Research report

BIOLOGICAL SCIENCES

Immunology and Inflammation

Cross-reactivity of a pathogenic autoantibody to a tumor antigen in $GABA_A$ receptor encephalitis

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ABSTRACT

Encephalitis associated with antibodies against the neuronal gamma-aminobutyric-acid-A receptor ($GABA_A$-R) is a rare form of autoimmune encephalitis. The pathogenesis is still unknown but autoimmune mechanisms were surmised. Here we identified a strongly expanded B cell clone in the cerebrospinal fluid of a patient with $GABA_A$-R encephalitis. We expressed the antibody produced by it, and showed by ELISA and immunohistochemistry that it recognizes the $GABA_A$-R. Patch-clamp recordings revealed that it tones down inhibitory synaptic transmission and causes increased excitability of hippocampal CA1 pyramidal neurons. Thus, the antibody likely contributed to clinical disease symptoms. Hybridization to a protein-array revealed the cross-reactive protein "LIM-domain-only protein 5" (LMO5), which is related to cell-cycle regulation and tumor growth. We confirmed LMO5-recognition by immunoprecipitation and ELISA, and showed that CSF samples from two other patients with $GABA_A$-R encephalitis also recognized LMO5. This suggests that cross-reactivity between $GABA_A$-R and LMO5 is frequent in $GABA_A$-R encephalitis and supports the hypothesis of a paraneoplastic etiology.

KEYWORDS

autoimmune encephalitis, gamma-aminobutyric-acid-A receptor ($GABA_A$-R) encephalitis, paraneoplastic encephalitis, autoantibody.

SIGNIFICANCE

Antibodies recognizing the neuronal gamma-aminobutyric-acid-A receptor ($GABA_A$-R) cause severe encephalitis by triggering internalization of the antibody-receptor complexes in inhibitory synapses, which leads to hyperexcitability and dysfunction of neuronal networks. From the cerebrospinal fluid of a patient with $GABA_A$–R encephalitis, we cloned a highly expressed antibody and showed that it binds the $GABA_A$-R and influences signal transduction in neurons explaining clinical symptoms. Using several experimental techniques, we confirmed that the antibody cross-reacts to an onco-protein, which is known to be involved in several malignancies. We showed that cross-reactivity to this onco-protein may also be detected in two other $GABA_A$-R patients, suggesting that such cross-reactivity is presumably a key event in the pathogenesis of $GABA_A$-R encephalitis.

INTRODUCTION

A shared feature of autoimmune encephalitis syndromes are autoantibodies recognizing either intracellular antigens or extracellular epitopes of cell surface antigens (1-5). Intracellular antigens are primarily released by cellular immune responses and later bound by autoantibodies whereas cell surface antigens are assumed to be targets of direct humoral immune responses. For both processes, paraneoplastic mechanisms are surmised, and tumors are indeed often detected in conjunction with both types of autoimmunity albeit at different frequencies. However, in some patients a tumor is identified neither at the onset nor during the course of the neurological disease. Here, the immune reaction may have already erased the malignancy, or have kept it below the diagnostic detection limit (6, 7).

Encephalitis associated with autoantibodies against the gamma-aminobutyric-acid-A receptor ($GABA_A$-R) has recently been described (8-11). Anti-$GABA_A$-R antibodies cause $GABA_A$-R cross-linking and internalization of the antibody-receptor complex with a selective reduction of postsynaptic $GABA_A$-R clusters at inhibitory GABAergic synapses (9, 12). This is supposed to cause hyperexcitability and dysfunction of neuronal networks, and thus clinical disease symptoms (1). The immunopathogenesis is heterogeneous and may include viral as well as tumoral triggers of $GABA_A$-R encephalitis: viral infections of the CNS may represent triggers of the disease in some patients (13,14), whereas peripheral tumors are clinically detectable in about one third of all patients at the time of $GABA_A$-R encephalitis diagnosis (14). The malignancies comprise thymoma, Hodgkin´s lymphoma, non-Hodgkin´s lymphoma, multiple myeloma, small cell lung cancer, and rectal cancer (8-10,13,15,16).

Here we describe cloning and functional analysis of a pathogenic antibody from the cerebrospinal fluid (CSF) B cells of a patient with “idiopathic” $GABA_A$-R encephalitis, who has been described earlier and was termed "index patient 2" (IP2) (9). In the CSF and the hippocampus of this patient, we have recently detected a strongly expanded CD8+ T cell clone (17). We expressed the dominant antibody from the CSF B cells of patient IP2 (termed Ab-IP2) recombinantly and showed that it binds recombinant α1-subunits of the $GABA_A$-R, recognizes hippocampal neuropil structures, and dampens phasic GABAergic inhibitory synaptic activity and increases excitability in hippocampal CA1 pyramidal neurons. Hybridization to a protein array and confirmation by ELISA and immunoprecipitation revealed that Ab-IP2 also recognized "LIM-domain only protein 5" (LMO5). We detected cross-reactivity between $GABA_A$-R and LMO5 also in CSF samples from two other patients with $GABA_A$-R encephalitis. Strikingly, all LMO proteins are related to tumor growth and spread (18) and this has also been demonstrated specifically for LMO5 (19-24). This may hint towards a paraneoplastic pathogenesis (induced by detectable or occult tumors) and linked T and B cell responses as a major principle in the immunopathogenesis of $GABA_A$-R encephalitis.

RESULTS

Repertoire analysis reveals an expanded B cell clone in the CSF of patient IP2

B cell receptor repertoire analysis by next generation sequencing revealed an almost monoclonal expansion of an IGHV3-21*01 heavy-chain (H-chain) with an IgG1 Fc region in addition to several non-expanded H-chains. Some of them had closely related sequences that differed only in one or few amino acids suggesting antigen-driven affinity maturation by somatic hypermutation. For the light-chains, we found a strongly expanded IGKV3-20*01 κ-chain with a short CDR3 region, and again a background of weakly or non-expanded, diverse L-chains. The very strong expansions of a single H- and a single κ-chain suggest that these chains were pairing to yield antibody Ab-IP2 (*SI Appendix* **Fig. S1**). An essentially monoclonal expansion of a cognate B cell clone suggests a highly focused immune response to dominant antigen(s).

Ab-IP2 recognizes $GABA_A$-R

To study the function of Ab-IP2, we cloned, expressed, and purified the recombinant Ab-IP2 (rAb-IP2). Since the amino acid sequences of the extracellular domain of the α1-subunit (amino acids 28 to 251) of the human $GABA_A$-R ($GABA_A$-R-α1ex) in humans, mice, and rats are identical (except for Leu4), we stained serial sections from rat hippocampus with rAb-IP2 (**Fig. 1A**), a commercial anti-$GABA_A$-R antibody 62-3G1 (**Fig. 1B**) and with the negative control antibody rOCB-MS3-s1 (25) (**Fig. 1C**). The staining patterns of rAb-IP2 and 62-3G1 were identical, indicating that rAb-IP2 indeed might recognize GABA-$_A$R in the hippocampal neuropil. We validated this assumption by an ELISA, in which recombinant $GABA_A$-R-α1ex was specifically recognized by rAb-IP2 in a dose-dependent manner (**Fig. 1D**). $GABA_A$-R-α1ex was not recognized by the control antibody rOCB-MS3-s1 (25). As a further validation, we showed by flow cytometry of HEK293Expi cells that were transfected with the α1- and β3-subunits of $GABA_A$-R, that rAb-IP2 recognizes these $GABA_A$-Rs (*SI Appendix* **Fig. S1**).

To study effects of rAb-IP2 on neuronal synaptic signaling and excitability, we performed electrophysiological recordings in hippocampal CA1 pyramidal neurons using acute mouse brain slices (**Fig. 2**), characterized by high expression levels of the α1-subunit of $GABA_A$-R (26). Preincubation of slices with rAb-IP2 for 2 hours, significantly decreased the number of spontaneous inhibitory postsynaptic currents (sIPSCs) occurring in 10 minutes compared to slices incubated with the negative control antibody rOCB-MS3-s1 (25) (**Fig. 2A** and **B**). Incubation with rAb-IP2 also reduced the amplitude of sIPSC compared to slices incubated with rOCB-MS3-s1, although this change did not reach significance threshold (**Fig. 2A and C**). Next, hippocampal CA1 pyramidal neurons were challenged with a series of depolarizing current steps at the individual RMPs and the number of elicited action potentials (APs) was recorded. Notably, no differences in RMPs were found between the rAb-IP2- and rOCB-MS3-s1-incubated slices (**Fig. 2D**). Moreover, analysis of the passive electrical properties of CA1 pyramidal neurons, namely the input resistance (R_{in}) and the whole-cell capacitance, as major determinants of intrinsic neuronal excitability, did not differ between rAb-IP2- and rOCB-MS3-s1-incubated slices (**Fig. 2E** and **F**). However, preincubation of slices with rAb-IP2 reduced the AP firing threshold in CA1 pyramidal neurons compared to rOCB-MS3-s1-incubated slices. Moreover, at a given depolarizing current step, the number of APs in CA1 pyramidal neurons was higher in rAb-IP2-incubated compared to rOCB-MS3-s1-incubated slices (**Fig. 2G** and **H**).

Cross-reactivity of rAb-IP2 to LMO5

To test whether rAb-IP2 is cross-reactive to other antigens, which might eventually have initiated immune responses, we hybridized rAb-IP2 to a commercial "ProtoArray" microarray that contained ~9.400 different human proteins expressed in insect cells. We observed strong signals of rAb-IP2 with several intracellular proteins, including some with LIM domains. LMO5 yielded the strongest signals, while other LMO family members, such as CSRP1, were also recognized although with lower affinity (**Fig. 3A**). Because all proteins spotted on the ProtoArray were produced in insect cells, we confirmed recognition of LMO5 by rAb-IP2 through immunoprecipitation using commercial recombinant LMO5 produced in HEK293 cells (**Fig. 3B**). CSRP1 was not immunoprecipitated, consistent with the much lower affinity of rAb-IP2 to CSRP1 as compared to LMO5 seen by array hybridization (**Fig. 3A**). Furthermore, rAb-IP2 recognized LMO5 produced in *E. coli* in an ELISA in a dose-dependent manner (**Fig. 3C**). Taken together, in three independent experiments we showed that rAb-IP2 specifically recognized LMO5.

LMO5 is recognized by CSF samples from other patients with anti-GABA$_A$-R encephalitis

To investigate whether cross-reactivity between GABA$_A$-R and LMO5 was unique to patient IP2, we tested CSF samples from IP2 and two other patients with "idiopathic" GABA$_A$-R encephalitis (GABA$_A$-R-1 and -2). Further, we included three subjects with other forms of antibody associated CNS diseases (AACNSD-1 to -3), three with non-inflammatory neurological diseases (NIC-1 to -3), and five with multiple sclerosis (MS-1 to -5). We could not establish a presumably more sensitive cell-based assay by expressing LMO5 fused to transmembrane domains at the surface of eukaryotic cell lines. We failed presumably because LMO5 (synonym: "Cysteine and glycine-rich protein 2", CSRP2) is a small intracellular protein with 16 reduced cysteine-residues of 193 amino acids in total (https://www.uniprot.org/uniprot/Q16527), which will form disulfide bonds at the cell surface leading to denaturation of the protein. Furthermore, intracellular overexpression is hampered by the function of LMO5 as a cell-cycle regulator. Therefore, we produced LMO5 in *E. coli*, purified it to homogeneity, and tested its recognition by CSF supernatants in an ELISA (**Fig. 4**). In two CSF samples from other patients with GABA$_A$-R encephalitis (GABA$_A$-R-1 and GABA$_A$-R-2), LMO5 was recognized with two-fold higher signals than the highest signals in most other samples. This finding was reproducible in two independent experiments, each performed in duplicate. These results suggest that cross-reactivity between GABA$_A$-R and LMO5 is not a unique feature of Ab-IP2 but a more general feature of GABA$_A$-R encephalitis.

Surprisingly, however, CSF from patient IP2 did not recognize LMO5 in this assay - probably because the CSF sample used here was taken briefly before death, i.e. it was not identical to the sample used for initial diagnosis (9). In the time between initial diagnosis and death, patient IP2 had received escalated immunotherapy including immediately antibody-depleting treatments (ref 9, *SI Appendix* **Table S1**). In the CSF sample drawn at initial diagnosis, the detection limit of Ab-IP2 was 1:320 (ref. 9, *SI Appendix* **Table S1**). In our CSF sample, however, it was decreased to 1:32, i.e. it was tenfold lower. Furthermore, the LMO5-ELISA used here is not as sensitive as the cell-based assay that was used for detecting GABA$_A$-R binding. MS-3 and MS-5 also recognized LMO5, which is expressed at high levels in brain tissue (https://www.proteinatlas.org/ENSG00000175183-CSRP2/tissue). CNS tissue destruction caused by long-lasting inflammation has presumably released LOM5 from the cytosol of destroyed cells leading to antibody responses against this obviously highly immunogenic protein (19-24). Thus, high anti-LMO5 antibody titers in MS-3 and MS-5 are consistent with our earlier observation that recognition of intracellular ubiquitous proteins is often seen in MS subjects with oligoclonal bands (25). Notably, samples of GABA$_A$-R-1 and

$GABA_A$-R-2 were obtained at the disease onset and exhibited no oligoclonal bands (*SI Appendix* **Table S1**) indicating a more specific immune response. Except MS-3 and MS-5, all other control CSF samples from subjects with AACNSD, NID, and MS yielded considerably lower signals than $GABA_A$-R-1 and $GABA_A$-R-2.

DISCUSSION

Analysis of the B cell receptor repertoire in the CSF of patient IP2 revealed a strongly expanded B cell clone that produces the dominant antibody Ab-IP2. By three independent experiments we could demonstrate that rAb-IP2 recognizes the extracellular domain of $GABA_A$-R: First, we observed identical neuropil staining patterns in immunohistochemistry of hippocampal tissue sections as with a commercial anti-$GABA_A$-R antibody. Although not a strict proof, this suggests that rAb-IP2 recognizes $GABA_A$-R. Second, rAb-IP2 strongly and specifically bound to the extracellular domain of the α1-subunit of the human $GABA_A$-R in an ELISA in a dose-dependent manner. Third, we could show that rAb-IP2 is functional because it reduced the amplitude and frequency of spontaneous postsynaptic GABAergic events in hippocampal CA1 pyramidal neurons. This is consistent with a reduction of postsynaptic $GABA_A$-R clusters at GABAergic inhibitory synapses and a dampening net effect on the interneuron network activity. Both effects lead to a strongly reduced AP firing threshold and increased AP firing of hippocampal CA1 pyramidal neurons. These disinhibiting effects on neuronal function may form the basis of the clinical symptoms of patients with $GABA_A$-R encephalitis (8-10).

We identified LMO5 as a major target of rAb-IP2, and in addition, we found reactivity to several other proteins with lower affinities. Some, but not all of these target proteins, contained LIM domains. Although the "original" antigen that recruited Ab-IP2 in patient IP2 will remain elusive, it is tempting to speculate that LIM-domain proteins might have been released from (clinically occult) peripheral tumor cells following anti-tumoral immunity most probably initiated by CD8+ T cells. Of note, we have recently described a CD8+ T cell clone that was strongly expanded in the CSF and in the hippocampus of patient IP2 but not in the operculo-insular cortex (17). Thus, a CD8+ T cell mediated early anti-tumor response in the periphery might have released intracellular proteins and cryptic epitopes of onco-proteins might then have initiated production of Ab-IP2, which accidentally was cross-reactive to $GABA_A$-R in the CNS. The parallel expansions of a B- and T cell clone might indicate a functional link between these two main constituents of the adaptive immune system both in the periphery and the CNS, although the antigens recognized by the B- and T-cell clones do not necessarily have to be identical. However, an initiation of the adaptive immune response within the CNS (eventually induced by an (occult) viral infection (27) with CD8+ T cell mediated tissue destruction, release of intracellular LMO5, and formation of Ab-IP2, which then cross-reacts with the $GABA_A$-R appears less likely but cannot formally be excluded. Thus, the association of $GABA_A$-R encephalitis with clinically detectable peripheral tumors in about one third of all patients (14) and the possibility of an even higher portion of patients with clinically occult peripheral malignancies strongly argues in favor of the initiation of the autoimmune response by a neoplastic process in the periphery.

Cross-reactivity between a neural cell-surface and an intracellular onco-protein points towards such a paraneoplastic pathogenesis. All LIM-domain proteins are located intracellularly, often involved in regulating cell growth, and thus related to tumor growth and metastasis (28). In particular, all LMO proteins are involved in tumor outgrowth (15). Indeed, LMO5 has recently been related as an onco-protein to a variety of hematological malignancies (19-21,23) and solid tumors (22), and might also be involved in other malignancies. Likewise, the association of $GABA_A$-R encephalitis with hematological and solid tumors has been described before (8-10, 13-16). The pattern of cross-reactivity between $GABA_A$-R and LMO5 observed for rAb-IP2 might be quite common in $GABA_A$-R encephalitis as we could detect anti-LMO5 reactivity in two other patients with $GABA_A$-R encephalitis. The signals were not very strong and variable in the control CSF samples, but the signals found for $GABA_A$-R-1 and $GABA_A$-R-2 in two independent experiments were reproducibly increased twofold and significantly higher than in controls. Although our cohort is small, and more patients need to be tested, our data suggest that cross-reactivity between $GABA_A$-R and LIM-domain proteins is not restricted to patient IP2. Detecting such common patterns in the pathogeneses of patients with $GABA_A$-R encephalitis might help to develop rational strategies for early diagnosis and therapy.

MATERIALS AND METHODS

Clinical samples

Clinical data of patients with $GABA_A$-R encephalitis and control subjects with other forms of antibody associated CNS diseases, multiple sclerosis, or non-inflammatory neurological diseases are given in **Table S1**.

To test CSF samples for antibodies against $GABA_A$-R, we used a cell-based assay with fixed HEK293 cells expressing either α1- and β3-subunits or α1-, β3- and γ2-subunits of the $GABA_A$-R or a mock control. This assay was shown to be equally sensitive to a cell-based assay with living HEK293 cells (16).

Clinical data of patient IP2 are described (9). The CSF sample analyzed here was collected briefly before death. CSF samples of other patients with $GABA_A$-R encephalitis were collected within the German Network for Research on Autoimmune Encephalitis (https://generate-net.de/). CSF samples from patients with MS, AACNSD, and NID were collected at the Ludwig Maximilians University of Munich. The study was approved by the ethics committees of the University of Valencia, Spain (PT13/0010/0026), Munich, Germany (project 163-16), Kiel, Germany (AZ 13-162), and Münster, Germany (AZ 2013-682-b-S). All participants gave written informed consent prior to study conduct, according to the principles of the Declaration of Helsinki.

B cell receptor repertoire analyses by next generation sequencing

To analyze the B cell receptor repertoire, RNA was isolated from 1.5 ml of a fresh CSF sample and transcribed into cDNA. Reverse transcription and two rounds of PCR amplification were carried out as described (29) except that the reverse inner primers for the second PCR were tagged with multiplex identifiers for sample identification. Nested multiplex PCR products for IgG-H-, κ- and λ-chains obtained from each sample were purified with QIAquick Gel Extraction Kit (QIAGEN) and pooled in equimolar concentrations. The mixtures were further processed by the sequencing service at FISABIO, Valencia, Spain, where adaptors A and B were ligated and the samples sequenced with a Roche GS FLX sequencer and Titanium chemistry, which yields long enough reads to identify full length Vn(D)nJ-sequences of the H- and L-chains. Productively rearranged Ig-sequences were analyzed using the program IMGT/HighV-QUEST tool.

Expression and characterization of rAb-IP2

The IGHV3-21*01 and IGκV3-20*01 chains were obtained by gene-synthesis (Invitrogen) or amplified from cDNA, respectively. They were cloned into the expression plasmid pTT5, which adds V5- and His_6-tags to the H-chains, co-transfected and expressed in HEK-293E cells, purified by immobilized metal affinity chromatography (IMAC), and characterized as described (25).

Recognition of $GABA_A$-R was confirmed by immunohistochemistry, ELISA, and flow cytometry. Immunohistochemistry was performed on hippocampal slices from Wistar rat brain. The tissue was fixed with 4% paraformaldehyde overnight in freezing compound medium, sectioned, and dried. After heat induced epitope-retrieval, slides were blocked with 2% bovine serum albumin, 10 % fetal calf serum (Sigma-Aldrich), 0.1 % Tween20 (Karl Roth), 1% hu/mouse/rb serum in PBS (Dako) and stained with rAb-IP2 (50 µg/ml), the mouse anti-$GABA_A$-R antibody MAB339 (Merck, 1:500) that recognizes the α-subunits of $GABA_A$-R, and with the negative control antibody rOCB-MS3-s1 (50 µg/ml). As secondary antibodies the antibodies Alexa-Fluor-488 goat anti-mouse IgG (Invitrogen, 1:1000), and goat anti-human IgG-AF488 (Invitrogen, 1:1000), respectively, were used. Nuclei were stained with DAPI and autofluorescent background was reduced using Sudan Black B (Sigma-Aldrich, 0.1 % in 70% ethanol). Signal was visualized using an inverted Leica DMi8 epifluorescence microscope.

To validate recognition of $GABA_A$-R by ELISA, the extracellular domain of the α1-subunit of the human $GABA_A$-R ($GABA_A$-R-α1ex) was produced in *E. coli* as fusion protein with a thioredoxin-His_6 tag. From the soluble fraction, $GABA_A$-R-α1ex was purified by immobilized metal affinity chromatography (IMAC), coated over night to Costar ELISA plates at 15 µg/ml at 4 °C, and exposed to varying concentrations of rAb-IP2 or the control antibody rOCB-MS3-s1 (25) for 1 hour at 37 °C. For detection we used a rabbit anti-human IgG H- and L-chain HRP-conjugated secondary antibody (Abcam, 1:10,000). Signals were detected using 3,3′,5,5′-tetramethylbenzidine (Invitrogen) at 450 nm.

For flow cytometry, HEK293Expi cells (Thermo Fisher) were transiently transfected with different molar ratios of the plasmids pTriEx-1-GABARA1 and pTriEx-1-GABARB3p, which contain either cDNA of the $GABA_A$-R α1- or β3-subunit. The cells were grown in Expi293™ Expression Medium (Thermo Fisher) for 48 hours, washed three times with PBS containing 5µM $ZnCl_2$ and incubated with 5 µg/ml of either rAb-IP2, MAB339, or rOCB-MS3-s1 for 30 min on ice. After three washing steps, the cells were incubated with either goat anti-human IgG-AF-488 (1:500, Invitrogen) or goat anti-mouse IgG-AF-488 (1:500, Invitrogen) for 30 min on ice, resuspended in FACS-buffer containing TO-PRO™-1 iodide (1:6000, Thermo

Fisher) and analyzed using a BD FACSVerse™ (BD Biosciences) and the software FlowJo™ (Tree Star).

Functional recognition of $GABA_A$ receptor by rAb-IP2

Functional recognition of $GABA_A$-R was validated by electrophysiology in acute coronal brain slices from C57BL/6 mice. The mice were anesthetized (4% isoflurane in O_2), the brain was quickly removed and acute coronal brain slices were obtained by cutting 300 μm thick slices using a vibratome (Leica, Germany). Recordings were performed on visually identified pyramidal neurons of the CA1 hippocampal region. Slices were continuously perfused with an extracellular solution (artificial cerebrospinal fluid, ACSF) containing 120 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 22 mM $NaHCO_3$, 20 mM $C_6H_{12}O_6$, 2 mM $CaCl_2$, 2 mM $MgSO_4$, set to pH of 7.35 with carbogen. Next, in order to test its effect on neuronal function, brain slices were incubated for 2 h with 4.84 μg/ml rAb-IP2 or rOCB-MS3-s1 as control. Control slices were kept under the same conditions in the absence of rAb-IP2. Recordings were performed using glass-pipette electrodes pulled from borosilicate glass (GC150TF-10; Clark Electromedical Instruments, Pangbourne, UK), connected to an EPC-10 amplifier (HEKA Elektronik, Lambrecht, Germany). Typical electrode resistance was 4-5 MΩ, with a series resistance in the range of 5-15 MΩ (compensation ≥ 25%). All recordings were governed by Patchmaster software (HEKA Elektronik) and corrected for the liquid junction potential.

Spontaneous GABAergic synaptic activity was recorded in whole cell configuration in the voltage clamp mode using pipettes filled with a KCl-based, high chloride intracellular solution containing 10 mM NaCl, 110 mM KCl, 11 mM EDTA, 10 mM HEPES, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 15 mM phosphocreatin, 3 mM Mg-ATP, 0.5 mM Na-GTP, set to pH 7.25 with KOH and osmolality of 295 mOsmol/kg). Spontaneous GABAergic activity was analysed by recording for 10 min and then calculating number and amplitude of sIPSCs obtained by clamping the cells at a potential of -70 mV in the presence of the glutamate receptor blockers DNQX (10 μM), an AMPA/kainate-receptor-blocker, and AP-5 (10 μM), a NMDA-receptor blocker. Analyses were performed using Mini Analysis (Statsoft, USA) and Fitmaster (HEKA, Germany).

RMP and APs were recorded in whole cell configuration in the current clamp mode using pipettes filled with a K-gluconate-based intracellular solution containing 10 mM NaCl, 88 mM K-gluconate, 20 mM K_3-citrate, 10 mM HEPES, 3 mM BAPTA, 15 mM phosphocreatine, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 3 mM Mg-ATP, 0.5 mM Na-GTP, set to pH

7.25 with KOH and osmolality of 295 mOsmol/kg). The RMP was registered in current clamp mode by adjusting the injected (DC) current to 0 pA. For experiments evaluating the AP firing pattern of hippocampal neurons, a series of depolarizing current steps (from +20 to +160 pA, increment of 20 pA and duration of 2.5 s) was performed at RMP by adjusting the DC current. Number and amplitude of elicited APs were analyzed as described before (30). The input resistance (R_{in}) and the whole cell capacitance of CA1 pyramidal neurons were obtained as described before (31)

Recognition of LMO5 by rAb-IP2 and by CSF samples from other patients and controls

rAb-IP2 and the control antibody r8-18C5 were hybridized to ProtoArrays (Invitrogen) as described (25).

Recognition of LMO5 was validated by immunoprecipitation and ELISA. For immunoprecipitation, rAb-IP2, or the control antibodies r8-18-C5, and OCB-MS3-s1 (both ref. 20) were bound to magnetic Protein G beads (ThermoFisher), incubated with 2 µg LMO5 or CSRP1 produced in HEK293 cells (both Origene), and washed 6 times with PBS. Antigens were eluted with SDS loading buffer. Eluates were detected by Western blotting as described (25).

For ELISA, cDNA of human LMO5, extended for a 3C-proteinase cleavage site, a V5 tag and a His_6 tag at its C-terminus, was cloned into pET19b, and expressed in *E. coli* BL21 at 30 °C in presence of 10 µM $ZnCl_2$. After lysis by ultrasound and ultracentrifugation, LMO5 was purified from the supernatant in presence of 20 mM HEPES (pH 7.5), 350 mM NaCl, 20 µM $ZnCl_2$ and 0.1% β-mercaptoethanol ± 250 mM imidazole by IMAC, dialyzed against 20 mM HEPES (pH 7.5), 150 mM NaCl, 5 µM $ZnCl_2$, 0.1% β-mercaptoethanol and digested by His_6-tagged HRV-3C protease (Sigma). The digestion products were applied to another IMAC column, untagged LMO5 eluted at the above buffer with 75 mM imidazole, and dialyzed as above. For control samples, exactly the same purification was performed from *E. coli* BL21 transfected with pET19b that lacked LMO5, but carried all tags described above. For ELISA, high binding 96 well plates (Costar) were coated with 10 µg/ml LMO5 or corresponding fractions from the mock-plasmid purification. After blocking by 50% Blocker Casein in Tris buffered saline (TBS) (Thermo Fisher) in dialysis buffer for 2.5 h, either 0 to 1,000 µg/ml rAb-IP2 or 1:10 diluted CSF samples from patients, controls, or no CSF (all diluted in TBS + 5% Blocker Casein in TBS) were added for 2 h. Plates were washed with TBS-T (TBS + 0.01% Triton X100), followed by incubation in goat-anti-human IgG HRP antibody (Dianova, 1:5000 diluted TBS + 5% Blocker Casein (TBS)) and washing steps. The ELISA was developed by

TMB Substrate Solution (Invitrogen) and absorbance measured at 450 nm. For analysis, data of the samples without CSF were subtracted from the data of patients and controls. The sensitivity limit of this assay was 0.5 µg/ml as determined by titrating purified rAb-IP2.

Statistics

For the electrophysiological experiments, statistical significance was analyzed using Prism6 (GraphPad) two-way factorial ANOVA in case of multiple comparisons, followed by Bonferroni post-hoc test, while comparisons of two groups was performed using Student's t-test. For ELISA experiments, standard deviations of the mean are given. Statistical significance was calculated with GraphPad Prism 6 by unpaired t-test and a value of $P < 0.05$(*), $P < 0.01$(**) and $P < 0.001$(***) was considered statistically significant.

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Conflict of interest

The authors declare no competing financial interests.

Author contributions

SBr performed antibody cloning and expression, array hybridization, immunoprecipitation, and rAb.IP2-ELISA experiments; MC, AFM, and AMH performed electrophysiology experiments;

SW performed ELISA experiments with human CSF samples; SBr and SW cloned, expressed, and purified LMO5 variants; KH performed immunohistochemistry experiments; DG performed flow cytometry experiments; EB performed PCR and NGS experiments; LK provided reagents; CA, HP, LAG, SBi, FL, BT, TK, BC, and NM provided samples and clinical data; SGM and NM supervised electrophysiology experiments; RH, MC, BC, SGM, and NM partly designed the research and contributed to data analysis and writing; KD, EB, SBr, AB, MC, AFM, AMH, SW, and BC analyzed the data; KD, EB, and SGM initiated and designed the research and wrote the paper. All authors edited, reviewed, and approved the manuscript.

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Figure Legends

Figure 1: rAb-Ip2 recognizes the extracellular domain of $GABA_A$-R-α1.

Immunohistochemical staining of FFPE fixed rat hippocampus. **(A)** Staining with rAb-IP2 (green). Nuclei are stained with DAPI. Isotype control staining was negative. Scale bar: 100 µm. **(B)** Staining with the commercial antibody 62-3G1 to the $GABA_A$-R-α1 subunit (green). **(C)** Staining with the negative control antibody rOCB-MS3-s1. **(D)** The ELISA shows that rAb-IP2 recognizes recombinant $GABA_A$-R-α1ex produced in *E. coli* in a concentration dependent manner (o). Control antibody rOCB-MS3-s1 does not show any reactivity to $GABA_A$-R-α1ex (**o**). Error bars indicate standard error of the mean, n=4. Statistical significance was calculated with GraphPad Prism 6 by unpaired t-test. *P < 0.05,** P < 0.01,*** P < 0.001.

Figure 2: Electrophysiological effects of rAb-IP2 on murine hippocampal CA1 pyramidal neurons.

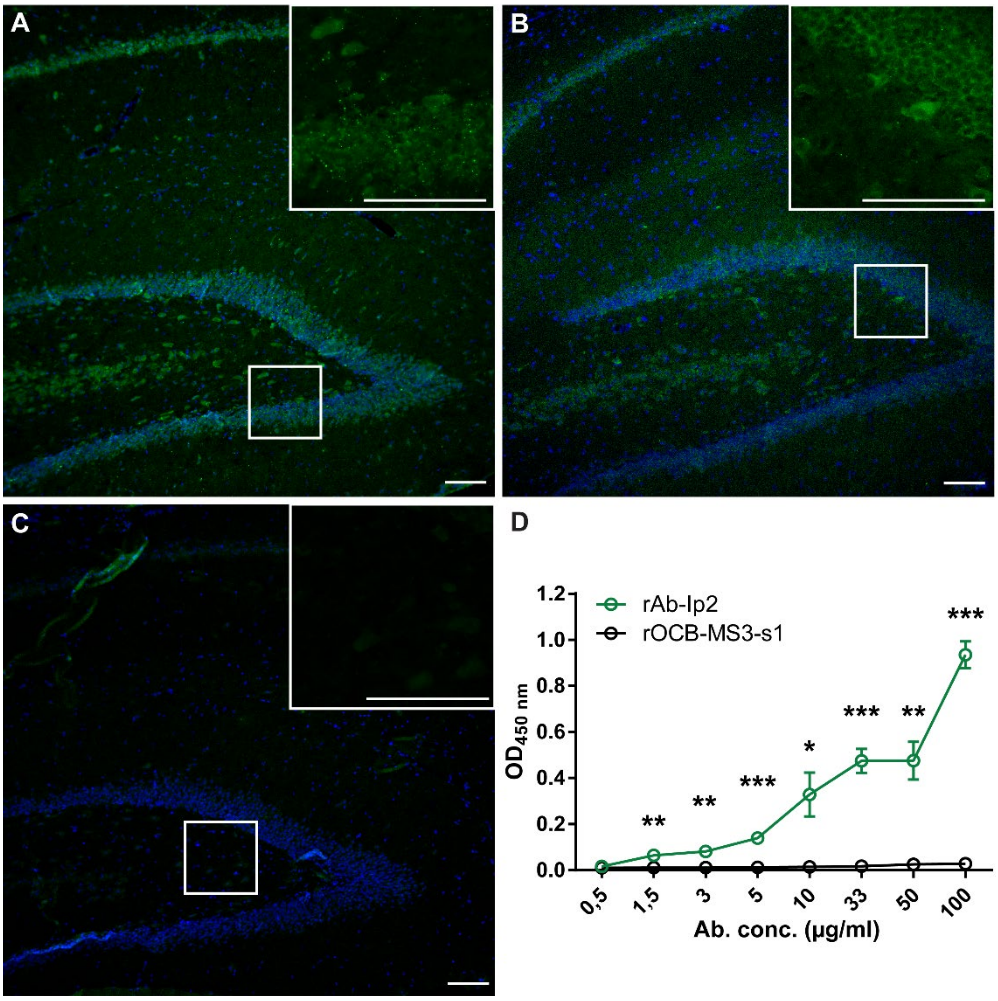
(A) Exemplary traces showing spontaneous inhibitory postsynaptic currents (sIPSCs) recorded from rOCB-MS3-s1- (control; left) and rAb-IP2-incubated (right) pyramidal neurons in the hippocampal region CA1. **(B)** Scatter plot showing that incubation with rAb-IP2 leads to a significant decrease of the number of sIPSCs recorded in an overall period of 10 minutes in comparison to control (rAb-IP2: 371.8 ± 65.8 n=6; rOCB-MS3-s1: 1316.0 ± 171.8 n=5; unpaired Student's t-test: t=5.511 df=9, p = 0.0004). **(C)** Bar graphs show a tendency to reduced amplitudes of sIPSCs in rAb-IP2-incubated pyramidal neurons in comparison to controls (rAb-IP2: 19.19 ± 0.60 pA n=3; rOCB-MS3-s1: 23.9 ± 5.6 pA n=4; Mann Whitney test: p = 0.4). **(D)** Scatter plot showing the resting membrane potential (RMP) of rAb-IP2-incubated neurons and controls (rAbIP2: -67.20 ± 1.56 n=10; rOCB-MS3-s1: -64.5 ± 1.83 mV n=8; unpaired Student's t-test: t=1.128 df=16, p = 0.2764). **(E)** Scatter plot showing the input resistance (R_{in}) of rAb-IP2-incubated neurons and controls (rAb-IP2: 145.6 ± 10.3 MΩ n=9; rOCB-MS3-s1: 104.3 ± 20.2 MΩ n=7, respectively; Mann Whitney test: p = 0.11). **(F)** Scatter plot showing the whole-cell capacitance of rAb-IP2-incubated neurons and controls (rAb-IP2: 16.12 ± 1.44 pF n=10; rOCB-MS3-s1: 14.07 ± 1.82 pF n=8, respectively; unpaired Student's t-test: t=0.8988 df=16, p = 0.382). **(G)** Bar graphs showing quantification of action potentials (APs) generated in response to depolarizing current steps of increasing intensity (from +20 to +240 pA, 20 pA increment, duration of 2.5 ms) in rAb-IP2-incubated pyramidal neurons and controls (Mixed design Two-Way ANOVA, main effect of rAb-IP2- vs. rOCB-MS3-s1-incubation: $F_{(1,165)}$ =

6.538, $p = 0.02$, Sidak's multiple comparisons test for rAb-IP2- vs. rOCB-MS3-s1: at 120 pA, $p = 0.0459$; at 140 pA, $p = 0.0127$; at 160 pA, $p = 0.0115$; at 180 pA, $p = 0.0055$; at 200 pA, $p = 0.0076$ and at 220 pA, $p = 0.0257$). Of note, for both groups, the input/output relation reflected by the proportional increase of the number of APs in response to the increasing current intensity did not differ, indicating a lower threshold for AP firing in rAb-IP2-incubated cells rather than a defective AP generation in rOCB-MS3-s1-incubated cells (main effect of input/output relation: $F_{(11,\ 165)} = 24{,}90$, $p< 0{,}0001$, Sidak´s multiple comparisons test: no significance between groups). **(H)** Exemplary traces showing APs generated in response to a +140 pA current step at resting membrane potential in rAb-IP2-incubated (red) and rOCB-MS3-s1-incubated (control; black) pyramidal neurons.

Figure 3: Identification of LMO5 as antigen of Ab-IP2

(A) Hybridization of rAb-IP2 to a protein array (32). Sections of the developed array are shown. The first column lists the antibodies used, the second column the detected target antigens, the third column the signals of the antigens, and the fourth column the signals of the secondary antibody alone. The color code ranges from black (no reactivity) to red (medium reactivity) and white (strong reactivity). All samples were spotted in duplicates. The upper row shows the detection of LMO5 (synonym CSRP2) by rAb-IP2. The middle row shows the detection of the homologous CSRP1 by rAb-IP2. Both proteins are recognized specifically as the secondary antibody alone yields no or a much weaker signal on the array. The lowest row shows recognition of the positive control antigen major oligodendrocyte glycoprotein (MOG) by the MOG-specific antibody r8-18C5. LMO5 and MOG were detected with high affinity, whereas CSRP1 was detected with low affinity. **(B)** Validation of LMO5 recognition by rAb-IP2 by immunoprecipitation. Recombinant proteins LMO5 (lanes 1 to 3) and CSRP1 (lanes 4 to 6) were produced in HEK293 cells and precipitated with antibodies rOCB-MS3-s1 (lanes 1 and 4), rAb-IP2 (lanes 2 and 5), and r8-18C5 (lanes 3 and 6). Only LMO5 could be precipitated by rAb-IP2. The blot is representative for three independent experiments. **(C)** Validation of LMO5 recognition by rAb-IP2 by ELISA. LMO5 was coated to plates and detected by rAb-IP2 between 0 and 1000 µg/ml. rAb-IP2 recognition of recombinant LMO5 produced in *E. coli* occurred in a dose dependent manner. The shown ELISA is representative for two independent experiments. The parameter for the linear equation are $y = 0{,}0009x + 0{,}0942$, and the correlation coefficient is $R^2 = 0.9954$ with $P < 0.001$.

Figure 4: Detection of anti-LMO5 antibodies in other patients and controls.
CSF samples from IP2, two other patients with $GABA_A$-R encephalitis ($GABA_A$-R-1 and -2), three with other forms of antibody associated CNS diseases (AACNSD-1 to -3), three with NIC (NIC-1 to -3), and five with MS (MS-1 to -5) were tested in an ELISA experiment. Clinical details are given in Table S1. CSF samples were diluted 1:10. We could also detect significant signals when the samples from $GABA_A$-R-1 and $GABA_A$-R-2 were diluted 1:20 or 1:40, respectively. Relative absorbance units were measured as optical density at 450 nm (OD450nm). A representative experiment of two independent assays is shown. Assays were performed in duplicate. Error bars indicate standard deviations of duplicates.


A
B
C
D
rAb-Ip2
rOCB-MS3-s1
OD450 nm
Ab. conc. (µg/ml)
0,5
1,5
3
5
10
33
50
100
1.2
1.0
0.8
0.6
0.4
0.2
0.0
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*
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**
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Figure 1

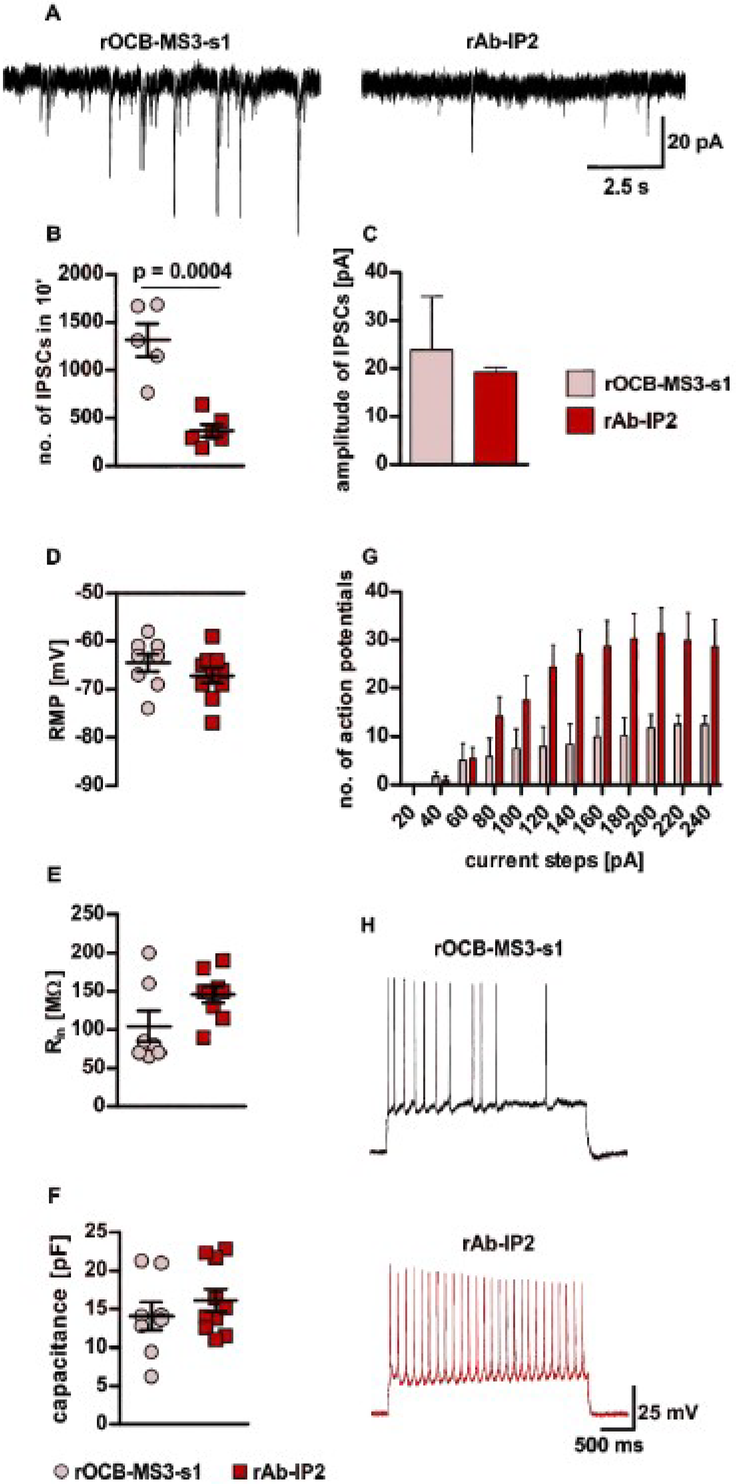
A
rOCB-MS3-s1
rAb-IP2
20 pA
2.5 s
B
p = 0.0004
no. of IPSCs in 10'
2000
1500
1000
500
0
C
amplitude of IPSCs [pA]
40
30
20
10
0
rOCB-MS3-s1
rAb-IP2
D
RMP [mV]
-50
-60
-70
-80
-90
G
no. of action potentials
40
30
20
10
0
20 40 60 80 100 120 140 160 180 200 220 240
current steps [pA]
E
R_{in} [MΩ]
250
200
150
100
50
0
H
rOCB-MS3-s1
rAb-IP2
25 mV
500 ms
F
capacitance [pF]
25
20
15
10
5
0
rOCB-MS3-s1
rAb-IP2

Figure 2

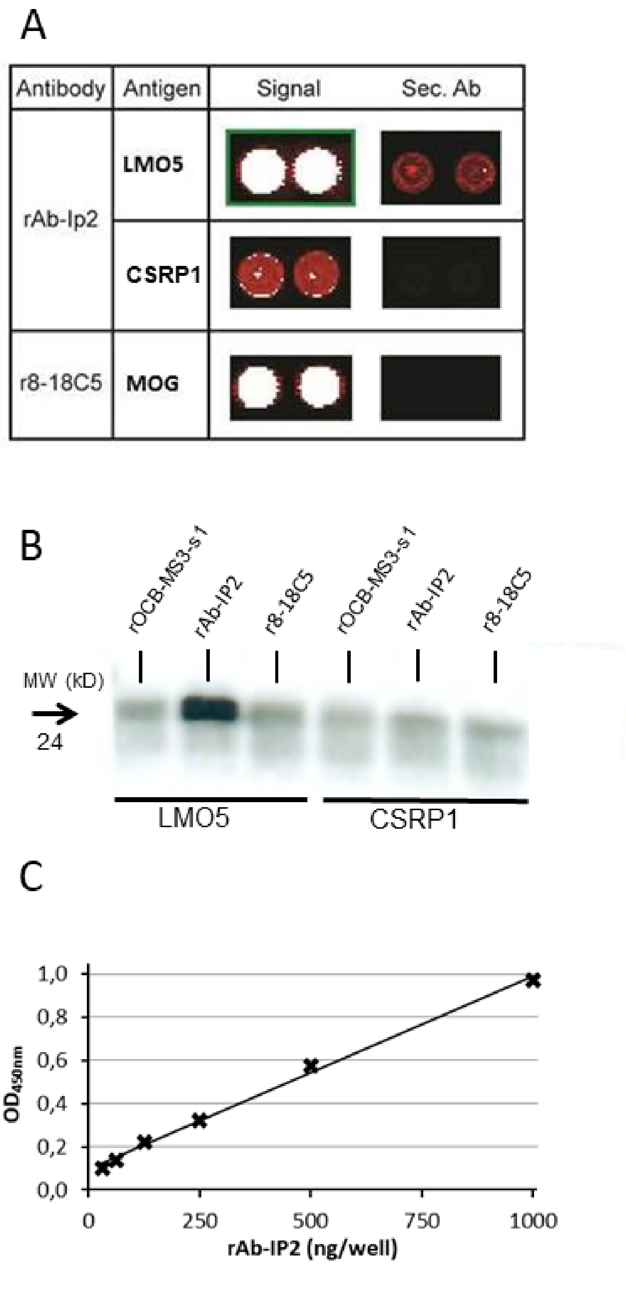
A
Antibody
Antigen
Signal
Sec. Ab
rAb-Ip2
LMO5
CSRP1
r8-18C5
MOG
B
rOCB-MS3-s1
rAb-IP2
r8-18C5
rOCB-MS3-s1
rAb-IP2
r8-18C5
MW (kD)
24
LMO5
CSRP1
C
1,0
0,8
0,6
0,4
0,2
0,0
OD450nm
0
250
500
750
1000
rAb-IP2 (ng/well)

Figure 3

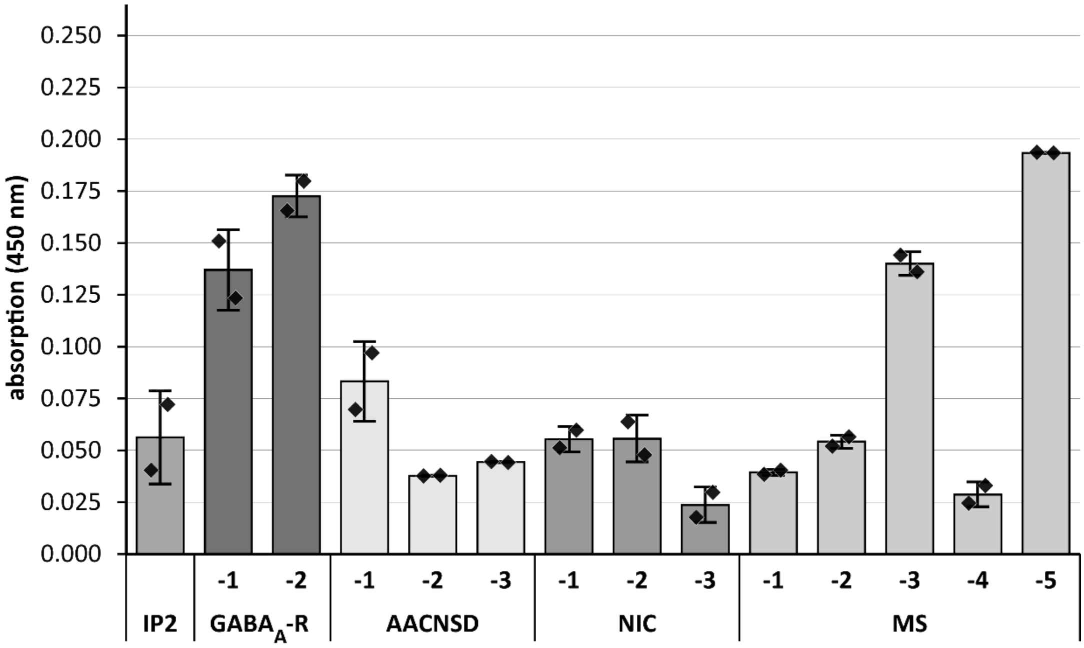
0.250
0.225
0.200
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0.000
absorption (450 nm)
-1
-2
-1
-2
-3
-1
-2
-3
-1
-2
-3
-4
-5
IP2
GABAA-R
AACNSD
NIC
MS

Figure 4

Supporting Information for

Cross-reactivity of a pathogenic autoantibody to a tumor antigen in $GABA_A$ receptor encephalitis

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This PDF file includes:

Heavy chain: IGHV3-21*01

CDR1 CDR2

EVQLVESGG.GLVKPGGSLRLSCAASGFTS....SFYSMNWVRQAPGKGLEWVSSISSS..SSYIYYADSVK.

CDR3

GRFTISRDNAKNSLYLQMSSLRADDTAVYYCAR.VGIMEWSDPRFVDEK. YGMDVWGQGTTVTVSS

Light chain: IGKV3-20*01

CDR1 CDR2

EIVLTQSPGTLSLSPGERATLSCRASQSVS.....SRYLAWYQQKPGQAPRLLIYGA.......SSRATGIP.

CDR3

DRFSGSG..SGTDFILTISRLEPEDFAVYYCQQYGSSL................YTFGQGTKLEIK

Figure S1: Sequence of the expanded antibody Ab-IP2 from patient IP2.

Amino acid sequences of the dominant H- and κ-chains of antibody Ab-IP2 are shown. Amino acid mutations introduced by somatic hypermutation and recombination are printed in red letters. The canonical cystein (C) residues and the conserved sequences in the J-regions (WGXG and FGXG) are highlighted in grey. The complementarity determining regions (CDR) 1 to 3 are indicated.

Figure S1

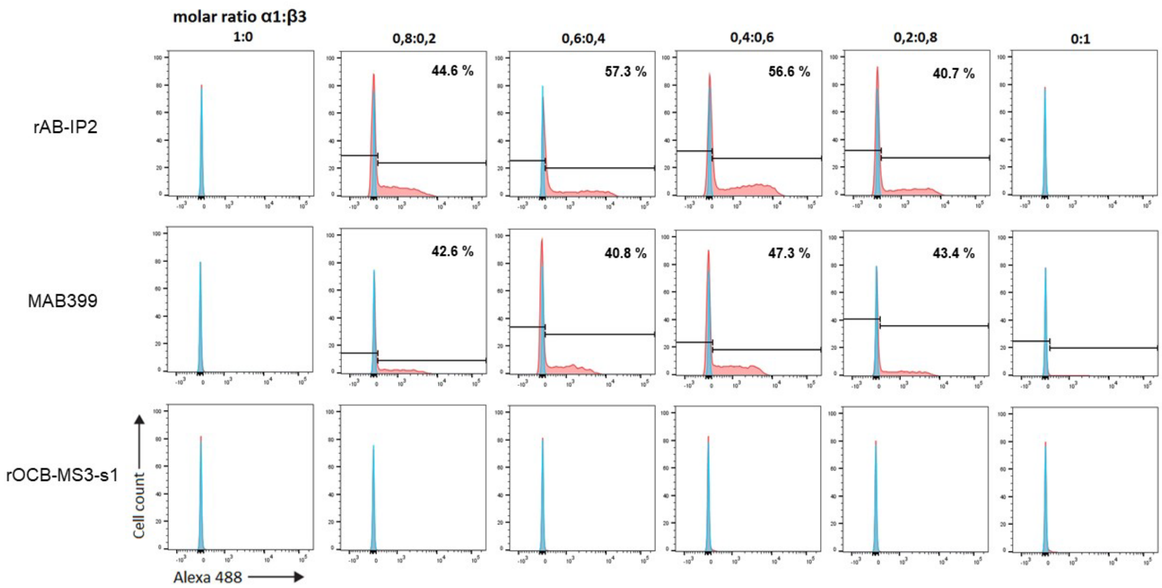

Figure S2: rAB-IP2 recognizes GABA$_A$-R

HEK293Expi cells, which grow in suspension, were transiently transfected with expression plasmids encoding the α1- and β3-subunits of GABA$_A$-R at different molar ratios shown in the uppermost lane. Cells were analyzed by flow cytometry for binding of rAb-IP2 (upper panel), the commercial anti-GABA$_A$-α1 antibody MAB399 (middle panel), and the negative control antibody rOCB-MS3-s1 (lowest panel). The percentages of positive cells are indicated in the plots. Binding of rAb-IP2 and MAB399 to transfected HEK293Expi cells was observed at different molar ratios of the α1- and β3-subunits, while no binding was observed, when only one subunit was transfected.

Figure S2

Subject	Disease type	Age	Sex	OCBs	Disease-specific antibody titers (CBA) at diagnosis	Immunotherapy preceding biosampling	Further clinical remarks
IP2	$GABA_A$-R Enc.	51	m	yes	Serum: $GABA_A$-R > 1:1280; CSF: $GABA_A$-R > 1:320	IVIG, PLEX, Cyclophosphamide, Rituximab	ITP, AT (1)
$GABA_A$-R-1	$GABA_A$-R Enc.	34	m	no	Serum: $GABA_A$-R 1:100; CSF: $GABA_A$-R 1:10	none	
$GABA_A$-R-2	$GABA_A$-R Enc.	47	f	no	Serum: $GABA_A$-R 1:320; CSF: $GABA_A$-R 1:10	none	
MS-1	RRMS	38	f	yes	none	none	anti-phospholipid antibody syndrome
MS-2	RRMS	21	f	yes*	none	none	
MS-3	RRMS	23	f	yes	none	none	
MS-4	PPMS	43	m	yes*	none	none	
MS-5	PPMS	45	m	yes	none	none	
Enc-1	NMDAR Enc.	20	f	yes*	Serum: NMDA-R 1:3.2; CSF: NMDA-R negative	Rituximab	3 cycles, 13 6, and 0.5 months before sampling
Enc-2	LGI-1 Enc.	62	m	yes*	Serum: LGI-1 1:800; CSF: LGI-1 positive	PLEX, Prednisolone	Both 2 months before sampling
Enc-3	GAD Enc.	49	f	no	Serum: GAD65 78 U/ml; CSF: GAD65 139 U/ml	Rituximab Azathioprin	5 cycles ~every 6 months, starting 28 month before sampling. starting 13 months before sampling, ongoing
NIC-1	IIH	43	f	no	none	none	
NIC-2	IIH	57	f	no	none	none	
NIC-3	IIH	58	m	no	none	none	

Table S1: Clinical characteristics of all subjects. Columns 1 and 2 list the subjects and their disease types. We analyzed CSF samples from patient IP2, three other cases with idiopathic anti-GABAA-R encephalitis ($GABA_A$-R-1 and -2), five cases with MS (MS-1 to -5), three cases with other forms of antibody associated CNS diseases (AACNSD-1 to -3), and three non-inflammatory disease controls (NID-1 to -3). Abbreviations are: RRMS = relapsing-remitting

MS; PPMS = primary progressive MS; NMDAR Enc = anti-N-Methyl-D-Aspartate-receptor encephalitis; LGI-1 Enc. = anti-leucine-rich-glioma-inactivated-1 associated encephalitis; GAD Enc. = anti-glutamic acid decarboxylase encephalitis; IIH = idiopathic intracranial hypertension. Column 3 lists the ages of the subjects, column 4 their sex, column 5 presence or absence of CSF-specific OCBs, column 6 the disease-specific antibody titers determined by cell-based-assays at diagnosis, column 7 the immunotherapies at the time point of lumbar puncture (LP), and column 8 further clinical remarks. Further abbreviations are: AT = autoimmune thyreoditis; CBA = cell-based-assay; ITP = idiopathic thromocyopenic purpura; IVIG = intravenous immunoglobulines; PLEX = plasma exchange; OCB = oligoclonal bands: * = in these subjects, OCBs were detected in CSF and in serum, indicating non-intrathecal OCB production.

Table S1

SI Reference

1. M. Petit-Pedrol et al., Encephalitis with refractory seizures, status epilepticus, and antibodies to the GABAA receptor: a case series, characterisation of the antigen, and analysis of the effects of antibodies. Lancet Neurol 13:276-286 (2014).