using KOD FX (Toyobo, Osaka, Japan). No band was detected at the expected position of the full-length emerlin transcript, and a unique shorter band was extracted from the gel (QIAquick Gel Extraction Kit, Qiagen, Venlo, Netherlands) and sequenced. The first 65 nucleotides in exon six were found to be absent, and the reading frame was shifted. The protein was changed because the frame was shifted from position 150 to a premature stop codon at 187 (p.Arg150fs), producing a truncated emerlin protein lacking the transmembrane region and C-terminal tail (Fig. 3C, D).

## Discussion

We studied the clinical, pathological, and genetic features of a Japanese patient with X-linked EDMD. We observed a partial deficiency of emerlin in the skeletal muscle with severe arrhythmia and continuous serum CK elevation, but he only exhibited slight muscular involvement and mild joint contracture. A splice acceptor site mutation, c.450-2A>G (p.Arg150fs), was identified in the *EMD* gene of this patient.

The *EMD* gene on chromosome Xq28 contains six exons

and encodes an emerlin protein with 254 amino acids. Emerlin consists of a large hydrophilic nucleoplasmic domain (residues 1-222), a transmembrane region (residues 223-243), and a short C-terminal tail (residues 244-254) (7). It is a ubiquitously expressed protein located at the inner nuclear membrane of most cells in the body with the highest mRNA expression in skeletal and cardiac muscle, and it has been proposed to have functions in gene expression, RNA processing, cell signaling, and chromatin dynamics (2, 8).

The 22-year-old patient in this study suffered a complete atrioventricular conduction block, and a permanent pacemaker was implanted to prevent lethal cardiac arrest. His mother and sisters were also affected with arrhythmia, but they did not show evidence of skeletal muscle abnormalities. Previous reports have documented that such symptomatic female carriers have very low emerlin levels (<5% of normal) due to skewed X-inactivation (9). A pathological study of cardiac muscle performed by local doctors revealed nonspecific mild interstitial fibrosis. Slight muscular involvement was observed during both the clinical and imaging examinations. The cause of this proband was unclear until his persistent high CK level was noticed and cardiomyopathy suspected, at which point a skeletal muscle specimen was obtained.

The histochemical pathology of the skeletal muscle indicated fiber size variation with scattered internalized nuclei. An immunohistochemical study with the NCL-Emerlin monoclonal antibody targeted to 220 amino acids near the N-terminus of emerlin revealed a significant reduction of nuclear emerlin, and a diagnosis of X-linked EDMD was made. After direct sequencing of the *EMD* gene, an A>G transversion at 1,644 (c.450-2A>G) was observed, which should eliminate the splice acceptor site before exon six. In order to

identify the candidate acceptor site, we performed RT-PCR using mRNA extracted from the patient's frozen skeletal muscle tissue. A truncated transcript with 187 residues generated by aberrant splicing was detected. The new AG splice acceptor site, which occurs 65 bp after the wild-type site in the *EMD*, shifted the frame from position 150 to a premature stop codon at position 187. Most of the truncated emerlin lacked the transmembrane segment and the C-terminal tail, thus suggesting that it might fail to target the endoplasmic reticulum and would thus be rapidly degraded (10). Interestingly, this c.450-2A>G splice site mutation was reported in another unrelated patient in Japan in 1999 (6). The initial symptom of the first patient was neck contracture, but our patient initially experienced cardiac involvement. In fact, immunostaining and immunoblotting studies performed in the previous patient revealed the absence of emerlin and a premature stop codon generated at residue 235. The differences between these two patients with same mutation suggest that there could be other potential mechanisms involved in protein expression and mRNA splicing.

In an additional immunohistochemical study, we employed two additional emerlin-targeted antibodies, H-12 (targets residues 3 to 254) and C-20 (targets a peptide near the C-terminus). In the present patient, emerlin expression was detected by H-12 staining (but at a much lower level than normal) but not by C-20 staining. In this case, a skin sample could have been helpful in further verifying the status of emerlin. We concluded that the truncated emerlin in the present patient could react with antibodies targeting a peptide near the N-terminus (NCL-Emerlin and H-12) at much lower levels, but it could not bind the antibody targeted to the C-terminus (C-20). In addition, the nucleoplasm showed stronger staining than the cytoplasm, but this was not as strong as that observed at the nuclear edge, thus indicating that emerlin may have been expressed in the nucleoplasm. An immunoelectron microscopy study might be more reliable for identifying emerlin expression. The reason why the mutated version of emerlin without the transmembrane segment was detectable on the nuclei requires further research.

According to the UMD-EMD mutations database (<http://www.umd.be/EMD/>), 94 different mutations of the *EMD* gene have been reported in 298 records, and 15 of these are splice site mutations (15.96%). It is notable that some other X-linked EDMD patients with truncated emerlin longer than 187 amino acids presented with complete absences of emerlin as detected with the NCL-Emerlin antibody from the same company (11-13). To evaluate the emerlin protein expression, it would be more reliable to use various antibodies targeted to different domains. An antibody targeting the C-terminus could therefore be useful for detecting truncated emerlin, which accounts for most *EMD* mutations.

Mutations in emerlin can cause different phenotypes, even within the same family. In patients with distal premature stop codons, which do not evoke a nonsense-mediated decay mechanism, the phenotypes present as a typical

triad (6, 12-16) or cardiac phenotype (11, 17). Rare cases of a reduction in emerlin due to a missense mutation may have a milder phenotype (18). The cardiac muscle is supposed to be less tolerant of emerlin deficiency than the skeletal muscle (19). A lack of or decrease in emerlin levels in the heart may alter electrical resistance and cardiomyocyte adhesion, which could lead to conduction delay or block (17). Emerlinopathy also includes a limb-girdle muscular dystrophy phenotype (20, 21). In the present case with partial emerlin deficiency, although a complete atrioventricular conduction block was observed, there was only slight muscle weakness and joint contracture. The truncated protein remaining in the nucleoplasm may have contributed to the relatively benign phenotype, especially in the skeletal muscle.

In conclusion, we identified a splice site mutation before the last exon of *EMD* in an X-linked EDMD patient, and he showed evidence of a truncated protein at both pathological and genetic levels. We detected partial expression of a truncated emerlin protein without the transmembrane region and C-terminal domain by immunohistochemical staining with antibodies against different emerlin domains. We recommend the use of multiple antibodies that bind different emerlin domains in order to obtain a comprehensive view of the protein. The anti-emerlin antibody C-20 may be useful for detecting the absence of the C-terminus. Our findings also suggest that all young patients with significant cardiac conduction defects of unknown etiology be assessed to exclude the presence of EDMD, even if they have either no skeletal muscle or only slight skeletal muscle or joint involvement.

#### Author's disclosure of potential Conflicts of Interest (COI).

Hiroshi Takashima: Patent royalties/licensing fees, Achena Diagnosis

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#### References

1. Emery AE, Dreifuss FE. Unusual type of benign x-linked muscular dystrophy. *J Neurol Neurosurg Psychiatry* **29**: 338-342, 1966.
2. Bione S, Maestrini E, Rivella S, et al. Identification of a novel X-linked gene responsible for Emery-Dreifuss muscular dystrophy. *Nat Genet* **8**: 323-327, 1994.
3. Bonne G, Di Barletta MR, Varnous S, et al. Mutations in the gene

- encoding lamin A/C cause autosomal dominant Emery-Dreifuss muscular dystrophy. *Nat Genet* **21**: 285-288, 1999.
4. Raffaele Di Barletta M, Ricci E, Galluzzi G, et al. Different mutations in the LMNA gene cause autosomal dominant and autosomal recessive Emery-Dreifuss muscular dystrophy. *Am J Hum Genet* **66**: 1407-1412, 2000.
  5. Helbling-Leclerc A, Bonne G, Schwartz K. Emery-Dreifuss muscular dystrophy. *Eur J Hum Genet* **10**: 157-161, 2002.
  6. Funakoshi M, Tsuchiya Y, Arahata K. Emerin and cardiomyopathy in Emery-Dreifuss muscular dystrophy. *Neuromuscul Disord* **9**: 108-114, 1999.
  7. Ellis JA, Brown CA, Tilley LD, et al. Two distal mutations in the gene encoding emerin have profoundly different effects on emerin protein expression. *Neuromuscul Disord* **10**: 24-30, 2000.
  8. Holaska JM, Wilson KL. An emerin "proteome": purification of distinct emerin-containing complexes from HeLa cells suggests molecular basis for diverse roles including gene regulation, mRNA splicing, signaling, mechanosensing, and nuclear architecture. *Biochemistry* **46**: 8897-8908, 2007.
  9. Manilal S, Recan D, Sewry CA, et al. Mutations in Emery-Dreifuss muscular dystrophy and their effects on emerin protein expression. *Hum Mol Genet* **7**: 855-864, 1998.
  10. Yates JR, Bagshaw J, Aksmanovic VM, et al. Genotype-phenotype analysis in X-linked Emery-Dreifuss muscular dystrophy and identification of a missense mutation associated with a milder phenotype. *Neuromuscul Disord* **9**: 159-165, 1999.
  11. Vytopil M, Vohanka S, Vlasinova J, et al. The screening for X-linked Emery-Dreifuss muscular dystrophy amongst young patients with idiopathic heart conduction system disease treated by a pacemaker implant. *Eur J Neurol* **11**: 531-534, 2004.
  12. Astejada MN, Goto K, Nagano A, et al. Emerinopathy and laminopathy clinical, pathological and molecular features of muscular dystrophy with nuclear envelopathy in Japan. *Acta Myol* **26**: 159-164, 2007.
  13. Paradas C, Márquez C, Gallardo E, et al. X-linked Emery-Dreifuss muscular dystrophy and vacuoles: an immunohistochemical characterization. *Muscle Nerve* **32**: 61-65, 2005.
  14. Ognibene A, Sabatelli P, Petrini S, et al. Nuclear changes in a case of X-linked Emery-Dreifuss muscular dystrophy. *Muscle Nerve* **22**: 864-869, 1999.
  15. Hong JS, Ki CS, Kim JW, et al. Cardiac dysrhythmias, cardiomyopathy and muscular dystrophy in patients with Emery-Dreifuss muscular dystrophy and limb-girdle muscular dystrophy type 1B. *J Korean Med Sci* **20**: 283-290, 2005.
  16. Niebroj-Dobosz I, Marchel M, Madej A, et al. Circulating autoantibodies to troponin I in Emery-Dreifuss muscular dystrophy. *Acta Myol* **27**: 1-6, 2008.
  17. Sakata K, Shimizu M, Ino H, et al. High incidence of sudden cardiac death with conduction disturbances and atrial cardiomyopathy caused by a nonsense mutation in the STA gene. *Circulation* **111**: 3352-3358, 2005.
  18. Ellis JA, Yates JR, Kendrick-Jones J, Brown CA. Changes at P183 of emerin weaken its protein-protein interactions resulting in X-linked Emery-Dreifuss muscular dystrophy. *Hum Genet* **104**: 262-268, 1999.
  19. Wehnert M, Muntoni F. 60th ENMC international workshop: non X-linked Emery-Dreifuss muscular dystrophy 5-7 June 1998, Naarden, The Netherlands. *Neuromuscul Disord* **9**: 115-121, 1999.
  20. Muntoni F, Lichtarowicz-Krynska EJ, Sewry CA, et al. Early presentation of X-linked Emery-Dreifuss muscular dystrophy resembling limb-girdle muscular dystrophy. *Neuromuscul Disord* **8**: 72-76, 1998.
  21. Ura S, Hayashi YK, Goto K, et al. Limb-girdle muscular dystrophy due to emerin gene mutations. *Arch Neurol* **64**: 1038-1041, 2007.

# Identification and Characterization of GABA<sub>A</sub> 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<sub>B</sub> receptor, and LGI1. However, it remains unclear how many synaptic autoantigens are yet to be defined. Using immunoproteomics, we identified autoantibodies against the GABA<sub>A</sub> 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  $\beta$ 3 subunit of the GABA<sub>A</sub> receptor. The  $\beta$ 3-subunit-containing GABA<sub>A</sub> 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  $\beta$ 3 subunit was knocked down. Our developed multiplex ELISA testing showed that both patients had similar levels of GABA<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> receptor antibodies play a central role in the patients' symptoms. Therefore, this study establishes anti-GABA<sub>A</sub> receptor encephalitis and expands the pathogenic roles of GABA<sub>A</sub> receptor autoantibodies.

**Key words:** autoantibody; autoimmune encephalitis; cognitive impairment; GABA<sub>A</sub> 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<sub>B</sub> 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<sub>A</sub> receptor mediates most of the fast inhibitory synaptic transmission in the brain and is composed of heteropentameric assemblies of different subunit subtypes [ $\alpha$  (1–6),  $\beta$  (1–3),  $\gamma$  (1–3),  $\delta$ ,  $\epsilon$ ,  $\theta$ ,  $\pi$ , and  $\rho$  (1–3)] to form chloride ion channels (Macdonald and Olsen, 1994; Jacob et al., 2008; Rudolph and Knoflach, 2011). The majority of GABA<sub>A</sub> receptors contain two  $\alpha$  subunits, two  $\beta$  subunits, and one  $\gamma$  or  $\delta$  subunit. The GABA<sub>A</sub> 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<sub>A</sub> receptor subunits, including  $\alpha$ 1,  $\beta$ 3,  $\gamma$ 2, and  $\delta$ , cause genetic epilepsy syndromes (Macdonald et al., 2010) and genetic loss of the  $\beta$ 3 subunit in mice causes seizures and learning and memory deficits (DeLorey et al., 1998). Therefore, although the GABA<sub>A</sub> receptor can be a strong candidate affected in autoimmune CNS disorders, GABA<sub>A</sub> receptor antibodies have not yet been reported.

Here, using a nonbiased proteomic method, we identified autoantibodies against the GABA<sub>A</sub> receptor in two patients with encephalitis. The patients' GABA<sub>A</sub> receptor antibodies specifically caused downregulation of GABA<sub>A</sub> receptors. The present study establishes a pathogenic role of GABA<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> receptor  $\alpha$ 2 (catalog #600-401-D45 RRID:AB\_11182018; Rockland Immunochemicals),  $\alpha$ 5 (catalog #AB9678 RRID:AB\_570435; Millipore),  $\beta$ 3 (catalog #ab4046 RRID:AB\_2109564; Abcam),  $\gamma$ 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<sub>A</sub> receptor  $\alpha$ 1 (catalog #75-136 RRID:AB\_2108811; NeuroMab),  $\beta$ 1 (catalog #75-137 RRID:AB\_2109406; NeuroMab),  $\beta$ 2/ $\beta$ 3 (extracellular epitope, catalog #MAB341 RRID:AB\_2109419; Millipore),  $\beta$ -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<sub>A</sub> receptor  $\alpha$ 1 (NM\_183326),  $\alpha$ 2 (NM\_001135779),  $\alpha$ 5 (NM\_017295),  $\beta$ 1 (NM\_012956), and  $\beta$ 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<sub>A</sub> receptor  $\gamma$ 2 (Fang et al., 2006).

Rat GABA<sub>A</sub> receptor  $\beta$ 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- $\beta$ 3-211, 5'-AGCATCGACATGGTTTCTGAA-3' (an alternative sequence: miR- $\beta$ 3-347, 5'-TCTGGGTGCCTGACACATATT-3'; both sequences yielded the same results) and miR-LacZ ( $\beta$ -galactosidase), 5'-GACTACACAATCAGCGATTT-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  $\beta$ -actin promoter. The resultant miR constructs were validated for the knock down of cotransfected rat GABA<sub>A</sub> receptor  $\beta$ 3 expression in HEK293T cells by Western blotting (see Fig. 3A). Rat GABA<sub>A</sub> receptor  $\beta$ 3 subunit rescue construct [miR- $\beta$ 3-211-resistant  $\beta$ 3 (res $\beta$ 3) in pCAGGS], which has two different nucleotides in the target sequences, was generated using site-directed mutagenesis (5'-AACATCGACATCGCCAGCATTTGATATGGTTTCTGAAGTCAACAT-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 ( $5 \times 10^4$  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<sub>A</sub> receptor  $\gamma$ 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<sub>A</sub> receptor  $\beta$ 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- $\beta$ 3) with or without rescue  $\beta$ 3 construct by Lipofectamine 2000. At 5 d after transfection, live

neurons were incubated with anti-GABA<sub>A</sub> 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 ( $5 \times 10^5$  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<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> receptor subunit intensity was graphed.

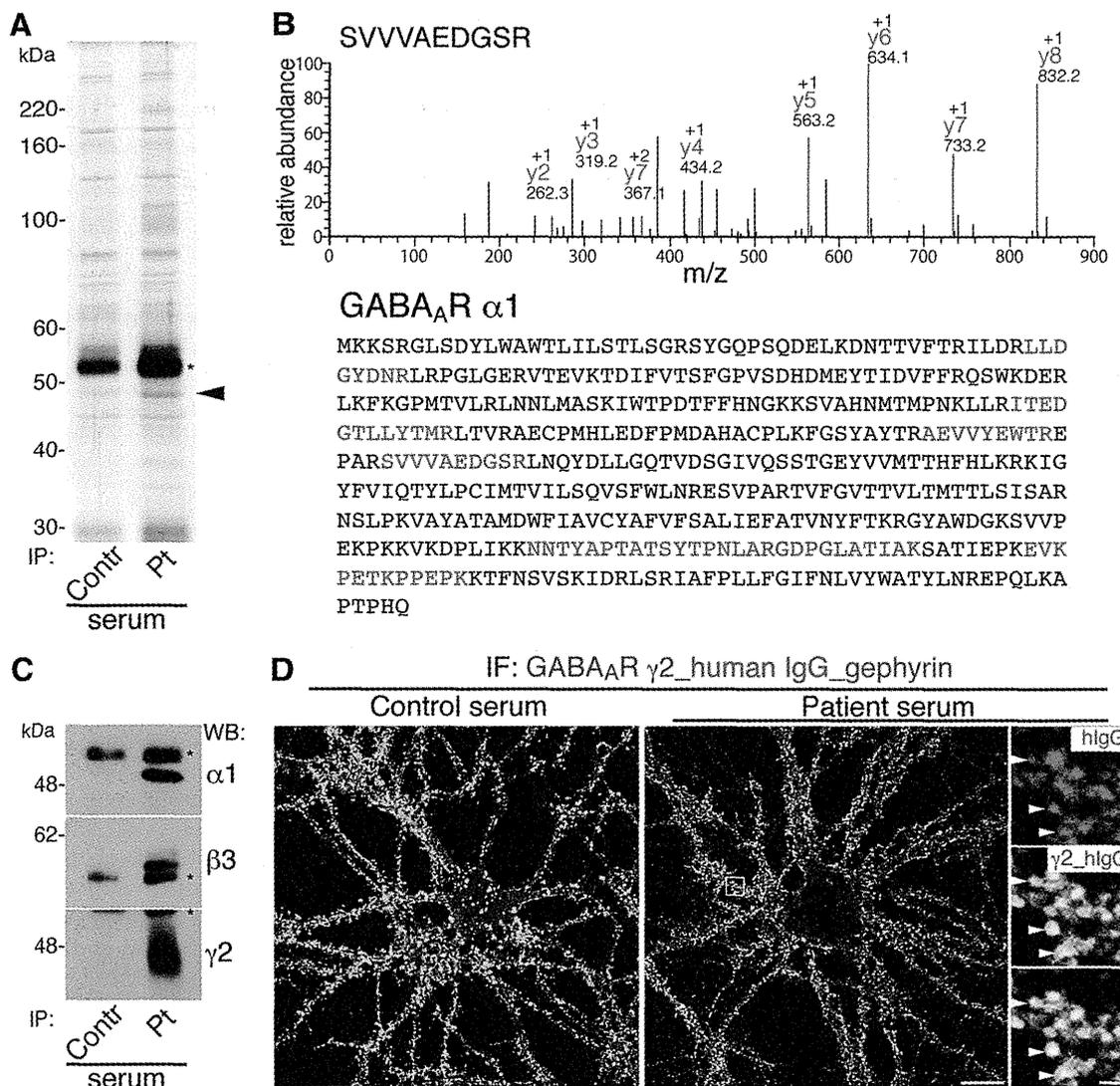
The identities of human and rat GABA<sub>A</sub> receptor  $\alpha 1$ ,  $\beta 3$ , and  $\gamma 2$  subunits in their amino acid sequence are 100%, 97%, and 99%, respectively; and that of the human and mouse  $\gamma 2$  subunit is 99%, suggesting that the results obtained by using rat or mouse GABA<sub>A</sub> 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<sub>A</sub> 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 ( $3 \times 10^4$  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<sub>A</sub> receptor autoantibodies.** To determine the effect of the patients' serum antibodies on the surface or synaptic GABA<sub>A</sub> receptor density, cultured rat hippocampal neurons ( $\sim 30$  DIV) were treated with the indicated serum samples for 2 d. 4.5  $\mu\text{l}$  of the serum was added daily to 300  $\mu\text{l}$  of each culture medium (final 3% concentration). After washing, live neurons were then incubated with an antibody to an extracellular epitope of GABA<sub>A</sub> 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<sub>A</sub> receptor  $\beta 3$  subunit antibody (the intracellular epitope) after cell permeabilization to visualize the GABA<sub>A</sub> receptors containing the  $\beta 3$  subunit. To quantify the synaptic GABA<sub>A</sub> receptors, we randomly chose dendrites and analyzed the number of GABA<sub>A</sub> receptor  $\gamma 2$  and  $\beta 3$  clusters along dendrites (20  $\mu\text{m}$  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 ( $1.5 \times 10^4$  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<sub>A</sub> receptor antibodies for 24 h.



**Figure 1.** Identification of GABA<sub>A</sub> 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<sub>A</sub> receptor  $\alpha$ 1 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<sub>A</sub> receptor  $\alpha$ 1 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  $\alpha$ 1,  $\beta$ 3, and  $\gamma$ 2 subunits of the GABA<sub>A</sub> 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<sub>A</sub> receptors at the cell surface of rat hippocampal neurons. The serum reactivity (red; human IgG) was well overlapped with surface-expressed  $\gamma$ 2 subunits of GABA<sub>A</sub> 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  $\mu$ m (1  $\mu$ m, magnified). IP, immunoprecipitation; Contr, control; Pt, patient; GABA<sub>A</sub>R, GABA<sub>A</sub> receptor; WB, Western blotting; IF, immunofluorescence; hlgG, human Ig.

Nine microliters of the serum was added to 300  $\mu$ l 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 CaCl<sub>2</sub>, 1.0 MgCl<sub>2</sub>, 21.0 NaHCO<sub>3</sub>, 0.6 NaH<sub>2</sub>PO<sub>4</sub>, and 10.0 glucose equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, 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 $\times$ /0.90 NA; Olympus). Patch-clamp electrodes (resistance, 3–6 M $\Omega$ ) 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 Na<sub>3</sub>-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  $\mu$ M tetrodotoxin (TTX), 20  $\mu$ M CNQX, and 50  $\mu$ M APV (Satake et al., 2004). To record AMPA-receptor-mediated miniature EPSCs (mEPSCs), neurons were held at  $-80$  mV in the presence of 1  $\mu$ M TTX, 10  $\mu$ M bicuculline, and 50  $\mu$ M 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 ( $\sigma^2$ ) 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<sub>A</sub> receptor antibodies**

Pt#	Age	Sex	At onset of encephalitis <sup>a</sup>		History		Autoantibodies at onset of encephalitis			Reference	
			Symptoms/signs	Brain MRI (T2)	Cancer and other remarks (age)	Autoimmune diseases (age); autoantibodies <sup>b</sup>	In original case report <sup>b</sup>	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 pM (serum)	Anti-AchR, 130 nm; anti-VGKC, 649 pM (serum)	Anti-GABA <sub>A</sub> 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 pM (serum)	Anti-GABA <sub>A</sub> R, anti-CASPR2, and anti-DCC (serum)	Chemotherapy for recurrent thymoma (carboplatin and etoposide)	Mental state partially improved	Ohshita et al., 2006

<sup>a</sup>The serum samples at the onset of encephalitis were used for the present screening.

<sup>b</sup>Anti-VGKC, normal <100 pM; anti-AchR, normal <0.2 nM.

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

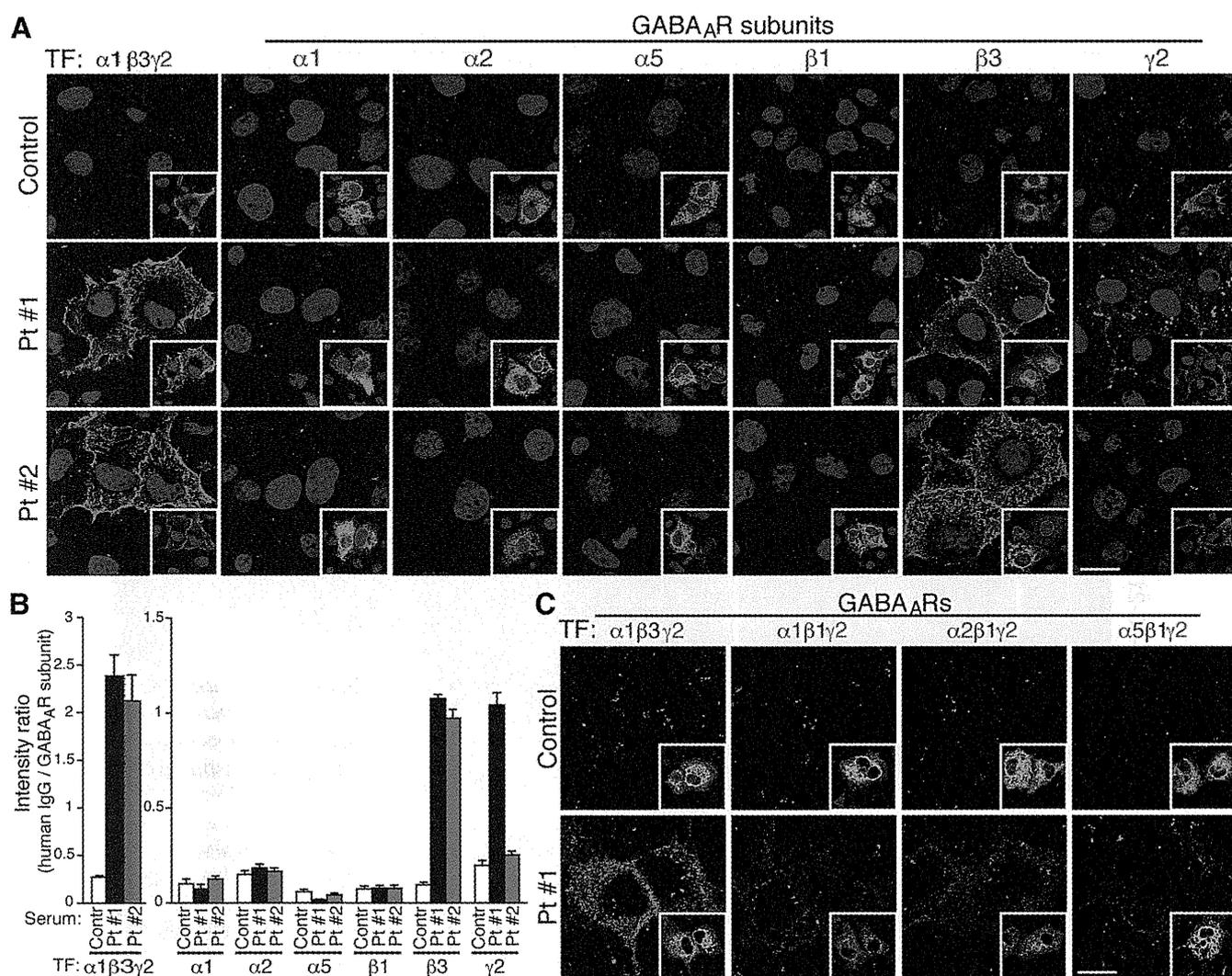
## Results

### Identification of GABA<sub>A</sub> 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<sub>A</sub> receptor  $\alpha$ 1 subunit: LLDGYDNR, ITEDGTLTYTMR, AEVVYEWTR, SVVVAEDGSR, NNTYAPTATSYTPNLAR, GDPGLATIAK, and EVKPETKPPEPK. The estimated molecular weight of rat GABA<sub>A</sub> receptor  $\alpha$ 1 (51.7 kDa) was close to that of p48. In addition, peptide fragments coinciding with those from the GABA<sub>A</sub> receptor  $\beta$ 3 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  $\alpha$ 1,  $\beta$ 3, and  $\gamma$ 2 subunits of GABA<sub>A</sub> receptor confirmed the immunoprecipitation of heteromeric GABA<sub>A</sub> receptors (Fig. 1C). Consistently, the serum antibodies showed overlapped signals with inhibitory synapses marked by GABA<sub>A</sub> receptor  $\gamma$ 2 and gephyrin antibodies in rat hippocampal neurons (Fig. 1D).

### GABA<sub>A</sub> receptor autoantibodies are directed to extracellular epitope of the $\beta$ 3 subunit

To determine whether the patient serum antibodies bind directly to the GABA<sub>A</sub> receptor and, if so, which of GABA<sub>A</sub> receptor subunits the antibodies recognize, the cell-based binding assay was performed. Because the native GABA<sub>A</sub> receptor is a heteropentamer composed of two  $\alpha$ , two  $\beta$ , and one  $\gamma$  subunits and the patient serum immunoprecipitated  $\alpha$ 1,  $\beta$ 3, and  $\gamma$ 2 subunits from hippocampal neurons (Fig. 1C),  $\alpha$ 1,  $\beta$ 3, and  $\gamma$ 2 subunits were coexpressed to display heteropentameric GABA<sub>A</sub> 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<sub>A</sub> receptors ( $\alpha$ 1/ $\beta$ 3/ $\gamma$ 2; Fig. 2A, left). Among 19 individual GABA<sub>A</sub> receptor subunits, we then examined the binding to individual GABA<sub>A</sub> receptor subunits ( $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 5,  $\beta$ 1,  $\beta$ 3, and  $\gamma$ 2), which are known to be included in GABA<sub>A</sub> receptor heteromers expressed in hippocampus (Pirker et al., 2000). The serum antibodies from Patient 1 strongly reacted to the cells expressing the  $\beta$ 3 subunit alone and weakly reacted to those expressing the  $\gamma$ 2 subunit alone, but did not to those expressing the  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 5, or  $\beta$ 1 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<sub>A</sub> receptor ( $\alpha$ 1/ $\beta$ 3/ $\gamma$ 2). 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<sub>A</sub> receptor  $\alpha$ 1/ $\beta$ 3/ $\gamma$ 2 and that the antibodies also strongly recognized the  $\beta$ 3 subunit, but neither the  $\alpha$ 1 nor the  $\gamma$ 2 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<sub>A</sub> 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<sub>A</sub> re-



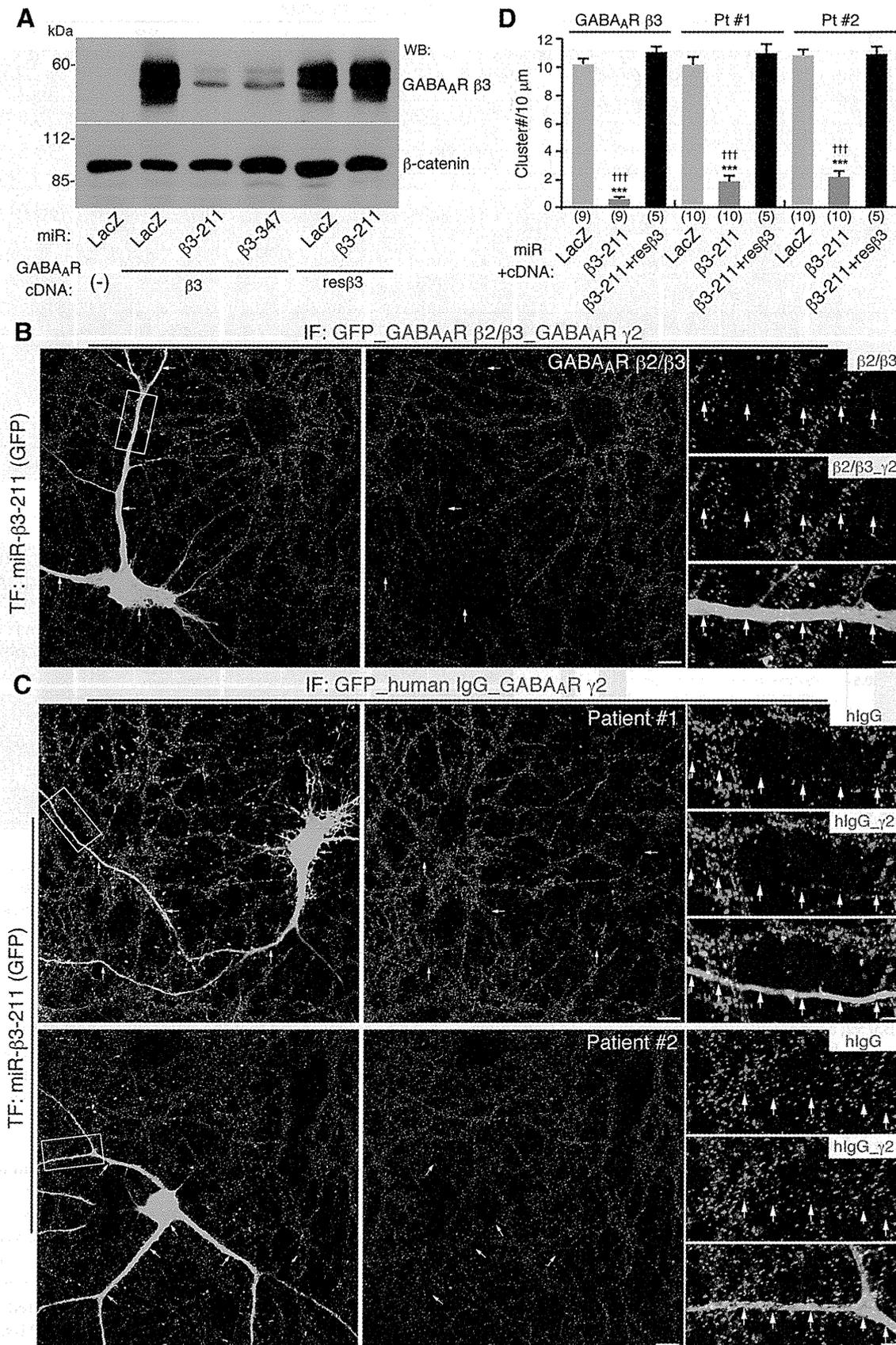
**Figure 2.** Patients' GABA<sub>A</sub> receptor antibodies are directed to extracellular epitope of  $\beta 3$  subunit. **A, B**, COS7 cells were transfected (TF) to surface express the indicated GABA<sub>A</sub> receptor subunits. Transfected cells were fixed and doubly stained with the patient sera (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<sub>A</sub> 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<sub>A</sub> receptors and tested for binding of serum antibodies (red). Transfected cells were detected by staining with the individual  $\alpha$  subunit (green) and  $\gamma 2$  subunit (blue) antibodies. Merged images are shown in insets. Scale bars, 20  $\mu$ m.

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<sub>A</sub> receptor subunits than  $\alpha 1/\beta 3/\gamma 2$ . Because neither serum antibody from the two patients bound to COS7 cells expressing the  $\alpha$  subunit alone, one may wonder whether the  $\alpha$  subunit might not have been efficiently expressed at the cell surface without other subunits. To further examine the possible involvement of  $\alpha$  subunit antibodies in the patient serum, COS7 cells were transfected with various combinations of three subunit genes of the GABA<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> receptor and that the extracellular part of the  $\beta 3$  subunit was the antigenic epitope recognized by the patients' GABA<sub>A</sub> 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<sub>A</sub> receptor containing $\beta 3$ subunit is the main target of the patient serum antibodies

We next investigated whether the GABA<sub>A</sub> 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<sub>A</sub> receptor  $\beta 3$  subunit were first validated by the reduced expression of exogenously expressed rat GABA<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub>-receptor-containing  $\beta 3$  subunit is a major target of the patients' antibodies in hippocampal neurons. **A**, Validation of miRNA constructs for the GABA<sub>A</sub> 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<sub>A</sub> receptor  $\beta 3$  and  $\beta$ -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<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> receptor containing the  $\beta 3$  subunit and that the patients had other autoantibodies in addition to GABA<sub>A</sub> receptor antibodies.

#### Coexisting antibodies with GABA<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> receptor  $\beta 3$  (Fig. 4*A*) and showed similar positive values for GABA<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> 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 =  $1.41 \pm 0.36$ ,  $n = 34$  patients; Patient A as a representative) than patients with neuromyotonia (no CNS symptoms) and LGI1 autoantibodies ( $0.65 \pm 0.16$ ,  $n = 10$  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<sub>A</sub> receptor containing the  $\beta 3$  subunit is a primary target of the patients' serum antibodies and is the main contributor to the patients' symptoms.

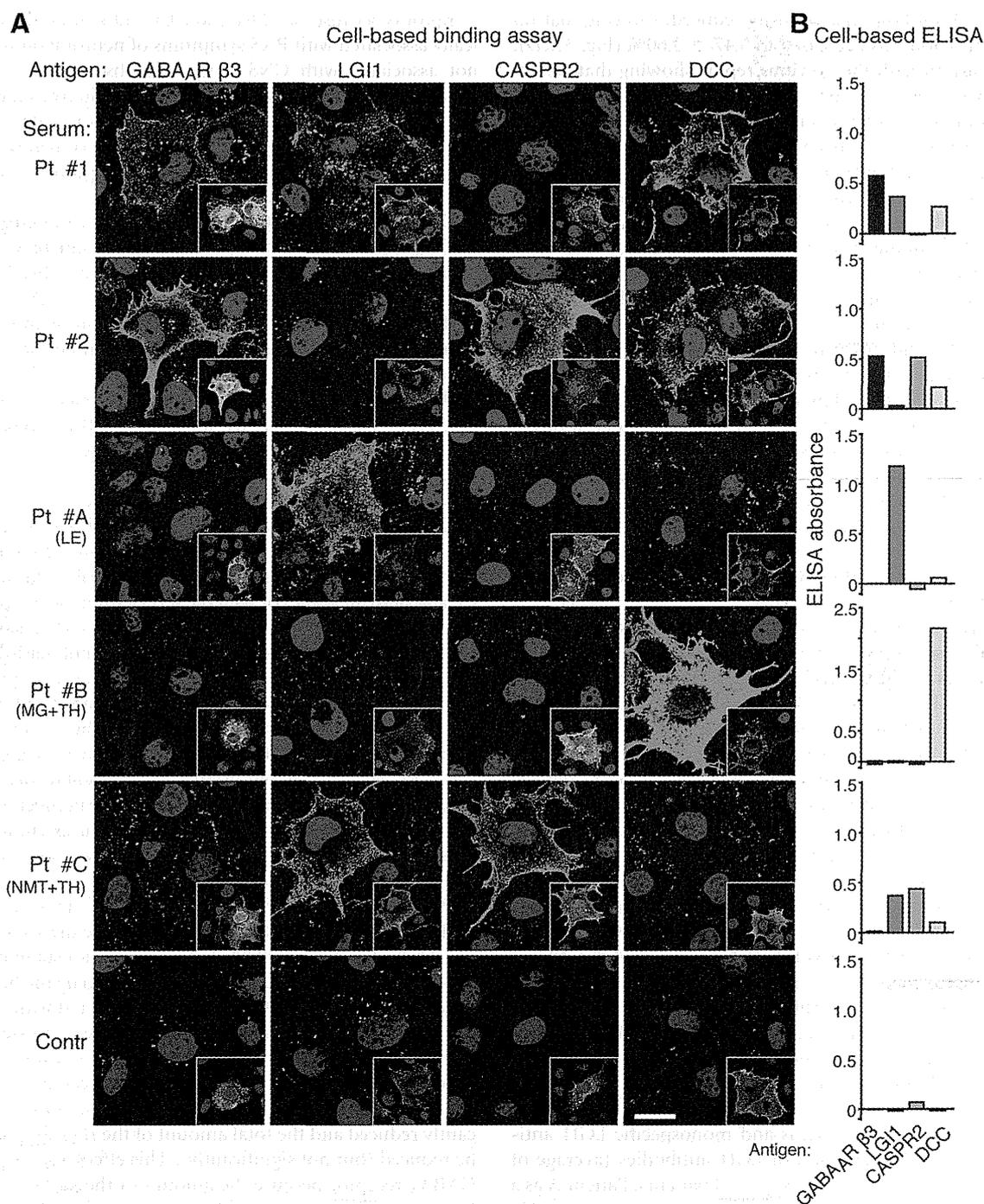
#### Patients' GABA<sub>A</sub> receptor antibodies reduce the number of both synaptic and surface GABA<sub>A</sub> receptor clusters

Next, we explored a mode of action of patients' GABA<sub>A</sub> 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<sub>A</sub> receptor antibodies reduce the number of synaptic GABA<sub>A</sub> receptors. When hippocampal neurons were treated with the serum from Patient 1 and Patient 2 for 2 d, the number of synaptic GABA<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> receptor antibodies (Fig. 4), did not affect the synaptic GABA<sub>A</sub> receptor clusters. The number of surface  $\gamma 2$  subunit clusters, including both synaptic and extrasynaptic GABA<sub>A</sub> receptors, was also heavily reduced by treatment with the serum from Patient 1 and Patient 2. The effect of the patients' serum on GABA<sub>A</sub> receptor clusters was not complement mediated because the heat-inactivated patient serum reduced the number of both synaptic and surface GABA<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> receptor because the amount of the surface GluA1 and N-cadherin was not affected. Together, these results indicate that GABA<sub>A</sub> receptor autoantibodies cause a selective decrease in GABA<sub>A</sub> receptor surface density and synaptic localization, probably by enhancing the receptor internalization.

To determine the relationship between GABA<sub>A</sub> 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<sub>A</sub> receptor antibodies (Fig. 5*C*, Table 1) and showed no effects on synaptic GABA<sub>A</sub> receptor density (Fig. 5*D*). In contrast, the sample of the same patient at the time of symptom presentation of encephalitis had elevated GABA<sub>A</sub> receptor antibodies instead of AChR antibodies and decreased synaptic GABA<sub>A</sub> 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  $\mu\text{m}$  (2  $\mu\text{m}$ , 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; †† $p < 0.001$  compared with miR- $\beta 3$ -211 + res $\beta 3$ . Error bars indicate SEM. The number of neurons examined is indicated in parentheses.



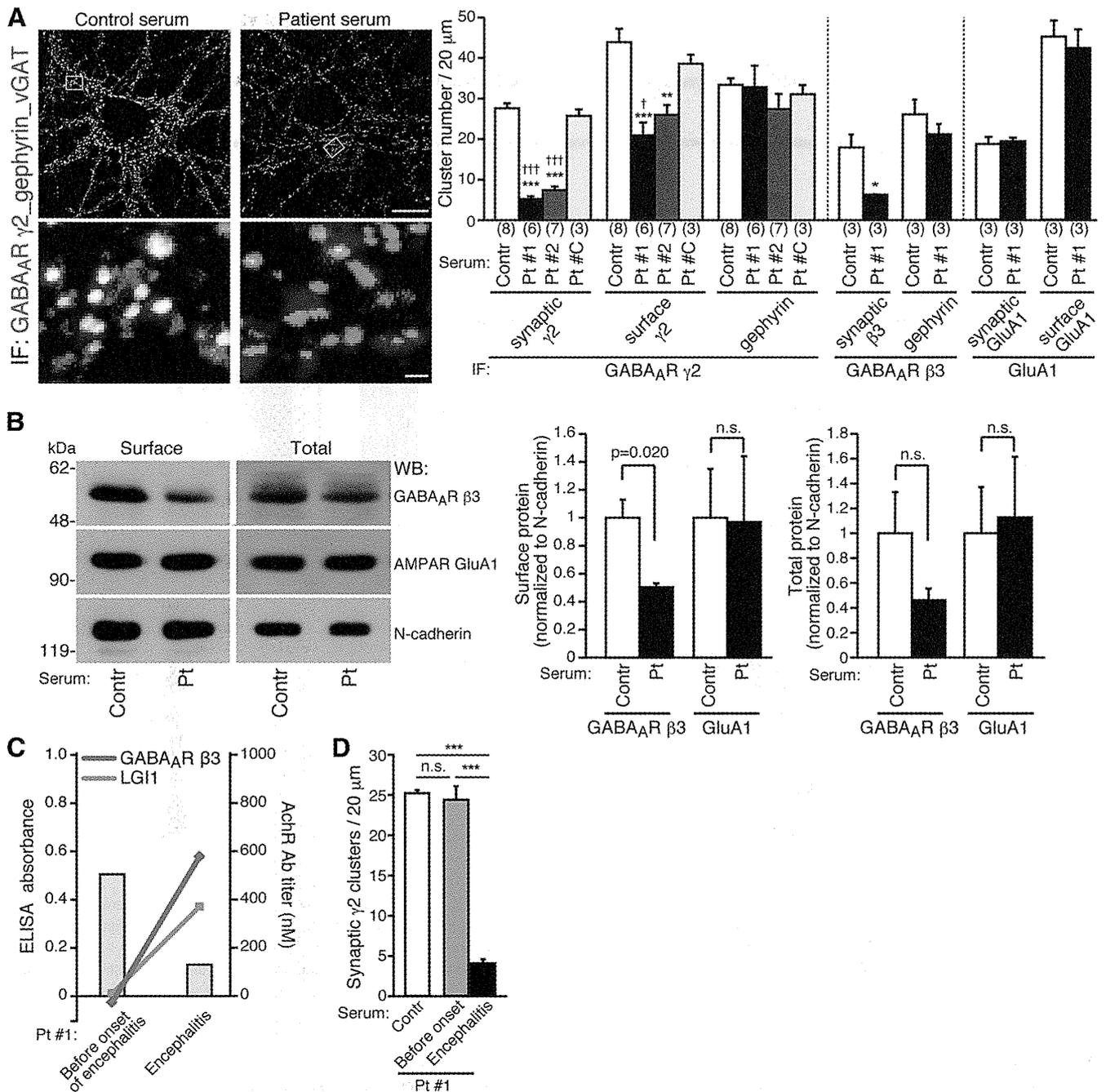
**Figure 4.** Identification of coexisting antibodies with GABA<sub>A</sub> receptor antibodies in the patient serum. **A, B**, Patient 1 and Patient 2 sera from their initial episodes of encephalitis were tested by cell-based binding assay (**A**) and cell-based ELISA tests (**B**) against the GABA<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> 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

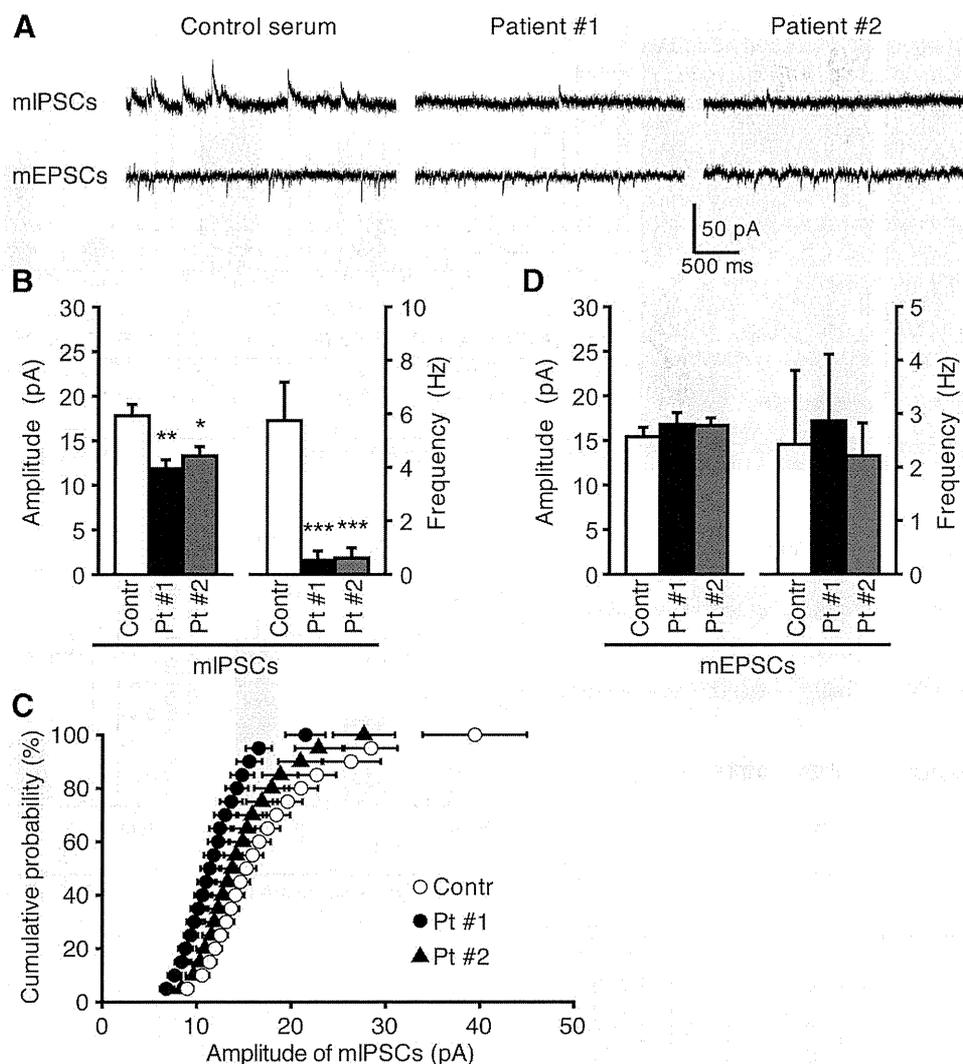
patch-clamp recording of mIPSCs in rat hippocampal neurons. We found a significant decrease in the mean amplitude of mIPSCs in patients' serum-treated neurons compared with that of control serum-treated neurons (Fig. 6A, B). This result is consistent with our immunocytochemical data (Fig. 5A) showing that the treatment of neurons with patients' serum reduced the number of synaptic clusters of GABA<sub>A</sub> 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<sub>A</sub> receptor antibodies specifically reduce synaptic and cell surface GABA<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> receptors were investigated (**D**). Average values for GABA<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> receptor function.



**Figure 6.** Patients' GABA<sub>A</sub> 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<sub>A</sub> receptor autoantibodies in autoimmune encephalitis

GABA is a major inhibitory neurotransmitter and exerts its functions through ionotropic GABA<sub>A</sub> receptors and metabotropic GABA<sub>B</sub> receptors. GABA<sub>B</sub> receptor was recently identified as an autoantigen associated with limbic encephalitis (Lancaster et al., 2010). However, antibodies to the ionotropic GABA<sub>A</sub> receptor have not been yet reported in any neuroimmunological disorders. Here, we found the autoantibodies to GABA<sub>A</sub> receptors in patients with autoimmune encephalitis and revealed a mode of action of the antibodies. One may wonder why GABA<sub>A</sub> 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<sub>A</sub> receptor subunits (all are ~50 kDa) in the SDS-PAGE gel, thereby hindering the detection of GABA<sub>A</sub> receptor subunits. Very recently, while this manuscript was under review, a related paper was published reporting the identification of GABA<sub>A</sub> 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<sub>A</sub> receptors were sometimes concurrently detected with autoantibodies to GAD65 or GABA<sub>B</sub> 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<sub>A</sub> receptor autoantibodies coexist with LGI1, CASPR2, or DCC antibodies. It will be worthwhile to test patients presenting with thymoma and encephalitis for GABA<sub>A</sub> receptor autoantibodies.

### Link between GABA<sub>A</sub> receptor autoantibodies and patient symptoms

We found that the GABA<sub>A</sub> receptor antibodies of both Patient 1 and Patient 2 targeted the  $\beta 3$  subunit of the GABA<sub>A</sub> receptor directly. Based on the previous genetic studies showing that mutations in the human GABA<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> receptor density, whereas the sample of the same patient before the onset of encephalitis had no detectable GABA<sub>A</sub> receptor antibodies and no effects on synaptic GABA<sub>A</sub> receptor density (Fig. 5C,D). To further strengthen the link between GABA<sub>A</sub> 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<sub>A</sub> receptor antibodies, indicating that not all patients with thymoma develop GABA<sub>A</sub> receptor antibodies. For example, the Patient B, with invasive thymoma and myasthenia gravis, had monospecifically DCC antibodies without GABA<sub>A</sub> receptor antibodies, whereas Patient C, with invasive thymoma and neuromyotonia, had LGI1 and CASPR2 antibodies without GABA<sub>A</sub> receptor antibodies (Fig. 4). Treatment of hippocampal neurons with these patients' serum did not affect synaptic GABA<sub>A</sub> 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,  $25.4 \pm 4.2$ ,  $p = 0.85$ , 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<sub>A</sub> receptor antibodies. Treatment with this patient serum did not affect the synaptic GABA<sub>A</sub> 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<sub>A</sub> receptor antibodies. This serum treatment did not affect the synaptic GABA<sub>A</sub> receptor clusters (cluster number for control serum,  $27.6 \pm 1.2$ ; for the patient serum,  $24.6 \pm 2.3$ ,  $p = 0.39$ , Student's  $t$  test;  $n = 3$  separate cultures). These overall results exclude the possibility that coexisting antibodies other than GABA<sub>A</sub> receptor antibodies mediate the effects and support the specific role of GABA<sub>A</sub> receptor antibodies in the patients' symptoms.

Two patients with GABA<sub>A</sub> 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<sub>A</sub> receptor mediated by GABA<sub>A</sub> receptor  $\beta 3$  antibodies may cause

seizures in human patients. However, at present, it seems too early to conclude that GABA<sub>A</sub> 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<sub>A</sub> receptor antibodies and specific CNS symptoms will be clarified in the future as the number of cases increases.

### Anti-GABA<sub>A</sub> receptor encephalitis as a new class of autoimmune encephalitis

The present study indicates that encephalitis associated with GABA<sub>A</sub> receptor antibodies shows different clinical features and mechanisms, at least from limbic encephalitis associated with monospecific LGI1 antibodies. Both cases with the GABA<sub>A</sub> 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<sub>A</sub> receptor density. Conversely, serum from Patient 1 showed a selective effect on GABA<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> receptor antibodies in the symptoms experienced by Patient 1. Therefore, it is conceivable that encephalitis with GABA<sub>A</sub> receptor antibodies might be distinguished as a new class of autoimmune encephalitis. In addition, we propose that clinical phenotypes of autoimmune anti-GABA<sub>A</sub> 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<sub>A</sub> receptor autoantibodies associated with autoimmune encephalitis and revealed their pathogenic role, downregulation of the GABA<sub>A</sub> receptor function. Given that many agonistic and antagonistic ligands bind to specific sites on the GABA<sub>A</sub> receptor, the fine epitope mapping of autoantibodies on the GABA<sub>A</sub> receptor  $\beta 3$  subunit may contribute to further understanding the pathogenic mechanism causing

abnormal neuronal excitation in the brain and developing therapeutic interventions.

## References

- Cash SS, Larvie M, Dalmau J (2011) Case records of the Massachusetts General Hospital. Case 34–2011: A 75-year-old man with memory loss and partial seizures. *N Engl J Med* 365:1825–1833. [CrossRef Medline](#)
- Dalmau J, Tüzün E, Wu HY, Masjuan J, Rossi JE, Voloschin A, Baehring JM, Shimazaki H, Koide R, King D, Mason W, Sansing LH, Dichter MA, Rosenfeld MR, Lynch DR (2007) Paraneoplastic anti-N-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma. *Ann Neurol* 61:25–36. [CrossRef Medline](#)
- Dalmau J, Gleichman AJ, Hughes EG, Rossi JE, Peng X, Lai M, Dessain SK, Rosenfeld MR, Balice-Gordon R, Lynch DR (2008) Anti-NMDA-receptor encephalitis: case series and analysis of the effects of antibodies. *Lancet Neurol* 7:1091–1098. [CrossRef Medline](#)
- DeLorey TM, Handforth A, Anagnostaras SG, Homanics GE, Minassian BA, Asatourian A, Fanselow MS, Delgado-Escueta A, Ellison GD, Olsen RW (1998) Mice lacking the beta3 subunit of the GABA<sub>A</sub> receptor have the epilepsy phenotype and many of the behavioral characteristics of Angelman syndrome. *J Neurosci* 18:8505–8514. [Medline](#)
- Fang C, Deng L, Keller CA, Fukata M, Fukata Y, Chen G, Lüscher B (2006) GODZ-mediated palmitoylation of GABA<sub>A</sub> receptors is required for normal assembly and function of GABAergic inhibitory synapses. *J Neurosci* 26:12758–12768. [CrossRef Medline](#)
- Fukata Y, Lovero KL, Iwanaga T, Watanabe A, Yokoi N, Tabuchi K, Shigemoto R, Nicoll RA, Fukata M (2010) Disruption of LGI1-linked synaptic complex causes abnormal synaptic transmission and epilepsy. *Proc Natl Acad Sci U S A* 107:3799–3804. [CrossRef Medline](#)
- Fukata Y, Dimitrov A, Boncompain G, Vielemeyer O, Perez F, Fukata M (2013) Local palmitoylation cycles define activity-regulated postsynaptic subdomains. *J Cell Biol* 202:145–161. [CrossRef Medline](#)
- Hughes EG, Peng X, Gleichman AJ, Lai M, Zhou L, Tsou R, Parsons TD, Lynch DR, Dalmau J, Balice-Gordon RJ (2010) Cellular and synaptic mechanisms of anti-NMDA receptor encephalitis. *J Neurosci* 30:5866–5875. [CrossRef Medline](#)
- Hutchinson M, Waters P, McHugh J, Gorman G, O’Riordan S, Connolly S, Hager H, Yu P, Becker CM, Vincent A (2008) Progressive encephalomyelitis, rigidity, and myoclonus: a novel glycine receptor antibody. *Neurology* 71:1291–1292. [CrossRef Medline](#)
- Irani SR, Alexander S, Waters P, Kleopa KA, Pettingill P, Zuliani L, Peles E, Buckley C, Lang B, Vincent A (2010) Antibodies to Kv1 potassium channel-complex proteins leucine-rich, glioma inactivated 1 protein and contactin-associated protein-2 in limbic encephalitis, Morvan’s syndrome and acquired neuromyotonia. *Brain* 133:2734–2748. [CrossRef Medline](#)
- Jacob TC, Moss SJ, Jurd R (2008) GABA<sub>A</sub> receptor trafficking and its role in the dynamic modulation of neuronal inhibition. *Nat Rev Neurosci* 9:331–343. [CrossRef Medline](#)
- Lai M, Hughes EG, Peng X, Zhou L, Gleichman AJ, Shu H, Matà S, Kremens D, Vitaliani R, Geschwind MD, Bataller L, Kalb RG, Davis R, Graus F, Lynch DR, Balice-Gordon R, Dalmau J (2009) AMPA receptor antibodies in limbic encephalitis alter synaptic receptor location. *Ann Neurol* 65:424–434. [CrossRef Medline](#)
- Lai M, Huijbers MG, Lancaster E, Graus F, Bataller L, Balice-Gordon R, Cowell JK, Dalmau J (2010) Investigation of LGI1 as the antigen in limbic encephalitis previously attributed to potassium channels: a case series. *Lancet Neurol* 9:776–785. [CrossRef Medline](#)
- Lancaster E, Dalmau J (2012) Neuronal autoantigens—pathogenesis, associated disorders and antibody testing. *Nat Rev Neurol* 8:380–390. [CrossRef Medline](#)
- Lancaster E, Lai M, Peng X, Hughes E, Constantinescu R, Raizer J, Friedman D, Skeen MB, Grisold W, Kimura A, Ohta K, Iizuka T, Guzman M, Graus F, Moss SJ, Balice-Gordon R, Dalmau J (2010) Antibodies to the GABA<sub>B</sub> receptor in limbic encephalitis with seizures: case series and characterisation of the antigen. *Lancet Neurol* 9:67–76. [CrossRef Medline](#)
- Lancaster E, Martinez-Hernandez E, Dalmau J (2011) Encephalitis and antibodies to synaptic and neuronal cell surface proteins. *Neurology* 77:179–189. [CrossRef Medline](#)
- Macdonald RL, Olsen RW (1994) GABA<sub>A</sub> receptor channels. *Annu Rev Neurosci* 17:569–602. [CrossRef Medline](#)
- Macdonald RL, Kang JQ, Gallagher MJ (2010) Mutations in GABA<sub>A</sub> receptor subunits associated with genetic epilepsies. *J Physiol* 588:1861–1869. [CrossRef Medline](#)
- McKeon A, Martinez-Hernandez E, Lancaster E, Matsumoto JY, Harvey RJ, McEvoy KM, Pittcock SJ, Lennon VA, Dalmau J (2013) Glycine receptor autoimmune spectrum with stiff-man syndrome phenotype. *JAMA Neurol* 70:44–50. [CrossRef Medline](#)
- Miyazaki Y, Hirayama M, Watanabe H, Usami N, Yokoi K, Watanabe O, Sobue G (2012) Paraneoplastic encephalitis associated with myasthenia gravis and malignant thymoma. *J Clin Neurosci* 19:336–338. [CrossRef Medline](#)
- Moscato EH, Jain A, Peng X, Hughes EG, Dalmau J, Balice-Gordon RJ (2010) Mechanisms underlying autoimmune synaptic encephalitis leading to disorders of memory, behavior and cognition: insights from molecular, cellular and synaptic studies. *Eur J Neurosci* 32:298–309. [CrossRef Medline](#)
- Ohkawa T, Fukata Y, Yamasaki M, Miyazaki T, Yokoi N, Takashima H, Watanabe M, Watanabe O, Fukata M (2013) Autoantibodies to epilepsy-related LGI1 in limbic encephalitis neutralize LGI1-ADAM22 interaction and reduce synaptic AMPA receptors. *J Neurosci* 33:18161–18174. [CrossRef Medline](#)
- Ohshita T, Kawakami H, Maruyama H, Kohriyama T, Arimura K, Matsumoto M (2006) Voltage-gated potassium channel antibodies associated limbic encephalitis in a patient with invasive thymoma. *J Neurol Sci* 250:167–169. [CrossRef Medline](#)
- Petit-Pedrol M, Armangue T, Peng X, Bataller L, Cellucci T, Davis R, McCracken L, Martinez-Hernandez E, Mason WP, Kruer MC, Ritacco DG, Grisold W, Meaney BF, Alcalá C, Sillevs-Smith P, Titulaer MJ, Balice-Gordon R, Graus F, Dalmau J (2014) Encephalitis with refractory seizures, status epilepticus, and antibodies to the GABA<sub>A</sub> receptor: a case series, characterisation of the antigen, and analysis of the effects of antibodies. *Lancet Neurol* 13:276–286. [CrossRef Medline](#)
- Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000) GABA<sub>A</sub> receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience* 101:815–850. [CrossRef Medline](#)
- Rudolph U, Knoflach F (2011) Beyond classical benzodiazepines: novel therapeutic potential of GABA<sub>A</sub> receptor subtypes. *Nat Rev Drug Discov* 10:685–697. [CrossRef Medline](#)
- Satake S, Imoto K (2014) Ca<sub>v</sub>2.1 channels control multivesicular release by relying on their distance from exocytotic Ca<sup>2+</sup> sensors at rat cerebellar granule cells. *J Neurosci* 34:1462–1474. [CrossRef Medline](#)
- Satake S, Saitow F, Rusakov D, Konishi S (2004) AMPA receptor-mediated presynaptic inhibition at cerebellar GABAergic synapses: a characterization of molecular mechanisms. *Eur J Neurosci* 19:2464–2474. [CrossRef Medline](#)
- Sillevis Smitt P, Kinoshita A, De Leeuw B, Moll W, Coesmans M, Jaarsma D, Henzen-Logmans S, Vecht C, De Zeeuw C, Sekiyama N, Nakanishi S, Shigemoto R (2000) Paraneoplastic cerebellar ataxia due to autoantibodies against a glutamate receptor. *N Engl J Med* 342:21–27. [CrossRef Medline](#)
- Vincent A, Lang B, Kleopa KA (2006) Autoimmune channelopathies and related neurological disorders. *Neuron* 52:123–138. [CrossRef Medline](#)

# Median neuropathy at the wrist as an early manifestation of diabetic neuropathy

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## Keywords

Carpal tunnel syndrome, Diabetic neuropathy, Median neuropathy

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## ABSTRACT

**Aims/Introduction:** To elucidate the clinical significance of median neuropathy at the wrist (MN) in patients with diabetes.

**Materials and Methods:** In total, 340 patients with diabetes who were hospitalized for glycemic control were enrolled in the present study. The diagnoses of MN and diabetic polyneuropathy (DPN) were based on electrophysiological criteria. A total of 187 patients were divided into four subgroups: patients without MN or DPN; patients with MN without DPN; patients with MN and DPN; and patients with DPN without MN. Intergroup comparisons of clinical characteristics and results of nerve conduction studies were carried out.

**Results:** A total of 71 patients had neither MN nor DPN; 25 had MN, but no DPN; 55 had MN and DPN; and 36 had DPN, but no MN. In comparison with the MN and DPN group, the MN without DPN group included more patients in the early phase of diabetes (diagnosed within the past 5 years) and fewer patients with diabetic microangiopathy. Comparative median nerve conduction studies showed significantly lower motor and sensory nerve conduction velocities, longer F-wave latencies, and smaller sensory nerve action potentials in patients with MN and DPN than in those without DPN.

**Conclusions:** MN in patients with diabetes could be attributed to an impairment in axonal function at common entrapment sites, and could be used to identify an early manifestation of diabetic neuropathy.

## INTRODUCTION

Entrapment disorders are highly prevalent in patients with diabetes<sup>1</sup>, the most common neuropathy being carpal tunnel syndrome (CTS)<sup>2</sup>, which is classified as a focal limb disease<sup>3</sup>. Generally, CTS is more common in females than in males, in obese individuals than in non-obese individuals and in the dominant hand versus the non-dominant hand<sup>4</sup>. The high prevalence of CTS among patients with diabetes might be as a result of repeated undetected trauma, metabolic changes, accumulation of fluid or edema within the confined space of the carpal tunnel and/or diabetic cheiroarthropathy<sup>2</sup>. CTS in patients with diabetes is frequently asymptomatic, and is identified through electrophysiological tests. Additionally, there is no demonstrable correlation between the appearance of CTS and the stage of diabetic polyneuropathy (DPN)<sup>5,6</sup>. The clinical

diagnosis of CTS depends on a combination of appropriate clinical symptoms; therefore, most clinicians require electrophysiological confirmation of median neuropathy at the wrist (MN) before establishing a diagnosis of CTS<sup>6</sup>.

Nerve conduction studies (NCS), the most sensitive diagnostic method for detecting MN and DPN, provide the only available technique to diagnose subclinical cases. NCS are also useful in differentiating entrapment from distal symmetric polyneuropathies<sup>2</sup>. In order to elucidate the clinical significance of MN in patients with diabetes, we compared clinical and electrophysiological data between patients with MN without DPN, and those with MN and DPN.

## MATERIALS AND METHODS

### Patients

The current study was carried out at the Kagoshima City Hospital, Kagoshima, Japan, between February 2007 and September

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2012. We recruited 340 patients (156 women and 184 men) with diabetes mellitus who were hospitalized for hyperglycemia management during the study period. The mean age of the patients was  $56.8 \pm 11.7$  years, with a mean duration of diabetes of  $9.3 \pm 7.8$  years; 309 patients had type 2 diabetes, whereas 31 patients had type 1 diabetes. Glycated hemoglobin (HbA1c) levels were  $9.8 \pm 2.2\%$ . Routine biochemical and hematological tests, NCS and screening for diabetic complications were carried out for all patients. All patients with a medical history of serious trauma to the limbs, excessive alcohol consumption, use of neurotoxic medication and/or evidence of neuromuscular disease were excluded from the study in order to omit neuropathy as a result of other etiologies. Patients aged over 75 years were excluded because sensory nerve action potentials (SNAPs) of the nerves are largely dependent on age. Informed consent was obtained from all patients before their inclusion in the study.

### Nerve Conduction Study

All patients underwent conventional sensory and motor NCS. The median, ulnar, tibial, and sural nerves were tested in the upper and lower limbs. All studies were carried out using a standard electromyography machine with stimulating and recording electrodes (Viking Select; Nicolet Biomedical Japan, Tokyo, Japan). SNAPs of the nerves in the upper limb were recorded orthodromically. Median motor distal latencies were recorded using an interelectrode distance of 8 cm (stimulating the wrist and recording the thenar), while median sensory peak latencies were measured using a stimulation-to-recording electrode distance of 14 cm (digit 2 to wrist). Stimulations in the palm were carried out with an electrode distance of 7 cm (palm to wrist), and skin temperature was maintained above  $32^\circ\text{C}$  on the forearm and  $31^\circ\text{C}$  on the mid-leg. Electrophysiological criterion for the diagnosis of MN was determined as a prolongation in either median motor latency or sensory palm latency ( $>4.5$  and  $1.8$  ms, respectively). Importantly, the criterion required that the difference in sensory onset latency between the palm to wrist and digit 2 to palm was higher than  $0.4$  ms<sup>7</sup>. DPN was defined as a distal symmetric sensorimotor polyneuropathy by using recommendations of the American Academy of Electrodiagnostic Medicine<sup>8</sup>, and was diagnosed if there was a reduction of SNAPs in the median nerve ( $<7$   $\mu\text{V}$ ), ulnar nerve ( $<6.9$   $\mu\text{V}$ ) and sural nerve ( $<5$   $\mu\text{V}$ ). The cut-off value of SNAPs was determined to be the lower limit of the normal range commonly used in our laboratory. Although diabetes can involve multiple common entrapment sites, median-ulnar or median-radial comparative studies were not carried out. Therefore, patients were excluded from the study if they showed absent motor or sensory potentials in the median and ulnar nerves, presented with mononeuropathy other than MN and/or if the MN group had pathological electrophysiological findings in the ulnar nerve. On the basis of the results of the NCS, patients were divided into four subgroups: patients without MN or DPN (MN-/DPN-); patients with MN without DPN (MN+/DPN-); patients with MN and DPN (MN+/

DPN+); and patients with DPN without MN (MN-/DPN+). The clinical characteristics of patients and results of the NCS were compared between groups.

### Statistical Analyses

Data are expressed as means and standard deviations. Statistical analyses were carried out using Excel 2011 (Microsoft, Redmond, WA, USA) with the add-in software Statcel 3 (OMS, Tokyo, Japan). Data were analyzed by one-way analysis of variance (ANOVA), and then statistical significance of differences among the four groups was calculated using Tukey–Kramer or Steel–Dwass post-hoc tests or  $\chi^2$  statistics followed by the Bonferroni adjustment. *P*-values  $<0.05$  were considered statistically significant.

### RESULTS

Of the 340 patients examined, just 187 were included in the current study. Incidentally, 153 patients were excluded from the study; 43 patients with absent motor or sensory potentials in the median and ulnar nerves, 51 patients with mononeuropathy other than MN, 40 patients with pathological electrophysiological findings in the ulnar nerve in the MN group, and 19 patients with pathological electrophysiological findings in the ulnar and sural nerve.

The demographic data for the 187 participants are provided in Table 1. A total of 71 patients had neither MN nor DPN; 25 had MN, but no DPN; 55 had MN and DPN; and 36 had DPN, but no MN. There was no significant difference in sex, body mass index or HbA1c levels between the four subgroups. Compared with the MN+ or -/DPN+ group, the MN+/DPN- group had diabetes for a significantly shorter period, and showed preserved urinary C-peptide immunoreactivity. Additionally, the prevalence of diabetic retinopathy and diabetic nephropathy was significantly lower in the MN+/DPN- group than in the MN+ or -/DPN+ group. Within the MN-/DPN- group and MN+/DPN- group, there was no significant difference in age, duration of diabetes, urinary C-peptide immunoreactivity or the prevalence of diabetic retinopathy and diabetic nephropathy. Among the patients with DPN, none of the clinical characteristics differed significantly between the MN+/DPN+ group and the MN-/DPN+ group. The percentage of patients having a diabetes duration of 5 years or less was significantly higher among MN-/DPN- group and MN+/DPN- group than among the MN+/DPN+ group and MN-/DPN+ group (Figure 1). In particular, there was a significant difference between the MN+/DPN- group and MN+/DPN+ group. The results of the median NCS for the 187 participants are provided in Table 2. When comparing the MN+/DPN- group with the MN+/DPN+ group, the latter group was found to have significantly lower motor and sensory nerve conduction velocities, as well as prolonged F-wave latencies than the former group. The amplitude of compound muscle action potentials (CMAP) was not different between the two groups, whereas the SNAPs were significantly smaller in the MN+/DPN+ group than in the MN+/DPN- group. Among the