

Glycine Receptor β -Targeting Autoantibodies Contribute to the Pathology of Autoimmune Diseases

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Abstract

Background and Objectives

Stiff-person syndrome (SPS) and progressive encephalomyelitis with rigidity and myoclonus (PERM) are rare neurologic disorders of the CNS. Until now, exclusive GlyR α subunit-binding autoantibodies with subsequent changes in function and surface numbers were reported. GlyR autoantibodies have also been described in patients with focal epilepsy. Autoimmune reactivity against the GlyR β subunits has not yet been shown. Autoantibodies against GlyR α 1 target the large extracellular N-terminal domain. This domain shares a high degree of sequence homology with GlyR β making it not unlikely that GlyR β -specific autoantibody (aAb) exist and contribute to the disease pathology.

Methods

In this study, we investigated serum samples from 58 patients for aAb specifically detecting GlyR β . Studies in microarray format, cell-based assays, and primary spinal cord neurons and spinal cord tissue immunohistochemistry were performed to determine specific GlyR β binding and define aAb binding to distinct protein regions. Preadsorption approaches of aAbs using living cells and the purified extracellular receptor domain were further used. Finally, functional consequences for inhibitory neurotransmission upon GlyR β aAb binding were resolved by whole-cell patch-clamp recordings.

Results

Among 58 samples investigated, cell-based assays, tissue analysis, and preadsorption approaches revealed 2 patients with high specificity for GlyR β aAb. Quantitative protein cluster analysis demonstrated aAb binding to synaptic GlyR β colocalized with the scaffold protein gephyrin independent of the presence of GlyR α 1. At the functional level, binding of GlyR β aAb from both patients to its target impair glycine efficacy.

Discussion

Our study establishes GlyR β as novel target of aAb in patients with SPS/PERM. In contrast to exclusively GlyR α 1-positive sera, which alter glycine potency, aAbs against GlyR β impair receptor efficacy for the neurotransmitter glycine. Imaging and functional analyses showed that GlyR β aAbs antagonize inhibitory neurotransmission by affecting receptor function rather than localization.

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Graphical Abstract

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Glossary

aAb = autoantibody; **GlyR** = glycine receptor; **Pat** = patient; **PERM** = progressive encephalitis with rigidity and myoclonus; **SPS** = stiff-person syndrome.

Introduction

Stiff-person syndrome (SPS) is a rare autoimmune disease of the CNS with a prevalence of 1:1.000.000. Severe cases of SPS are associated with progressive encephalitis with rigidity and myoclonus (PERM).¹ Most common symptoms include muscle spasms, stiffness of abdominal and limb muscles, exaggerated startle, and different forms of phobias.^{2,3}

So far, glycine receptor (GlyR) autoantibody (aAb) have been found to bind GlyR α subunits expressed in spinal cord neurons and tissue without subtype preferences.⁴ Epitope characterization identified a common N-terminal region in the GlyR α 1 subunit with residues A²⁹-G⁶² for aAb binding.⁵ GlyR aAbs are able to cross-link receptors followed by subsequent internalization.⁴ Moreover, GlyR aAb binding impairs receptor function by direct blocking of most likely structural transitions essential for ion channel opening.^{5,6}

There are 4 GlyR α subunits (α 1-4) and one β subunit with each subunit consisting of a large extracellular domain (ECD), 4 transmembrane domains (TM1-4) connected by loop structures, and a short extracellular C-terminus.⁷⁻⁹ These subunits form pentameric chloride channels composed of α -homomers or $\alpha\beta$ -heteromers.^{10,11} While GlyR homomers have been found at presynaptic sites involved in the control of glycine release¹² and at extrasynaptic sites at postsynapses, GlyR heteromers form the synaptically localized receptors in postsynaptic neurons. The GlyR β subunit in heteromeric GlyRs is essential because it harbors the binding site for the scaffold protein gephyrin, which stabilizes GlyR complexes at postsynaptic sites.¹³ A constant packing of 2,000 GlyRs μm^{-2} at spinal cord synapses throughout adulthood has been estimated.¹⁴

Besides its structural role, GlyR β also contributes to GlyR function.¹⁵ Genetic human and murine variants of GlyR β associated with startle disease, which share phenotypic symptoms with SPS, have been determined with functional impairments of glycine potency and efficacy accompanied by less synaptic localization.¹⁶⁻¹⁸

In this study, we identified 2 patients with aAbs not only binding GlyR α but also the GlyR β subunit. Using patient serum samples, we investigated whether and how the disease pathology of GlyR β SPS differs from GlyR α SPS at the molecular, cellular, and functional levels.

Methods

Patients

Fifty-eight patient serum samples were submitted to our laboratory. In 48 patients, SPS was suspected, and they were negative

for ant glutamate-decarboxylase and anti-amphiphysin aAb. In 10 patients, a focal epilepsy of unknown cause was present, and GlyR aAb were found by routine screening. None of the patients with epilepsy had motor symptoms, hyperekplexia, or startle reaction.¹⁹ Disease pattern of 2 patients with GlyR $\alpha\beta$ aAb (Pat31 and Pat36) in comparison with 1 patient with GlyR α aAb only (Pat11) are further described in the Table.

Ethical Statement

Experiments using patient material have been approved by the Ethics Committee of the Medical Faculty, University of Würzburg, Germany ("Glycine receptor autoantibodies and spinal disinhibition," 20190424 01).

Anxiety Questionnaires

Patients underwent a neurologic examination, and Pat11 and Pat36 were given questionnaires about chronic pain,^{20,21} anxiety (Liebowitz Social Anxiety Scale [LSAS]; Anxiety Sensitivity Index [ASI]),^{22,23} and heightened sensitivity inducing spasms and falls (see Table for results).²⁴

Cell Lines

HEK-293 cells (Human Embryonic Kidney cells; CRL-1573; ATCC—Global Bioresource Center) were used for in vitro experiments. Cells were grown in minimum essential medium (Life Technologies, Carlsbad, US) supplemented with 10% fetal bovine serum, L-glutamine (200 mM), and 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin at 37°C and 5% CO₂.

Primary Spinal Cord Neurons

Neurons were prepared from embryos of a novel generated hybrid mouse line *Glra1*^{spdot}/*Glr β* ^{eos} from *Glr β* ^{eos14} and *Glra1*^{spdot} (*oscillator*, JAX stock #000536, JAX:000536, Jackson Laboratory, Bar Harbor, US) at the embryonic stage 12–13. Experiments were approved by the local veterinary authority (Veterinäramt der Stadt Würzburg, Germany) and the Ethics Committee of Animal Experiments, i.e., Regierung von Unterfranken, Würzburg, Germany (license no.:55.2.2-2532.2-949-31). Mixed spinal cord neuronal cultures were prepared as previously described.²⁵ Genotyping for *Glr β* ^{eos} and *Glra1*^{spdot} was performed according to Maynard et al.¹⁴ Stainings and electrophysiologic measurements were performed after 16–18 days in culture.

Transfection of Cells

HEK-293 cells were transiently transfected by using a modified calcium phosphate precipitation method.¹⁸

Immunocytochemistry

Living transfected HEK-293 cells or primary neurons were incubated for 2 hours at 4°C with patient sera, healthy control serum (1:50), or commercial antibody against GlyR α 1

Table Patient Characteristics

	Patient 31: alpha & beta	Patient 36: alpha & beta	Patient 11: alpha
Sex	Male	Male	Male
Age at blood withdrawal	58 y	68 y	44 y
Disease duration	48 y	16 y	19 y
Diagnosis	Focal epilepsy	SPS/PERM	SPS
Symptom history	Focal epilepsy with restless legs syndrome, insomnia, diabetes mellitus, chronic renal failure	Brainstem myoclonus and exaggerated startle response sensitive to minor auditory and tactile stimuli, abnormal eye movements with diplopia and nystagmus	Recurrent lockjaw associated with limb stiffness, startle, and frequent falls
Tested negative for other aAbs	NMDAR, LGI1, Caspr2, GABA _A R, AMPAR, GAD	NMDAR, LGI1, Caspr2, GABA _A R, AMPAR, GAD	NMDAR, LGI1, Caspr2, GABA _A R, AMPAR, GAD
Current symptoms	Intact	Under current medication, no increased muscle tone, no paresis, normal gait	Under current medication, reduced frequency of symptoms
Medication	Carbamazepine 600 mg pramipexole 0.75 mg	Steroid pulse therapy at 4-wk intervals with 4 ×1,000 mg methylprednisolone Clonazepam 2.5 mg Pramipexol 2 mg levodopa/benserazid 100/25 mg as needed	Clonazepam 8 mg 1-1-1 Δ ⁹ -Tetrahydrocannabinol/cannabidiol (Sativex spray) as needed Immunoabsorption every 4 wk
Pain (graded chronic pain scale, scale 0–4)	0	0	3
Anxiety sensitivity index, scale 0–72)	n.d.	29	38
Social anxiety and avoidance	Moderate to severe	Very mild	Very mild
Scale of increased sensitivity^a, 0–7)		3, noise, somatosensory and emotional excitement	4, noise and visual, somatosensory, and emotional excitement

Abbreviation: PERM = progressive encephalomyelitis with rigidity and myoclonus; SPS = stiff-person syndrome.

^a Dalakas et al. 2017.⁴³

(146111, 1:500, Synaptic Systems, Göttingen, Germany). After fixation using 4% paraformaldehyde/4% sucrose in phosphate-buffered saline (PBS) at pH 7.4 for 20 minutes at room temperature (RT), cells were blocked and permeabilized with 5% goat serum/0.2% Triton-X-100 in PBS for 30 minutes. Primary antibodies against myc-tagged GlyRβ (303008, 1:250, Synaptic Systems), gephyrin (147111, 1:500, Synaptic Systems), synapsin (574778, 1:500, Merck), and pan-α-GlyR (146011, 1:250, Synaptic Systems) were incubated for 1 hour, followed by incubation with secondary antibodies (all 1:500, 111-546-003, 109-165-003, 115-175-146, and 111-175-006, all from Dianova, Hamburg, Germany) for 1 hour. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and mounted in Mowiol.

PreadSORption

Living HEK-293 cells transfected with the GlyRa1 subunit were incubated with patient sera, healthy control serum (1:50), or GlyRa1 antibody (146111, 1:500, Synaptic Systems) for 1 hour at RT. The supernatant containing unbound antibodies was transferred to another coverslip with 3

repetitions. Finally, the supernatant was transferred to HEK-293 cells transfected with the zebrafish GlyRa1 and human GlyRβ subunit.

ELISA Neutralization With GlyRa1 ECD

GlyRa1 ECD preparation and enzyme-linked immunosorbent assay (ELISA) were performed as described previously.²⁶ Spinal cord neurons were stained afterward with patient serum (1:50).

Immunohistochemistry

Spinal cords were extracted from anesthetized *Glra1*^{+/+}/*Glrb*^{eos/eos} and *Glra1*^{spdot/spdot}/*Glrb*^{eos/eos} mice, bedded in Tissue-Tek and immediately frozen on dry ice. A cryostat (CM1950, Leica, Wetzlar, Germany) with a chamber temperature of −20°C was used to cut spinal cord sections of 9 μm thickness. Sections were mounted on SuperFrost Plus slides (03-0060, Langenbrinck, Niederrohrdorf, Switzerland).

Sections were fixed with ice-cold 2% paraformaldehyde in PBS at pH 7.4 for 30 seconds at RT. After washing, sections

were shortly dipped in 50 mM NH₄Cl for quenching and incubated in 0.1 mM glycine for 30 minutes. For blocking, 10% goat serum in PBS at pH 7.4 was used followed by primary antibody incubation with patient serum (1:50) and an anti-mEos-Cy3 (N3102-SC3-L, 1:200, Nanotag, Göttingen, Germany) overnight (ON) at 4°C. Secondary antibody goat-anti-human-IgG-Alexa-Fluor-647 (1:500, JIM-109-605-006, Biozol, Eching, Germany) was incubated for 1 hour at RT. Nuclei were stained with DAPI for another 10 minutes. Sections were covered with Fluor Save Reagent (345789, Calbiochem, Darmstadt, Germany).

Pentameric Structure of GlyR

The cryo-EM structure (7MLY⁹) of the pentameric GlyR with a subunit stoichiometry of 4α:1β was used to generate structural images. Figures were prepared with the help of Pymol (pymol.org, version 2.0.7).

μSPOT Synthesis

GlyR subunit ECDs (UniProtKB: P23415, P23416, O75311, Q5JXX5, P48167) were displayed in microarray format as 15mer overlapping peptide library. μSPOT²⁷ peptide microarrays were synthesized using a MultiPep RSi robot (CEM, Matthews, US) on cellulose discs containing 9-fluorenylmethoxycarbonyl-β-alanine (Fmoc-β-Ala) linkers (average loading: 130 nmol/disc—4mm diameter). Synthesis was performed by deprotecting the Fmoc-group using 20% piperidine in dimethylformamide (DMF). Peptide chains were elongated using a coupling solution consisting of amino acids (0.5 M) with oxyma (1 M) and diisopropylmethanediimine (1 M) in DMF (1:1:1). Coupling steps were conducted 3 times (30 minutes), followed by capping (4% acetic anhydride in DMF). Side chains were deprotected using 90% trifluoroacetic acid (TFA), 2% dichloromethane (DCM), 5% H₂O, and 3% triisopropylsilane (TIPS, 150 μL/well) for 1 hour at RT. Afterward, the deprotection solution was removed, and the discs were solubilized ON at RT using a solvation mixture containing 88.5% TFA, 4% trifluoromethanesulfonic acid, 5% H₂O, and 2.5% TIPS. The resulting peptide-cellulose conjugates (PCCs) were precipitated with ice-cold ether and spun down at 2,000×g for 10 minutes at 4°C, followed by 2 additional washes with ice-cold ether. Resulting pellets were dissolved in DMSO. PCC solutions were mixed 2:1 with saline-sodium citrate buffer (150 mM NaCl, 15 mM trisodium citrate, pH 7.0) and transferred to a 384-well plate. For transfer of the PCC solutions to white-coated CelluSpot blank slides (76 × 26 mm, Intavis AG Peptide Services, Tübingen, Germany), a SlideSpotter was used.

Microarray Binding Assay

Microarray slides were blocked for 1 hour in 5% (w/v) milk powder, 0.05% Tween20, and PBS at pH 7.4. The slides were incubated for 30 minutes with positive and negative sera (1:500) or GlyRα1 and GlyR pan-α antibody (1:2500) in blocking buffer. IgG antibodies were detected using goat-anti-human or goat-anti-mouse-IgG-HRP (31410, 1:2500, 31430, 1:5000, Thermo Fisher, Waltham, US). The readout was

detected with an Azure imaging system c400 using Super-Signal West Femto substrate (Thermo Scientific). Microarray binding intensities were quantified with FIJI using the “microarray profile” plugin (OptiNav Inc, Bellevue, US).

Neutralization in Microarray Format

Cleavable peptides were synthesized with an additional rink amide linker at the C-terminus of the identified epitope. Microarray slides were blocked using 5% (w/v) milk powder, 0.05% Tween20, and PBS at pH 7.4 for 1 hour. Serum samples from patient 36 were preincubated with cleaved peptide in the amount corresponding to 2 and 4 cellulose discs containing Fmoc-β-Ala linkers. Peptides were resuspended in 100 μL of PBS buffer, and 5 μL of serum was added subsequently. The samples were mixed at 1000 rpm (RT) for at least 30 minutes. A control without peptide was treated in the same manner. Solutions were added to 2.5 mL of blocking solution and incubated for 30 minutes on the slides. Microarray binding intensities were quantified with FIJI and normalized against the untreated slide.

Electrophysiologic Recordings

Patch-clamp analysis was performed on transfected HEK-293 cells or mixed primary neuronal cultures using whole-cell recordings. Experiments were performed at 21°C. Recording pipettes were pulled from borosilicate capillaries with open resistances of 3.5–5.5 MΩ and filled with internal buffer in mM (120 CsCl, 20 N(Et)₄Cl, 1 CaCl₂, 2 MgCl₂, 11 EGTA, 10 HEPES for HEK-293 cells; 140 CsCl, 1 EGTA, 10 HEPES, and 6 D-Glucose for neurons; pH 7.2, adjusted with CsOH). For determination of maximal current amplitudes (I_{max}) and dose-response curves (EC₅₀ values), glycine was applied in a concentration series of 10–1000 μM in external buffer in mM (137 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES for HEK-293; 130 NaCl, 3 KCl, 1.5 CaCl₂, 2 MgCl₂, 10 HEPES, 6 D-Glucose, and 10 TEA-Cl for neurons; pH 7.35, adjusted with NaOH). Glycine solutions were applied by the Octaflow II system (ALA Scientific Instruments, Farmingdale, US). Following recordings, 50 μM picrotoxinin (Sigma Aldrich, Darmstadt, Germany) + 100 μM glycine were applied. Homomeric GlyRα, but not heteromeric GlyRαβ, are blocked by picrotoxinin.²⁸ This test was used to discriminate between homomeric and heteromeric receptors. Current responses were amplified with an EPC-10 amplifier and measured at a holding potential of –60 mV using Patchmaster Next software (HEKA Elektronik, Reutlingen, Germany).

Western Blot Analysis

Spinal cord, brainstem, and cortex samples were extracted from deeply anesthetized male and female mice and directly frozen at –80°C. Lysate were prepared using 1 mL of brain homogenisate buffer (20 mM HEPES, 100 mM potassium acetate, 40 mM KCl, 5 mM EGTA, 5 mM MgCl₂, 5 mM DTT, 1% TritonX-100, 1 mM PMSF, and protease inhibitors (Roche, Basel, Switzerland). After a 15-minute centrifugation at 10,000×g at 4°C, supernatants were transferred and used for Western blots.

Protein samples were separated by SDS-PAGE using 11% (w/v) gels followed by transfer of proteins onto a nitrocellulose membrane (GE Healthcare, München, Germany). After blocking for 1 hour with 5% BSA in TBS-T (TBS with 1% v/v Tween20), membranes were incubated with primary antibodies anti-gephyrin, anti-GlyR pan- α , anti-GlyRa1, anti-VGAT (131003) all 1:1,000, Synaptic Systems), and anti-GAPDH (CB1001, 1:1,000, Merck) ON at 4°C. Proteins were visualized by horseradish peroxidase-coupled secondary antibodies (111-036-003 and 115-035-146, 1:15000, Dianova) and detected through chemiluminescence using clarity Western ECL substrate (170-5061, BioRad, Feldkirchen, Germany).

Experimental Design and Statistical Analysis

Images were captured using an Olympus Fluoview ix1000 microscope (UPLSAPO 60 \times oil objective) or a Zeiss Axio Imager 2 microscope (20 \times air objective). For image analysis and processing, the Fiji/ImageJ software was used.²⁹ Synaptic density/100 μ m was analyzed through the plug-ins NeuronJ and SynapscoutJ.

Data were analyzed using GraphPad Prism or Origin9 software and represented as mean \pm standard error of the mean (SEM).

The numbers of experiments (N; all experiments have been performed from 3 biological replicates or as stated otherwise) and cells (n) are listed in eTable 1 (links.lww.com/NXI/A969). Data were tested for outliers by ROUT ($Q = 1\%$). Normality of the data was reviewed by the Shapiro-Wilk normality test ($\alpha = 0.05$). Statistical significance was calculated using an unpaired 2-tailed Mann-Whitney test or an unpaired t test. p values are given in the result section or eTable 1. The 0-hypothesis was rejected at a level of $p < 0.05$.

Data Availability

Data that support the findings of this study are available from the corresponding author on reasonable request.

Results

The GlyR β Subunit Represents a Target for GlyR aAb

GlyR aAb have been identified to target a common sequence in the ECD of GlyRa subunits (Figure 1A).⁵ The adult receptor composition is 4 α :1 β heteromeric.^{8,9} The GlyRa ECD shares a high homology to the GlyR β subunit (Figure 1B). In this study, we investigated 58 serum samples from patients with SPS-like symptoms and focal epilepsy and tested them for binding to GlyR β (Figures 1A and 2, A–D). GlyR β subunit alone does not form functional channels^{8,9,30} and requires coexpressed α for transport to the plasma membrane (Figure 2B). Thirty samples harbored aAb against human GlyRa1 (eFigure 1A, links.lww.com/NXI/A966), whereas 28 displayed no binding. All GlyRa1-negative sera demonstrated also no binding to human GlyR β subunits (selected examples; eFigure 1B, eTable 2, links.lww.com/NXI/A970).

To analyze whether GlyRa1-positive sera bind GlyR β , we used zebrafish GlyRa1^{dr} to ensure transport and integration of GlyR β into the cellular membrane (Figure 2B). Previously, we have shown that most human serum samples harboring GlyRa aAb do not bind zebrafish α 1.⁵ Three patient sera of the 30 GlyRa1-positive sera bound to the zebrafish α 1 subunit and were thus excluded from further analysis. Testing of the remaining 27 human GlyRa1-positive sera resulted in GlyR β binding of Pat 31 and Pat 36 suggesting that indeed GlyR β represents a target for GlyR aAb (Figure 2D, eTable 2, links.lww.com/NXI/A970). Both patients had a confirmed autoimmune disorder. Pat31 had focal epilepsy without any other cause than GlyR aAb, and Pat36 had unequivocal SPS/PERM with good response to immunotherapy (Table). Detailed information is published elsewhere (Pat11,³¹ Pat31,^{19,32,33} and Pat36²⁶).

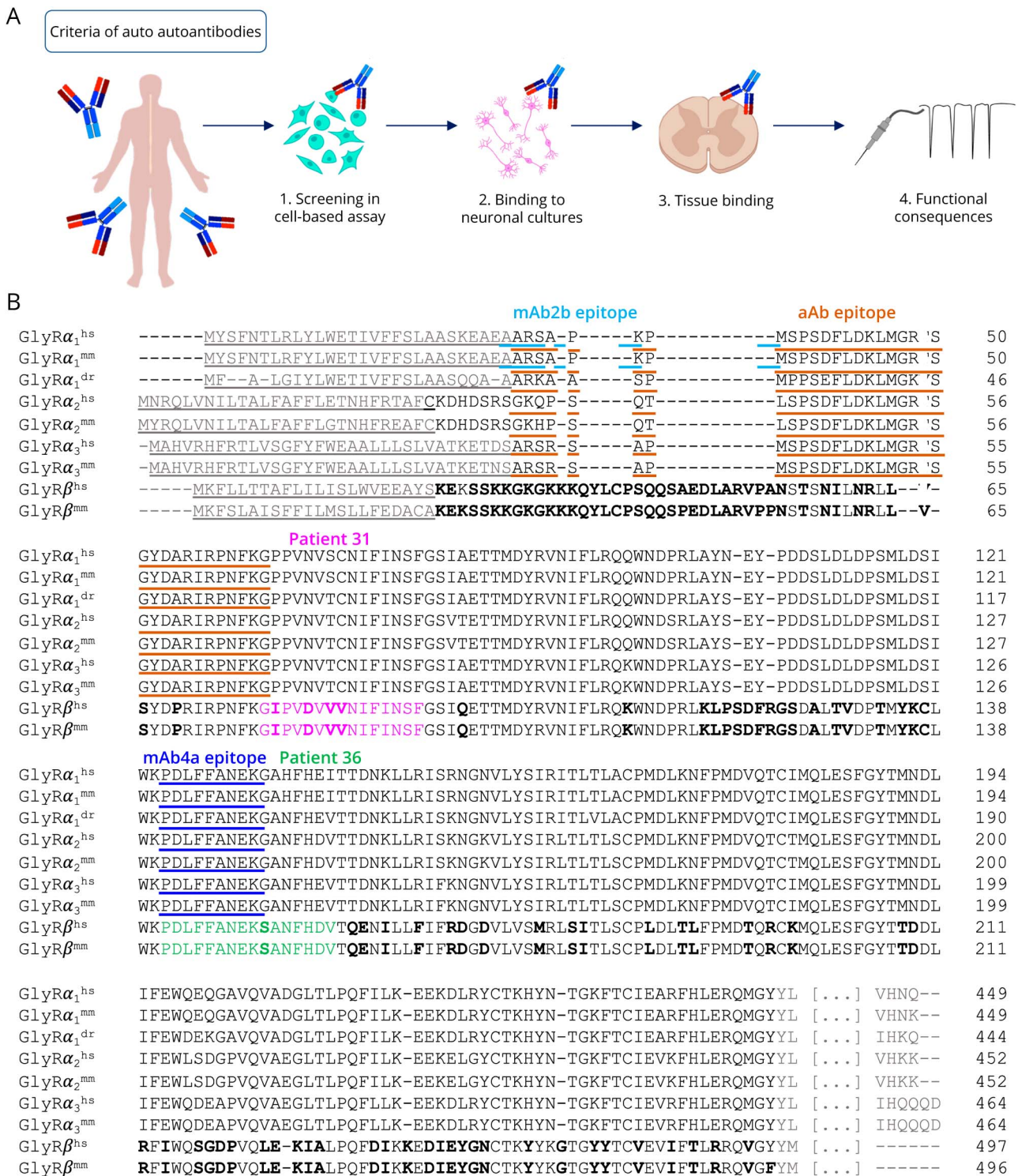
Single-nucleotide variations in the GlyR β subunit gene have been associated with an increased susceptibility to anxiety or panic disorders identified in a genome-wide association study with healthy human volunteers.^{34,35} However, anxiety scores of patients with GlyR β aAb (Pat31, Pat36) were not different from Pat11 with exclusively GlyRa aAb (Table).

Identification and Mapping of GlyR β Subunit aAb Epitopes

Peptide microarray-based screenings enable the identification, mapping, and validation of linear aAb epitopes (Figure 3A).³⁶ To identify possible binding regions of GlyR β aAb, GlyR subunits α 1-4 and β were displayed in form of 372 peptides (15 amino acids (AA) length, 10AA overlap, 5AA shift). The recapitulation of the reported epitopes of commonly used GlyR antibodies mAb2b and mAb4a confirms the microarray capacity to report binding epitopes within the structured GlyR ECD (Figure 3, B–D). The same arrays reported putative aAb binding sites for Pat31 and Pat36, which confirmed binding to the GlyR β subunit. Pat31 showed reactivity toward GlyRa1/ α 4/ β subunits, whereas Pat36 showed pan-GlyR subunit activity (Figure 3, B and C). A focused GlyR β library of 232 peptides (15AA length, 14AA overlap, 1AA shift) determined GlyR β sequences that may mediate aAb binding. An autoantibody epitope for Pat36 was mapped toward GlyR β residues ¹⁴¹PDLFFA-NEKSANFHDV¹⁵⁶ and for Pat31 toward ⁷⁷GIPVDVVVNI-FINSF⁹¹ (Figure 3D). The observation that GlyR β aAb binding of Pat 36 partially overlaps with the epitope of mAb4a suggests an elevated intrinsic immunogenic potential of this region. Both identified GlyR β epitopes are localized within surface-accessible GlyR β ECD β sheets (Figure 3, E and F).

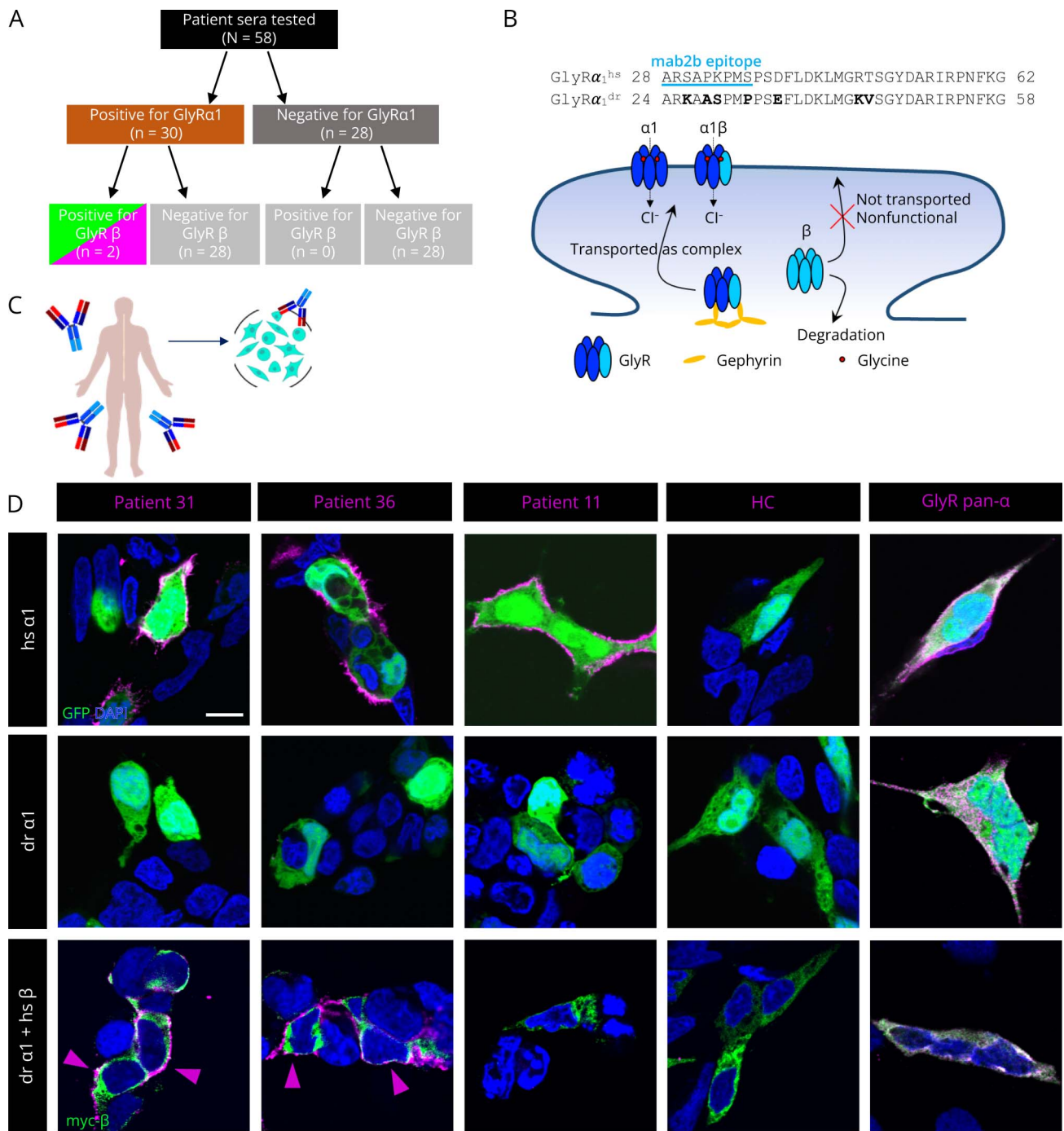
To confirm the microarray mapping and to verify the observed anti-GlyR β reactivity, we conducted on-chip neutralization experiments. In this study, serum of Pat36 was preincubated with increasing amounts of the soluble GlyR peptide (DSIWKPDLFFANEKG) that overlaps with the mapped binding site. The observed concentration-dependent signal reduction upon preadsorption (Figure 3G) supports the conclusion that the microarray binding signals resulted from sequence-specific GlyR β recognition of the patient aAb.

Figure 1 Overview of Reported GlyR Antibody and Autoantibody Epitopes



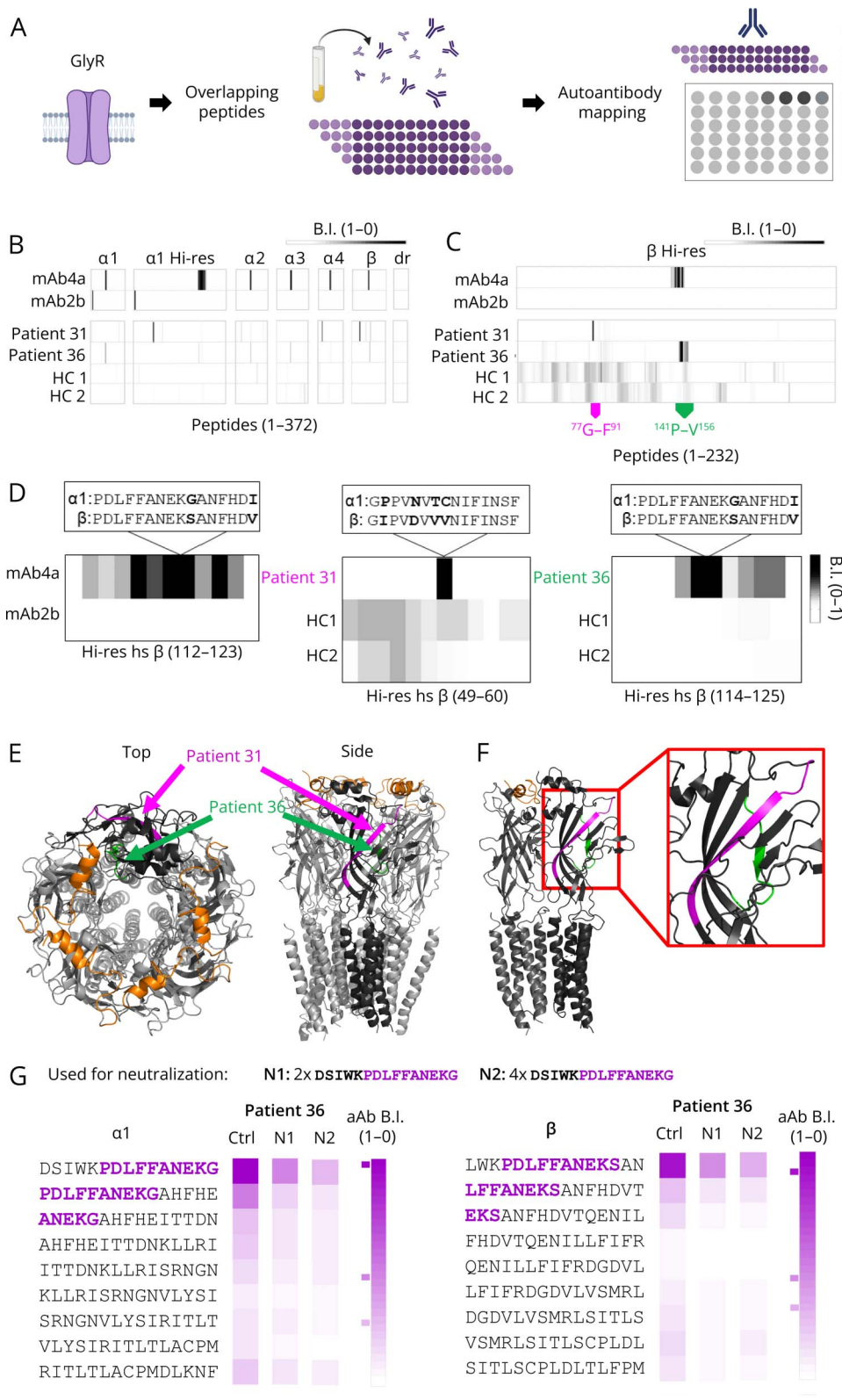
(A) Determination of autoantibody specificity by cell-based assays and neuronal and tissue binding and functional analysis in the presence of aAbs. (B) Alignment of GlyR subunits α1, α2, α3 and β from human and mouse and α1 from zebrafish concentrating on the ECD. Numbers of amino acids refer to nonmature protein. Labeled are the binding epitopes of the commercial GlyRa1 (mab2b) (cyan) and pan-α (mab4a) (blue) antibodies. In addition, the aAb epitope ²⁹A-⁶²G for GlyRa aAb binding is marked (brown).⁵ The here mapped sequences for aAb binding (green and magenta) are marked. Numbers of amino acids refer to nonmature protein.

Figure 2 Screening of Patient Samples Identifies Anti-GlyR β aAb



(A) Flowchart for the screening of sera from patients with SPS-like symptoms. Of 58 patient sera, 30 samples were positive for GlyR $\alpha 1$ aAb of which 2 were also positive for the GlyR β subunit. All patient serum samples negative for GlyR $\alpha 1$ aAb were also negative for binding to GlyR β . (B) Alignment of the postulated aAb epitope of human (hs) and zebrafish (dr) GlyR α . Scheme of the GlyR complex transport to the plasma membrane. GlyR β alone cannot form function ion channels and therefore is not transported to the membrane but degraded. (C) Principle of cell-based assay. (D) Binding of serum samples of Pat31, Pat36, Pat11, healthy control (HC) serum, and a commercial antibody against the GlyR α subunits to transfected HEK-293 cells. Cells were cotransfected either with GFP (green) and different GlyR α subunits from human (hs) and zebrafish (dr) or with zebrafish GlyR $\alpha 1$ and a myc-tagged human GlyR β . GlyR β is stained with an antimyc antibody (green, lower panel), binding of patient serum was verified with an anti-human-IgG-Cy3 antibody (magenta), and expression control of GlyR α is also demonstrated (magenta, right panel). Arrows point to binding of patient serum to transfected HEK-293 cells. Note, the detection of the GlyR β subunit through the myc antibody required cell fixation and permeabilization, leading to surface membrane and intracellular staining of GlyR β . Scale bar refers to 10 μm .

Figure 3 Epitope Mapping of GlyR β aAbs Through Peptide Microarrays



(A) Array workflow. GlyR α 1-4 and β sequences were extracted from UniProt. From those sequences, overlapping peptide libraries were synthesized in μ SPOT format. Peptide microarrays were incubated with serum samples from antiglycine receptor-positive patients. Epitope binding sequences were detected by chemiluminescence. (B) Shown are normalized heatmap binding intensities for Pat31 and Pat36, HC, and glycine receptor monoclonal antibodies incubated on the microarrays with all the different subunits displayed (α 1-4 and β). Each line corresponds to a peptide signal on the microarray. Pat31 shows binding on the α 1, α 4, and β subunits, whereas Pat36 shows pan-activity similarly to the mAb4a. (C-D) Detailed heatmap within the region of GlyR β binding by the pan-GlyR antibody mAb4a, and patients (Pat31, Pat36). (E) Crystal structure of the human GlyR heteropentamer (PDB: 7MLY)⁹ as top and side view with aAb epitopes marked (GlyR α orange, GlyR β magenta and green). (F) Right image shows GlyR α - β dimer interface. (G) Each GlyR subunit was displayed as overlapping peptides with 5 amino acids shift. Sera were probed without peptide (Ctrl) or by using increasing cleavable peptide amounts (N1 and N2—purple shades—correspond to the peptide amounts of 2-4 cellulose-cleavable discs that were preincubated with serum 36). Autoantibody binding was depicted as heatmap by normalizing detected intensities against the non-neutralized sera, where 1 corresponds to the α 1 signal for DSIWKPDLFFANEKG sequence.

Specific GlyR β aAbs Are Not Preabsorbed by GlyRa1

To confirm specific GlyR β binding of patient sera, GlyRa1 expressed in transfected HEK-293 cells were used for preadsorption of GlyRa1-specific aAbs from patient serum. As a second approach, incubation of patient serum with the purified and refolded GlyRa1 ECD coated to ELISA plates was used.²⁶ We transferred patient serum 3 times to transfected HEK-293 cells expressing only GlyRa1 for 1 hour followed by live staining of the remaining supernatant on HEK-293 cells expressing GlyRa1 and GlyR β . As control, we used an exclusively GlyRa1-positive serum with the same titer as Pat31 and Pat36 for better comparison (titer 1:500).

Serum signals of Pat31 and Pat36 were already strongly reduced after the first transfer and completely abolished after the second one, whereas binding of Pat12 serum was no longer visible after 3 transfers (Figure 4A). Final transfer of the samples to GlyRa1 and GlyR β expressing cells revealed again binding of Pat31 and Pat36 sera but not binding of Pat12 serum arguing that GlyR β -specific aAbs still remained in the serum.

Then patient samples were incubated with the ECD of GlyRa1 bound to ELISA plates, and the remaining supernatant was used for live staining of spinal cord neurons (Figure 4B). Although Pat36 and Pat12 sera both bound very strongly to spinal cord neurons before the adsorption by GlyRa1 ECD, remaining signal was only detectable for Pat36 serum afterward. Together, these findings further confirm that both, Pat31 and Pat36, sera contain aAb targeting GlyR β and cannot be neutralized by GlyRa1. Due to insufficient material from Pat31, this and some following experiments were performed only with Pat36 serum.

GlyR β aAbs Bind Specifically to Endogenous β Subunits

The binding of GlyR β -positive patient sera to endogenous GlyRs was evaluated using murine mixed spinal cord cultures isolated from a mouse model that allows specifically the detection of GlyR β . *Gla1*^{spdot/spdot}/*Glr*^{eos/eos} animals result from crossing mEos4-tagged *Glr*^{b14} with *Gla1* mutant *oscillator* mice.³⁷ *Oscillator* mice carry a frameshift mutation resulting in lack of GlyRa1 in homozygous animals. Binding of Pat31 and Pat36 sera were detected in both *Gla1*^{+/+}/*Glr*^{eos/eos} (wild-type controls) and *Gla1*^{spdot/spdot}/*Glr*^{eos/eos} neurons (absence of GlyRa1). Pat12 serum bound to *Gla1*^{+/+}/*Glr*^{eos/eos} but not *Gla1*^{spdot/spdot}/*Glr*^{eos/eos} neurons lacking GlyRa1 (Figure 5A). HC serum showed no binding (eFigure 2, links.lww.com/NXI/A967).

The gephyrin signal serves as a postsynaptic marker and allows the quantification of GlyR β (mEos) in gephyrin-positive clusters (Figure 5A). A comparison of gephyrin and GlyR β between wild-type controls (*Gla1*^{+/+}/*Glr*^{eos/eos}) and homozygous *oscillator* neurons (*Gla1*^{spdot/spdot}/*Glr*^{eos/eos}) revealed significantly less GlyR β in *oscillator* neurons

(** $p = 0.004$), while gephyrin levels were indistinguishable between *oscillator* and wild-type controls ($p = 0.35$, Figure 5, B and C, eTable 1A (links.lww.com/NXI/A969)). The reduced GlyR β expression in *oscillator* neurons was not surprising because GlyRa1, the main partner of GlyR β , is absent. Of interest, no significant differences were identified in the synaptic localization of GlyR β between *Gla1*^{spdot/spdot}/*Glr*^{eos/eos} and *Gla1*^{+/+}/*Glr*^{eos/eos} neurons ($p = 0.86$, Figure 5D). The number of synapses targeted by Pat31 and Pat36 sera showed no differences between *Gla1*^{+/+}/*Glr*^{eos/eos} and *Gla1*^{spdot/spdot}/*Glr*^{eos/eos} mice ($p = 0.11$ and $p = 0.43$, respectively, Figure 5E, eTable 1A).

Lack of GlyRa1 in homozygous *oscillator* mice was further validated using an $\alpha 1$ -specific antibody (mAb2b) in Western blots of spinal cord, brainstem, and cortex tissue lysates (eFigure 3, A and B, links.lww.com/NXI/A968). Nevertheless, other GlyRa subunits ($\alpha 2$ and $\alpha 3$) were present in spinal cord (GlyR pan- α -positive protein samples, GlyRa2-positive staining of dissociated spinal cord neurons; eFigure 3A-B), and hence, binding of patient serum to other GlyRa subunits ($\alpha 2$ and $\alpha 3$) could be possible. However, cell-based analysis revealed no binding of Pat31 and Pat36 sera to GlyRa2 or $\alpha 3$ (eFigure 3C) confirming that binding of Pat31 and Pat36 aAbs is specific to GlyR β .

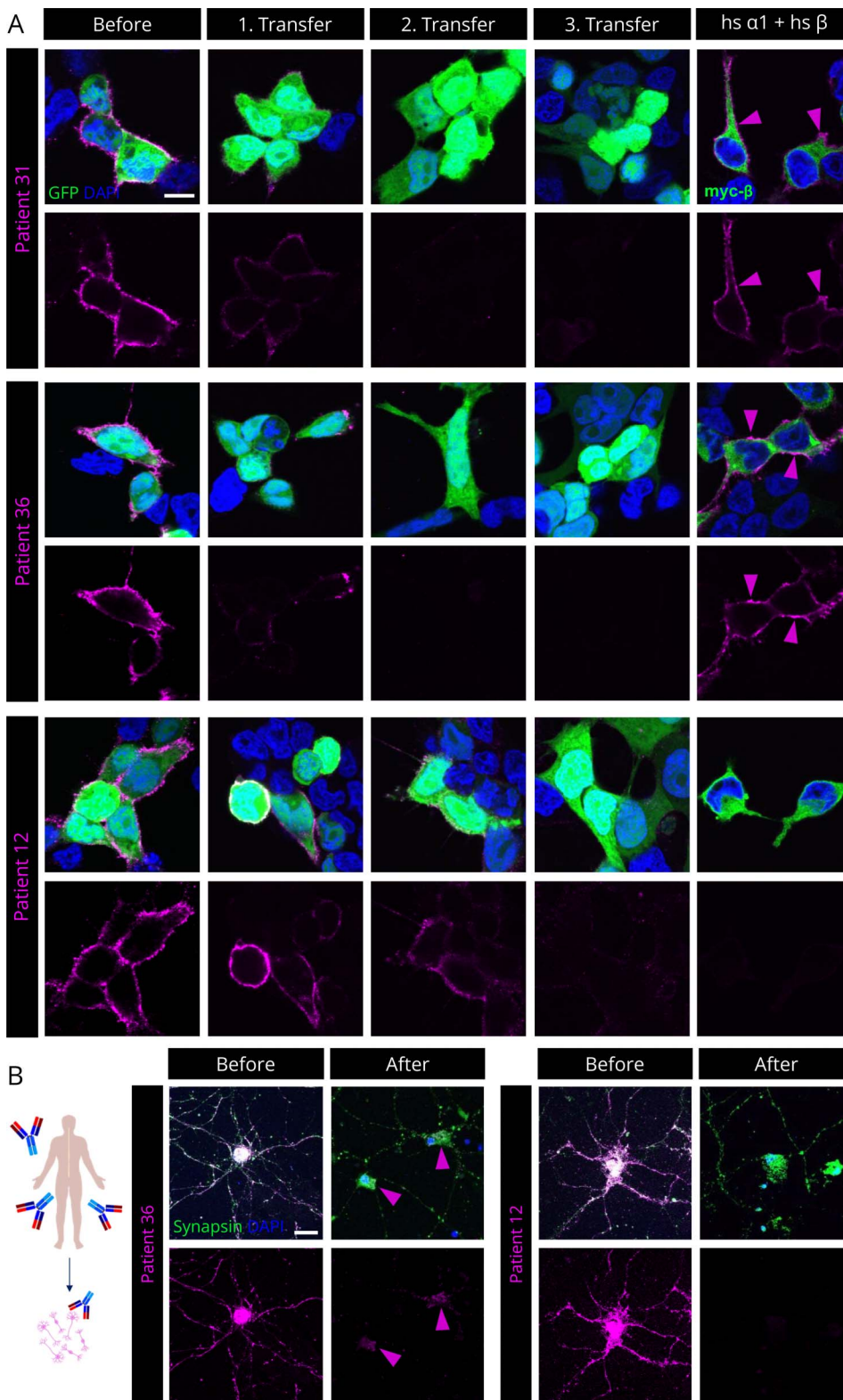
At last, binding of Pat36 serum to GlyR β was demonstrated by immunostaining of spinal cord sections of *Gla1*^{spdot/spdot}/*Glr*^{eos/eos} mice (Figure 5F). GlyR β signals in *Gla1*^{+/+}/*Glr*^{eos/eos} spinal cord were stronger than in *Gla1*^{spdot/spdot}/*Glr*^{eos/eos}. Similarly, serum staining was more intense to *Gla1*^{+/+}/*Glr*^{eos/eos} spinal cord while still present at *Gla1*^{spdot/spdot}/*Glr*^{eos/eos} dorsal and ventral horn spinal cord (Figure 5F, right images a₁, a₂, b₁, b₂). Our data clearly evaluated GlyR β targeting of aAb from some patients suggesting GlyR β as a new target for GlyR aAb in rare cases.

GlyR Ion Channel Function Is Altered Following Preincubation With GlyR β -Positive Patient Sera

To investigate physiologic consequences of GlyR β aAb targeting, ion channel function of GlyRs after preincubation with GlyR β -positive patient serum was tested to assess whether similar molecular alterations exist as demonstrated for aAb against GlyRa.⁵

Whole-cell patch-clamp measurements of mixed spinal cord neurons from *Gla1*^{spdot/spdot}/*Glr*^{eos/eos} and *Gla1*^{+/+}/*Glr*^{eos/eos} mice demonstrated almost absent glycine-induced I_{\max} for *Gla1*^{spdot/spdot}/*Glr*^{eos/eos} neurons lacking GlyRa1, while wild-type neurons showed large glycine-induced chloride currents (*Gla1*^{+/+}/*Glr*^{eos/eos}: 2.1 ± 0.3 nA; *Gla1*^{spdot/spdot}/*Glr*^{eos/eos}: 0.04 ± 0.01 nA, **** $p < 0.0001$) (Figure 6, A and B, eTable 1B, links.lww.com/NXI/A969). This excluded primary neurons from *Gla1*^{spdot/spdot}/*Glr*^{eos/eos} for investigation of the functional consequences of GlyR β aAb. To better discriminate between the effects of aAbs

Figure 4 Preadsorption of Patient Sera With GlyRa1 Offers Specific Detection of aAbs to GlyR β

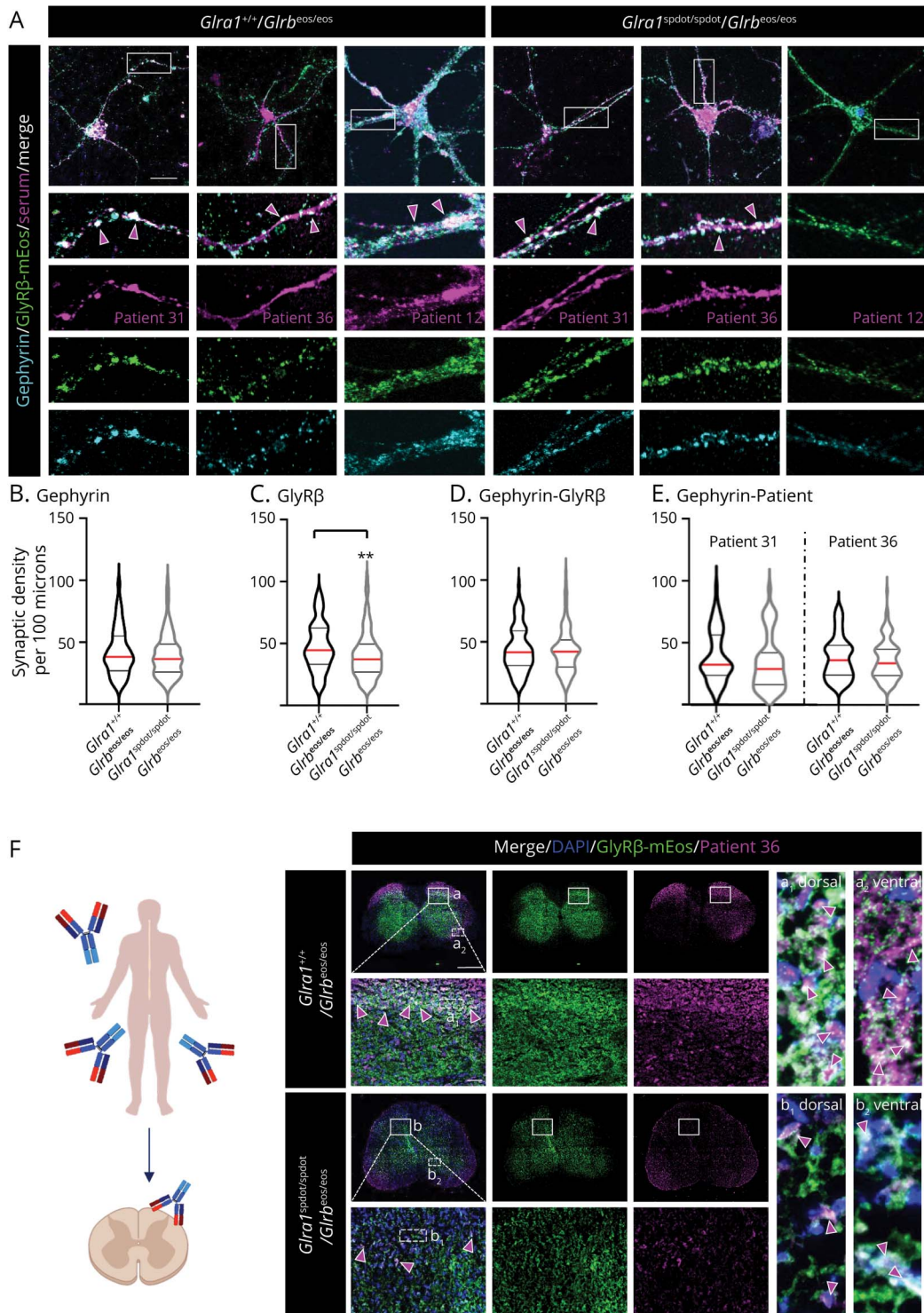


(A) Serum samples of Pat31, Pat36, and Pat12 were incubated on HEK-293 cells expressing the GlyRa1 subunit. After transfer of the supernatant for 3 times, cells cotransfected with zebrafish GlyRa1 and myc-tagged human GlyR β were stained. GlyR β is shown in green (right panel) and binding of patient serum in magenta. Arrows point to binding of patient serum to transfected HEK-293 cells after preadsorption. Note, while patient serum was added to living cells, the detection of the GlyR β subunit through the myc antibody required cell fixation and permeabilization, leading to also intracellular staining of GlyR β . Scale bar refers to 10 μ m. (B) aAb binding to primary neurons (left). Mixed spinal cord neuronal cultures were stained (right images) with patient serum before and after preadsorption of Pat36 serum by ELISA plates coated with GlyRa1 ECD. Synapsin (green) is used as synapse marker and serum binding is shown in magenta. Scale bar refers to 20 μ m.

against GlyRa1 and GlyR β , we turned back and transfected HEK-293 cells with either human GlyRa1^{hs} subunit alone, or coexpressed zebrafish GlyRa1^{dr} with human GlyR β . Similar to findings by Rauschenberger et al.,⁵ patient serum binding to

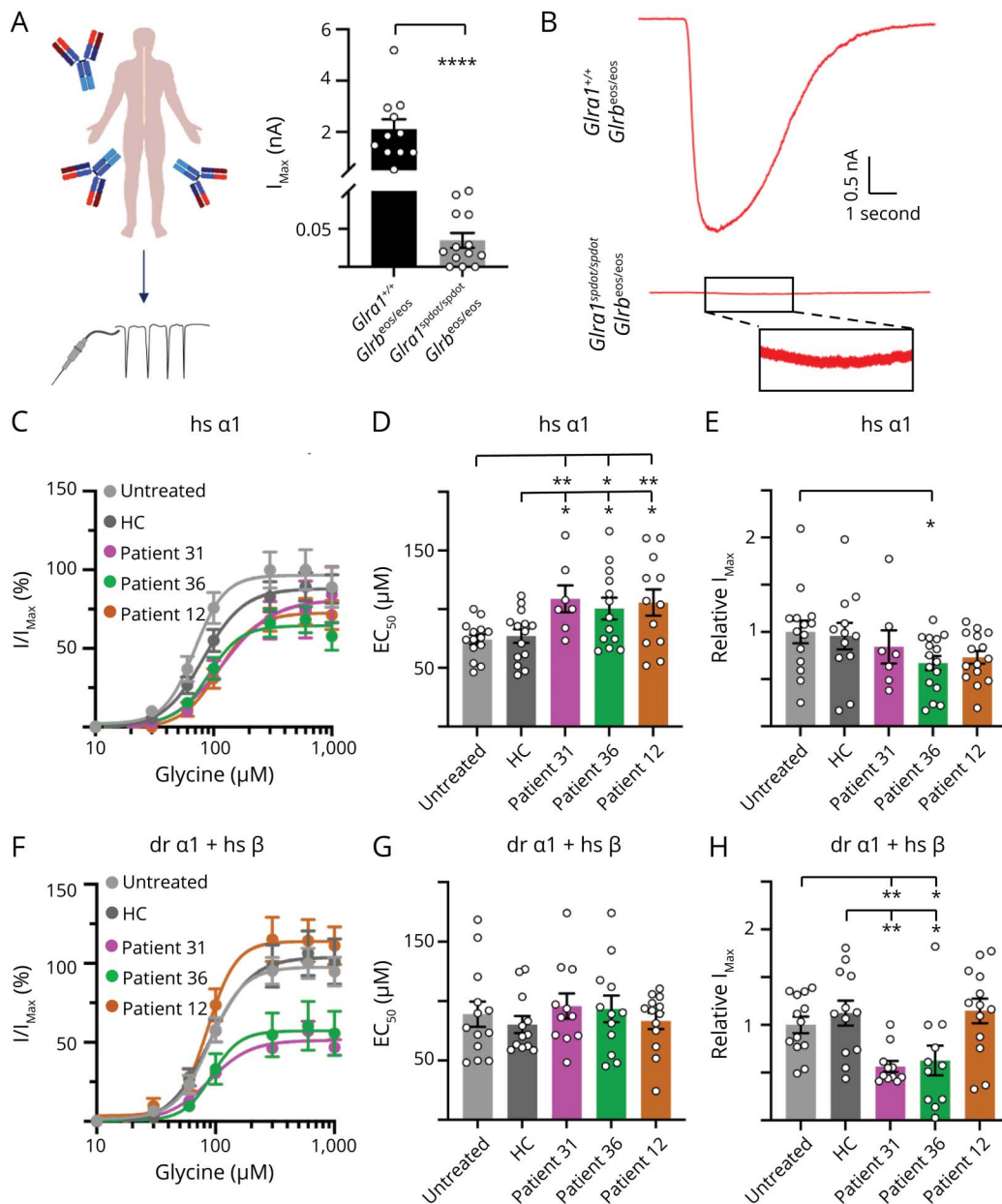
human GlyRa1 subunit led to a rightward shift in dose-response curves (untreated: 74 \pm 4 μ M; HC: 77 \pm 6 μ M; Pat31: 108 \pm 12 μ M, p = 0.003 compared with untreated and p = 0.01 compared with HC; Pat36: 100 \pm 9 μ M, p = 0.01

Figure 5 Patient Serum Binding to Neuronal GlyR β Subunits Confirms GlyR Beta-Specific Binding



(A) Immunocytochemical stainings with serum samples of Pat31, Pat36, and another patient serum exclusively binding to GlyR α 1 (Pat12) of mixed primary spinal cord neuronal cultures of *Glra1^{spdot/spdot}/Glrβ^{eos/eos}* and *Glra1^{+/+}/Glrβ^{eos/eos}* mice. *Glra1^{spdot/spdot}/Glrβ^{eos/eos}* and *Glra1^{+/+}/Glrβ^{eos/eos}* neurons were stained with antibodies against GlyR β mEos (green), human-IgG (magenta), and gephyrin (cyan). Arrows point to colocalizing GlyR β mEos and patient serum signals. Scale bars refer to 20 μ m and 5 μ m in magnification. (B-E) Quantification of synaptic density/100 microns in *Glra1^{spdot/spdot}/Glrβ^{eos/eos}* and *Glra1^{+/+}/Glrβ^{eos/eos}* neurons (n = 3). Data are shown in violin blots with a red line marking the median and black lines marking the quartiles. Levels of significance: ***p* < 0.01 (gephyrin: n.s. *p* = 0.35, n = 99 for *Glra1^{+/+}/Glrβ^{eos/eos}* and n = 123 for *Glra1^{spdot/spdot}/Glrβ^{eos/eos}*; GlyR β : ***p* = 0.004, n = 98 and n = 120; gephyrin-GlyR β : n.s. *p* = 0.86, n = 97 and n = 123; gephyrin-Pat31: n.s. *p* = 0.11, n = 70 and n = 56; gephyrin-Pat36: n.s. *p* = 0.43, n = 97 and n = 118). (F) Immunohistochemical stainings with Pat36 serum of *Glra1^{+/+}/Glrβ^{eos/eos}* and *Glra1^{spdot/spdot}/Glrβ^{eos/eos}* spinal cord slices with antibodies against GlyR β mEos (green) and human IgG (magenta). Arrows point to binding of patient serum to spinal cord slices colocalizing with GlyR β signal in the enlarged images (white rectangles in upper row; a and b). Scale bar refers to 500 μ m and 50 μ m in magnification. Further inlets (white dotted rectangles, a₁ dorsal, a₂ ventral, b₁ dorsal, b₂ ventral) are shown on the right with pink arrow heads pointing to colocalization between GlyR β and patient serum.

Figure 6 Electrophysiologic Characterization of GlyR α Ab Binding to the GlyR β Subunit



(A) Whole-cell patch-clamp measurements of glycine-induced maximal currents of *Glra1^{+/+}/Glrbeos/eos* and *Glra1^{spdot/spdot}/Glrbeos/eos* neurons. Data are shown in bar diagrams with mean \pm SEM and individual data points (*Glra1^{+/+}/Glrbeos/eos*; $n = 11$, *Glra1^{spdot/spdot}/Glrbeos/eos*; $n = 13$, **** $p < 0.0001$). (B) Exemplary maximal currents induced by 1 mM glycine of *Glra1^{+/+}/Glrbeos/eos* and *Glra1^{spdot/spdot}/Glrbeos/eos* neurons. (C) Dose-response curves of HEK-293 cells transfected with hsGlyRa1 to increasing glycine concentrations (10, 30, 60, 100, 300, 600, 1,000 μ M) either untreated or preincubated for 1 hour with Pat31, Pat36, Pat12, or healthy control (HC) serum. (D and E) Resulting EC_{50} values and maximal currents for HEK-293 cells transfected with human (hs) GlyRa1. Data are shown in bar diagrams with mean \pm SEM and individual data points (untreated: $n = 14$, Pat31: $n = 7$, Pat36: $n = 13$, Pat12: $n = 12$, HC: $n = 14$). (F) Dose-response curves of HEK-293 cells transfected with zebrafish (dr) GlyRa1 and hs GlyR β to increasing glycine concentrations (10, 30, 60, 100, 300, 600, 1,000 μ M) either untreated or preincubated for 1 hour with Pat31, Pat36, Pat12, or HC serum. (G–H) Resulting EC_{50} values and maximal currents for HEK-293 cells transfected with zebrafish (dr) GlyRa1 and hs GlyR β . Data are shown in bar diagrams with mean \pm SEM and individual data points (untreated: $n = 12$, Pat31: $n = 11$, Pat36: $n = 12$, Pat12: $n = 13$, HC: $n = 13$). Levels of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

compared with untreated and $p = 0.04$ compared with HC; Pat12: $106 \pm 11 \mu$ M, $p = 0.009$ compared with untreated and $p = 0.03$ compared with HC) and thus a reduced receptor potency (Figure 6, C and D). Binding of Pat36 serum but not Pat12 and Pat31 sera resulted in a slightly decreased maximal current (relative I_{max} to untreated: $100 \pm 12\%$; HC: $95 \pm 14\%$; Pat31: $84 \pm 18\%$; Pat36: $67 \pm 7\%$, $p = 0.02$ compared with untreated Pat12: $73 \pm 7\%$; Figure 6E, see also eTable 1C,

links.lww.com/NXI/A969). By contrast, binding of patient serum to GlyR β (zebrafish GlyRa1^{dr} with human GlyR β were transfected) did significantly decrease maximal currents after preincubation with both Pat31 ($56\% \pm 5.8\%$) and Pat36 ($52\% \pm 14.6\%$) sera compared with untreated ($100\% \pm 8.6\%$) and HC treated ($105\% \pm 13.8\%$) cells (Pat 31: ** $p = 0.001$ compared with untreated and ** $p = 0.002$ compared with HC; Pat 36: * $p = 0.02$ compared with untreated and * $p = 0.04$

compared with HC; Figure 6, F–H, see also eTable 1C) arguing for less glycine efficacy.

In sum, while binding of patient aAb to GlyR α subunits leads to reduced glycine potency, binding to GlyR β results in reduced maximal glycine-gated currents and hence reduced glycine efficacy. Therefore, GlyR aAbs targeting distinct GlyR subunits affect glycinergic function differently, thus arguing for a significant contribution of both GlyR α 1-specific and GlyR β -specific aAb to the disease pathology.

Discussion

GlyR aAbs are involved in the pathology of SPS and PERM. Binding of GlyR aAb mainly to GlyR α 1 but also to other α subunits has been described.⁴ Although the GlyR β subunit shares a high homology to GlyR α subunits in its ECD, no detection of GlyR β -specific aAb has been exhibited, yet. In this study, we found the GlyR β subunit as a novel target for GlyR aAb in 2 human patients experiencing SPS/PERM or focal epilepsy. Specificity for GlyR β was achieved using an N-terminal myc-tagged GlyR β variant coexpressed with zebrafish GlyR α 1 mainly not bound by patients with GlyR α 1-specific aAb.^{5,18} Among 58 patient sera investigated, 30 were positive for GlyR α 1 binding, 2 patients in addition targeted GlyR β specifically. Patient sera negative for GlyR α 1 binding did also not respond to GlyR β arguing most probably against an SPS/PERM phenotype with exclusively GlyR β aAb.

We confirmed specific binding of patient-derived GlyR β aAb at spinal cord neurons and tissue sections of a novel generated hybrid mouse line expressing an mEos-tagged GlyR β but lacked GlyR α 1 (*oscillator*).^{14,37} The signal obtained by the patient serum with GlyR β aAb colocalized with gephyrin, a direct interaction partner of GlyR β at synaptic sites. A 1:1 ratio of GlyR β to gephyrin at synapses has been demonstrated; however, the overall GlyR β amount in the hybrid line lacking GlyR α 1 is significantly lower.¹⁴ Other GlyR α subunits (α 2 or α 3) are possibly able to compensate for lack of GlyR α 1 at synaptic sites. Our patients with GlyR β aAb did, however, not bind mouse GlyR α 2 or α 3, confirming the specificity of patient aAb to GlyR β in spinal cord tissue.

Patients experiencing SPS/PERM show similar symptoms to patients with startle disease. Startle disease is due to variants in genes affecting glycinergic inhibition (*GLRA1* encoding the GlyR α 1 subunit, *SLC6A5* (GlyT2) and *GLRB* (GlyR β)).³⁸ *GLRB* variants decrease synaptic localization of heteromeric GlyRs or impair receptor function.^{16,18} Moreover, single-nucleotide variations in *GLRB* have been shown in humans with enhanced agoraphobic behavior.^{34,35} Enhanced anxiety has also been reported for patients with startle disease being afraid for unexpected acoustic stimuli.^{39,40} Our patients underwent questionnaires for anxiety, ASI, enhanced sensitivity to different stimuli, and pain. Patients with SPS exhibited a higher sensitivity score for noise, visual, somatosensory, and

emotional excitement in line with typical SPS symptoms. The patient with focal epilepsy exhibited moderate to severe anxiety. Several reports exhibited mRNA and protein expression of GlyR α and β in thalamic and midbrain areas, brain regions involved in anxiety circuits.^{14,41,42}

For GlyR α 1, a common N-terminal epitope has been determined by a chimeric approach making use of nonbinding to the zebrafish GlyR α 1 but to the human α 1.⁵ Although with limitations, another option to fine-map aAb binding sites is the use of peptide microarrays.^{27,36} Depending on the protein region targeted by the aAb, discontinuous or continuous epitopes have to be evaluated. Discontinuous epitopes require chemical approaches and/or experimental mimicking or computer-based predictions and are used if highly ordered structures are targeted, while continuous epitopes are usually observed when autoantibodies bind to disordered regions. Using a continuous overlapping peptide library, distinct GlyR β autoantibody binding sequences ⁷⁷GIPVDVVVNI-FINSE⁹¹ and ¹⁴¹PDLFFANEKSANFHDV¹⁵⁶ were identified. The determined binding sequence in the patient with focal epilepsy overlaps only partially between GlyR α and GlyR β . The second epitope ¹⁴¹P-¹⁵⁶V identified in patients with SPS/PERM is localized close to and overlapping with the binding site of mAb4a, a widely used commercial antibody that binds all GlyR α subunits and to some extent GlyR β . The observed pan reactivity is therefore mediated by an autoantibody that recognizes a conserved motif shared between subunits. The cell-based preadsorption experiment, however, indicates that there is an additional exclusive GlyR β -specific epitope. Whether the effect on efficacy is mediated by aAb binding to the mapped epitope or an epitope that could not be resolved in the array-based mapping analysis requires further studies, e.g., chimeric approaches. Binding of GlyR α 1-specific aAb from the same patient serum might also be enabled through other immunogenic regions as previously determined.⁵

Because it was recently shown that GlyRs assemble as 4 α :1 β , binding of an aAb against GlyR β most likely leads to cross-linking of 2 receptors leading subsequently to receptor internalization.^{8,9} Internalization of aAb-targeted GlyRs may thus underlie the observed reduced receptor efficacy. By contrast, functional alterations following aAb binding to exclusively the GlyR α subunit showed decreased receptor potency in line with previous observations and most probably due to conformational blocking.⁵ In addition, Crisp et al.⁶ demonstrated disrupted glycinergic neurotransmission in recordings from spinal cord neurons preincubated with patient sera and with Fab fragments suggesting that the functional impairment does not require cross-linking of receptors. Hence, our data provide evidence that patients harboring aAb against GlyR α 1 and β experience pronounced impairment of glycinergic inhibition by affected glycine efficacy and potency.

In this study, we show that GlyR aAb not only target GlyR α subunits but also in some cases GlyR β . With this novel contribution to the SPS/PERM disease pathology, we extend the

current knowledge of the molecular mechanism by substantially decreased inhibition at glycinergic synapses due to reduced glycine efficacy and potency. Whether the identified pathomechanism act in an additive manner or independent still needs to be verified. A detailed binding pattern investigation of similarities and differences in the aAb repertoire of patients will help to identify personalized aAb profiles and thus offer novel treatment options.

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Disclosure

The authors report no disclosures relevant to the manuscript. Go to [Neurology.org/NN](https://www.neurology.org/NN) for full disclosures.

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Inken Piro, MSc	Department of Neurology, University Hospital Wuerzburg, Germany	Major role in the acquisition of data; analysis or interpretation of data
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Appendix (continued)

Name	Location	Contribution
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