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Anti-VGLUT2 autoantibodies in neurological diseases

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ABSTRACT

Autoantibodies are important biomarkers for the diagnosis of autoimmune diseases that help to determine treatment strategies and to understand disease pathology. Despite the increasing numbers of neuronal autoantibody discoveries, there are still patients presenting with neurological autoimmune diseases and so far uncharacterized autoantibodies.

Between 12/2016 and 06/2024, we collected sera of 314 patients with a distinct uncharacterized IgG pattern in neuronal tissue indirect immunofluorescence assay (IIFA). By immunoprecipitation and mass spectrometry we identified vesicular glutamate transporter 2 (VGLUT2) as the autoantibody target and confirmed it in sera of 285/314 patients by recombinant IIFA with VGLUT2-expressing HEK293 cells, competitive inhibition assays and colocalization studies with a commercial antibody. The main diagnoses available of 87/285 patients (mean age 58, range 1–92) were encephalitis 25/87, dementia/cognitive impairment 17/87 and polyneuropathy 16/87. Detailed clinical data of 18 patients were collected retrospectively. The major symptoms of those index patients (mean age 56, range 2–78) involved cognitive changes (10/18) including memory impairment, aphasia and disorientation as well as sensorimotor disturbances (9/18) and gait abnormalities (9/18), accompanied by visual impairments (8/18). (Poly)neuropathy was observed in 10/18 cases. The majority of the anti-VGLUT2 index

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patients had coexisting diabetes mellitus type 2 or other chronic multisystem disorders. In 8/12 index patients, immunotherapy had beneficial effects, although only slight improvements could be obtained in most of the cases. All 17 analyzed index patient sera recognized a cytoplasmic epitope between amino acid 520–564 of VGLUT2, determined by immunoblot with recombinant antigen fragments.

Anti-VGLUT2 autoantibody-associated neurological diseases may represent a new type of autoimmune disorders that might benefit from immunomodulatory treatment and predominantly manifests with encephalitis, cognitive deficits and neuropathy.

1. Introduction

Autoimmune neurological syndromes can affect both the central and peripheral nervous system (Bhagavati, 2021). For both manifestations, it is assumed that cellular and autoantibody-mediated immune responses are involved in their pathogenesis. For central nervous system autoimmune diseases, an increasing number of autoantigens has been described, which can be localized either inside the cell or on the cell surface (Pruss, 2021). According to the associated autoantibody, the involved brain or spinal cord regions and the presence of a coexisting tumor, different syndromes were defined, including limbic encephalitis, cerebellar and rhombencephalitis, myelitis or demyelinating diseases. In line with the binding locations of those autoantibodies, affected patients can suffer from a wide range of symptoms, including cognitive impairments, psychiatric symptoms, epileptic seizures, visual impairments, speech or movement dysfunction.

Immune-mediated neuropathies, such as Guillain-Barré syndrome, chronic inflammatory demyelinating polyradiculoneuropathy, autoimmune nodopathies and multifocal motor neuropathy or paraproteinemic neuropathy are caused by peripheral nerve injury and characterized by progressive muscle weakness and/or sensory impairments (Shastri et al., 2023). Here, only a few disease-defining autoantibodies are well characterized, e.g., anti-ganglioside, anti-CNTN/CASPR1, anti-NF155, anti-NF186 or anti-MAG (Gupta et al., 2023; Stino et al., 2023; Wanleenuwat et al., 2020). In these cases, target antigens are mainly expressed at the node of Ranvier or in myelinating cells.

Autoimmune diseases affecting both the central and peripheral nervous system have been previously reported to lead to an overlapping spectrum of clinical symptoms. This is observed, e.g., in Miller-Fisher syndrome, a variant of Guillain-Barré syndrome, Bickerstaff brainstem encephalitis, combined central and peripheral demyelination or anti-Hu syndrome (Cortese et al., 2016; Dalmau et al., 1992; Kamm and Zettl, 2012; Leboyan et al., 2023). These conditions might develop when target antigens are expressed by central and peripheral nerve cells and the access of autoantibodies or T-cells to their targets is not inhibited by natural boundaries, such as the blood–brain-barrier, the blood-nerve-barrier or the myelin sheath. Anti-Hu, anti-CASPR2, anti-CRMP5, anti-amphiphysin or anti-NF155 autoantibodies represent some of the few examples of autoantibodies associated with peripheral and central nervous system disorders (Bhagavati, 2021; Kira, 2021).

Here, we describe the identification of vesicular glutamate transporter 2 (VGLUT2) as a novel autoantibody target in patients with neurological disorders with potential or confirmed autoimmune etiology affecting the central and/or peripheral nervous system. We report the detailed clinical and paraclinical features of 18 anti-VGLUT2 positive patients and the recognized autoantibody epitope of 17 anti-VGLUT2 positive patients.

2. Material and methods

2.1. Study design

A German reference laboratory for immunological analyses (Clinical Immunological Laboratory Prof. h.c. (RCH) Dr. med. W. Stöcker, Groß Grönau, Germany), receiving sera for the purpose of neural autoantibody testing at physicians' discretion, collected between 12/2016 and 06/2024 all sera which showed a distinct unclassified staining pattern on neuronal tissue IIFA and did not react with any of 30 different known neuronal antigens. All those sera of 314 patients were anonymized and provided to the authors for antigen identification. With one of those sera, with a particular strong reactivity in neuronal tissue IIFA (index serum 1, IS1, IgG tissue IIFA titer 1:3200), antigen identification was performed with immunoprecipitation and mass spectrometry. Subsequently, all 314 patients' sera were analyzed by recombinant IIFA with VGLUT2-expressing HEK293 cells. Anti-VGLUT2 autoantibodies could be detected in sera of 285/314 patients. The main diagnoses were available for 87/285 anti-VGLUT2 IgG positive patients. Detailed clinical and paraclinical data could be collected retrospectively by the participating centers for 18 patients [index patients, P1-18, mean age 56 (range 2–78), 61 % female]. One reason for the relatively low number of 18 well characterized index patients might be the fact that anti-VGLUT2 autoantibodies were identified in many cases retrospectively and that the patients had already left the hospital before the treating physicians were asked for detailed clinical data and a written informed consent of the patient. A second reason might be the circumstance that several samples were sent to the reference laboratory by other diagnostic labs which usually do not have access to detailed clinical information.

As control collectives, anonymized sera of 100 presumably healthy blood donors and 100 patients with chronic arthritis (ICD-10 codes: M05.30, M05.80, M05.90, M05.99, mean age 60, range 26–89, 61 % female) were analyzed in tissue IIFA and recombinant IIFA with VGLUT2-expressing HEK293 cells.

2.2. Study approval

All human samples and clinical investigations were done according to the Declaration of Helsinki principles. All patients whose detailed clinical data are reported in this study gave written informed consent to the publication. An approval from the Universitätsklinikum Düsseldorf, Charité – Universitätsmedizin Berlin and Hannover Medical School ethics boards was obtained (2021–1498, EA1/258/18 and 5582).

2.3. Indirect immunofluorescence assay (IIFA) with brain tissue and recombinant cells

IIFA was performed using slides with biochip mosaics of brain tissue cryosections (cerebellum of rat and monkey, hippocampus and adjacent thalamus of rat, whole brain of mouse) in addition to recombinant HEK293 cells separately expressing 30 different neuronal antigens [Hu, Yo, Ri, CV2, PNMA2, ITPR1, Homer 3, CARPVIII, ARHGAP26, ZIC4, DNER/Tr, GAD65, amphiphysin, recoverin, GABAB and GABAA receptor, glycine receptor, DPPX, IgLON5, glutamate receptors (types NMDA, AMPA, mGluR1, mGluR5, GLURD2), LGI1, CASPR2, AQP4, MOG and flotillin 1/2 (cut off 1:10), as well as Yo, CV2, ITPR1, Homer 3 and NCDN (cut off 1:100)] as previously described (Miske et al., 2023). Additionally, IIFA was performed using recombinant acetone-fixed HEK293 cells expressing VGLUT2 and empty vector-transfected HEK293 cells as control substrate (cut-off of 1:100).

IgG subclass determination was performed on all sera with sufficient material available (P1-11 and 13–18) and IgG subclass-specific FITC-labelled mouse anti-human IgG (Sigma-Aldrich F0767, F4516, F4641, F9890, final dilution of 1:25).

For colocalization experiments, biochips were incubated with anti-VGLUT2 mouse monoclonal antibody (1:200, Merck, AMAB91081) or anti-His (1:200, Merck, 70796–3) and Cy™3 AffiniPure™ goat anti-mouse IgG (H + L) (1:200, Jackson ImmunoResearch, 115-165-003) in addition to patient sera and Alexa Fluor® 488 AffiniPure™ goat anti-human IgG (1:500, ImmunoResearch, 109-545-008).

In competitive inhibition experiments, sera diluted 1:320 in PBS-Tween were pre-incubated for 1 h with lysate of recombinant HEK293 cells expressing VGLUT2 or, as control, empty vector transfected HEK293 cell lysate diluted 1:10 in PBS-Tween, before they were incubated in an IIFA on neuronal tissue sections. In addition, competitive inhibition experiments were performed with sera diluted 1:100 in PBS-Tween pre-incubated with lysates of recombinant E. coli cells expressing His-GST-VGLUT2 aa 520–564 or control buffer.

2.4. Indirect immunofluorescence assay (IIFA) with dorsal root ganglia

Dorsal root ganglia were freshly prepared from wildtype black six mice, fixated in 4 % PFA in PBS for 30 min on ice, frozen embedded in tissue-protection solution, cut into 8 µm thick cryosections and stained according to established protocols (Arlt et al., 2024). In brief, slides were thawed at room temperature, washed once with PBS, permeabilized with 0,25 % Triton X100 in PBS for 10 min, blocked with blocking solution (5 % normal goat serum, 2 % bovine serum albumin, 0,1% Triton X100 in PBS) for 30 min followed by overnight incubation of serum (final dilution 1:400) at 4 °C in blocking solution. After washing with PBS, secondary antibodies were applied (Alexa 488-labelled goat anti-human IgG, Dianova, 109–545–003, final concentration 1,5 µg/ml) for 2 h at room temperature. Costainings with a commercial anti-VGLUT2 antibody (SySy, 135403, final concentration 1 µg/ml) and Alexa 594-labelled anti-rabbit IgG (Jackson ImmunoResearch, 111-585-003, final concentration 3 µg/ml) were done sequentially to minimize cross-reactivity. Finally, slides were washed, cell nuclei were labeled by PBS-containing DAPI (final concentration 2 µg/ml) and mounted with Immu-Mount™. Slides were imaged on a widefield microscope. Images were taken as tile scans using NIH ImageJ 1.52i software.

2.5. Immunoprecipitation

The immunoprecipitation with 200 µl homogenate of rat brain and 30 µl serum in solubilization buffer (100 mmol/L tris-HCl pH 7.4, 150 mmol/L sodium chloride, 2.5 mmol/L EDTA, 0.5 % (w/v) deoxycholate, 1 % (w/v) Triton X-100 containing protease inhibitors) was performed as previously described (Miske et al., 2024). Immunoprecipitated proteins were separated by SDS-PAGE, visualized with Coomassie Brillant Blue (G-250) (Merck) gel staining and identified by mass spectrometric analysis as previously described (Miske et al., 2024).

2.6. Recombinant expression of full length VGLUT2 in HEK293

A cDNA clone coding for full length of human VGLUT2 was not available. Therefore, two synthetic gene fragments coding for amino acids 1–293 and 294–582 of human VGLUT2 were obtained from Eurofins Genomics Germany GmbH and digested with BsaI (New England Biolabs, R3733) resulting in 877 bp fragment-1 and 873 bp fragment-2 that were both gel-purified. By PCR a third fragment (VGLUT2 aa 294–582 without the stop codon) was amplified using the gene synthesis product VGLUT2 aa 294–582 as template and the DNA oligonucleotide primers VGLUT2 aa 294–582 sense and VGLUT2 aa 294–582 antisense (for sequences see Supplementary Table 1). The amplification product was digested with BsaI (New England Biolabs, R3733). The fragments VGLUT2 aa 1–293 and VGLUT2 aa 294–582 or VGLUT2 aa 1–293 and VGLUT2 aa 294–582 without stop codon were ligated with NcoI and XhoI linearized pTriEx-1 vector (Merck) resulting in VGLUT2 without a Histidin-tag (dHis) or VGLUT2 fused to a C-terminal octa Histidin-tag (H8).

VGLUT2 dHis or VGLUT2-His were expressed in HEK293 cells after Polyethylenimine-mediated transfection (Polysciences, 23966) according to the manufacturer's instructions. In order to prepare substrates for IIFA, HEK293 cells were seeded on sterile cover glasses, transfected and allowed to express VGLUT2 for 48 h, followed by acetone fixation for 10 min at room temperature. Alternatively, cells were transfected in standard T-flasks and harvested after 5 days. The cell sediment was extracted with solubilization buffer for further use in competitive inhibition experiments.

2.7. Recombinant expression of His-VGLUT2 fragments in E. coli

For epitope characterization, different fragments of human VGLUT2 (aa 1–71, 93–125, 266–310, 499–582, 520–582, 543–582, 565–582, 499–542, 499–564, 520–564) were amplified by PCR (for primer sequences see Supplementary Table 2).

The amplification products were digested with Esp3I (New England Biolabs, R0734) and ligated with NcoI and XhoI linearized and modified pET24d vector (Merck). The pET24d vector includes the sequence of an N-terminal octa histidine-tag (H8), a GST-tag and the cleavage site of the HRV-3C protease cleavage enzyme.

2.8. Immunoblot and VGLUT2 epitope mapping

E. coli expressing His-GST-VGLUT2 fragments aa 1–71, 93–125, 266–310, 499–582, 520–582, 543–582, 565–582, 499–542, 499–564, 520–564 were incubated with NuPAGE LDS sample buffer (ThermoFisher Scientific) containing 25 mmol/L dithiothreitol at 70 °C for 10 min. E. coli lysates were separated by SDS-PAGE (NuPAGE, ThermoFisher Scientific) and subsequently electrotransfered onto a nitrocellulose membrane according to the manufacturer's instructions (ThermoFisher Scientific). The membranes were blocked with 1:5 diluted sample buffer (EUROIMMUN Medizinische Labordiagnostika AG) for 15 min and incubated with the patient sera (dilution 1:200) or anti-His mouse monoclonal antibody (Merck, Germany, 1:2000), for 3 h, followed by a second incubation for 30 min with anti-human-IgG-Alkaline-Phosphatase (AP) (1:10, EUROIMMUN Medizinische Labordiagnostika AG) or anti-mouse-IgG-AP (Jackson ImmunoResearch, 1:2000) and staining with NBT/BCIP substrate (EUROIMMUN Medizinische Labordiagnostika AG).

3. Results

3.1. Identification of VGLUT2 as autoantibody target

Between 12/2016 and 06/2024, we collected sera of 314 patients, received by a reference laboratory for the purpose of autoantibody testing, showing a distinct pattern in neuronal tissue IIFA, characterized by a spotty staining of the granular layer and interrupted striped structures in the molecular layer of rat and monkey cerebellum (IgG titer 1:100–1:3.200) (Fig. 1A, B, E) as well as a weak fine granular fluorescence near the granular layer on hippocampal tissue sections (Fig. 1C, F). Additionally, a speckled reactivity of the adjacent thalamus was observed (Fig. 1D). IIFA with whole brain sections of mouse displayed a fine granular reactivity with further areas of the brain including midbrain, medulla, pons, hypothalamus, striatum and the cerebral cortex (Fig. 1G). All collected samples revealed no positive reactions in monospecific analyses with recombinant HEK293 cells expressing 30 neural autoantigens. To identify the target antigen, immunoprecipitation experiments with rat cerebellum followed by ESI mass spectrometry analysis were performed with one of the unclassified sera (index serum 1, IS1) and a control serum (CS). Vesicular glutamate transporter 2 (VGLUT2, UNIPROT acc. #Q9JI12) was identified in the eluate of IS1 but was absent in the control serum precipitate (Fig. 2A).

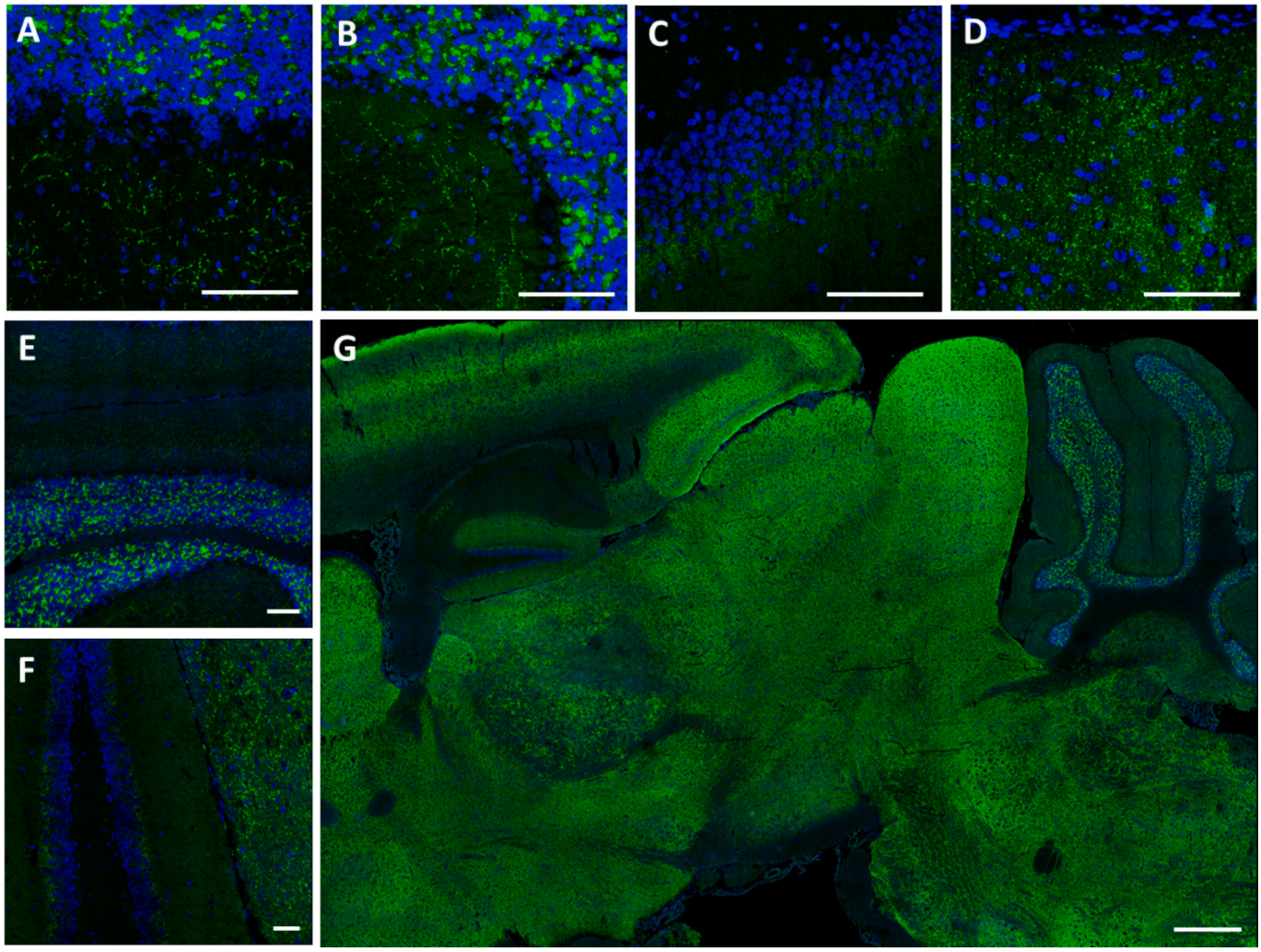

Fig. 1. Reactivity of anti-VGLUT2 index serum in indirect immunofluorescence assays with different neuronal tissues. Cryosections of cerebellum rat (A), cerebellum primate (B), hippocampus rat (C), thalamus rat (D), cerebellum mouse (E), hippocampus mouse (F) and whole brain mouse (G) were incubated with index serum 1 (IS1) in the first step, and with Alexa488-labelled goat anti-human IgG in the second step. Nuclei were counterstained by incubation with TO-PRO-3 iodide or DAPI. A spotty staining of the granular layer and interrupted striped structures in the molecular layer were observed. Hippocampal tissue sections showed a weak fine granular fluorescence of the inner molecular layer as well as a speckled reactivity of the adjacent thalamus. On whole brain mouse sections, additional areas of the brain including midbrain, medulla, pons, hypothalamus, striatum and the cerebral cortex were stained. Scale bars A-D: 100 μm, E: 100 μm, F: 50 μm, G: 500 μm.

3.2. Verification of VGLUT2 as target antigen

Correct antigen identification was confirmed with colocalization experiments in which an anti-VGLUT2 mouse monoclonal antibody showed an identical staining pattern on cerebellar tissue sections as IS1 (Fig. 2B). Moreover, the reaction of the IS1 on tissue could be abolished by pre-incubation with HEK293 lysate containing VGLUT2 (Fig. 2C). In recombinant IIFA with VGLUT2-expressing HEK293 cells (Fig. 2D), the index serum reacted with VGLUT2 (IgG titer 1:3200), but not with mock-transfected HEK293 cells. Remarkably, sera of 285/314 patients (91 %), with a similar reactivity in neuronal tissue IIFA as IS1, revealed positive reactions in IIFA with HEK293-VGLUT2 cells [IgG titers 1:100–1:10.000, mean age 58 (range 1–92), 52 % female], whereas only 1/100 sera from healthy donors showed a clumpy reactivity with HEK293-VGLUT2 cells above the cut-off of 1:100, but was negative in tissue IIFA (Supplementary Fig. 1). In addition, sera of patients with a non-neurological autoimmune pathology were analyzed (n = 100, diagnosed with rheumatoid arthritis, mean age 60, range 26–89, 61 % female). None of them reacted positive in anti-VGLUT2 tissue and recombinant IIFA with VGLUT2-expressing HEK293 cells.

A comparison of the frequency of anti-VGLUT2 autoantibodies with the frequency of established neuronal autoantibodies detected in the reference laboratory in 2023 revealed that anti-VGLUT2 positive sera were identified at approximately the same frequency as anti-LGI1 positive sera (Supplementary Table 3).

3.3. Anti-VGLUT2 positive patients present mainly with cognitive impairment, gait abnormalities and/or sensorimotor polyneuropathy

Of 87/285 (31 %) anti-VGLUT2 tissue and RC-IIFA positive patients the initial suspected diagnoses were available, with encephalitis (29 %), dementia or cognitive impairment (20 %) and polyneuropathy (18 %) being the most frequently ones, followed by epilepsy (10 %), cerebellar syndrome or movement disorders (7 %) and (tumefactive) multiple sclerosis (5 %) (for all initial diagnoses refer to Supplementary Table 4). Detailed clinical and paraclinical data could be retrospectively collected for 18/285 patients [index patients: mean age 56 (range 2–78), 61 % female, for summary refer to Table 1 and Supplementary Table 5]. Anti-VGLUT2 RC-IIFA IgG serum titers of the index patients were 1:320–1:3200 (all analyzed sera subclass IgG1). Most patients presented with cognitive impairments (10/18) including memory impairment, aphasia, impairment of word fluency, psychomotor retardation and

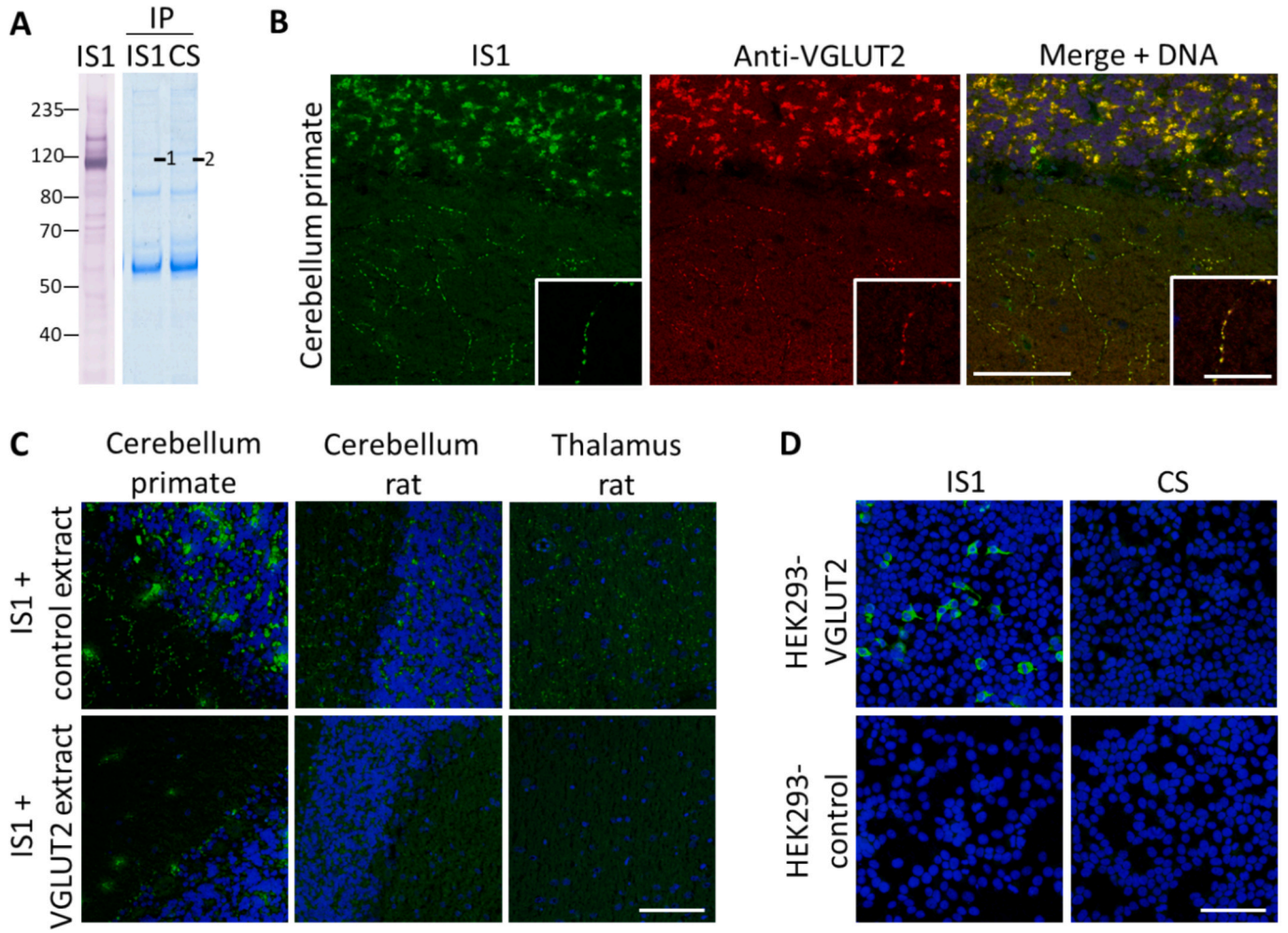

Fig. 2. Identification and verification of VGLUT2 as target autoantigen. A: Left panel: Immunoblot with cerebellar lysate incubated with index serum 1 (IS1, 1:200). Right panel: Coomassie-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis of immunoprecipitation (IP) eluates of IS1 or serum of a healthy control (CS) with homogenized rat cerebellum. Because IS1 showed a band at ~120 kDa in the cerebellar lysate immunoblot, bands at the size of ~120 kDa were selected for liquid chromatography/mass spectrometry (band 1 and 2), although, the Coomassie-stained gel did not show a visible band at this size. Mass spectrometry identified VGLUT2 in band 1 but not in band 2. B: Colocalization of IS1 and anti-VGLUT2 mouse monoclonal antibody on cerebellum with anti-human-IgG-Alexa488 and anti-mouse-Cy3 as secondary antibodies. A colocalization of the blotchy staining of the cerebellar granular layer and of the dotted and striped-like structures in the molecular layer was observed. C: Neutralization of autoantibody reaction on rat cerebellum with recombinant VGLUT2. IS1 was pre-incubated with extracts of HEK293 cells transfected with empty control vector or with plasmids harboring VGLUT2 cDNA in the first step, and with Alexa488-labelled goat anti-human IgG in the second step. The extract containing VGLUT2 abolished the immune reaction of IS1. D: Indirect immunofluorescence test using acetone-fixed VGLUT2 dHis or mock-transfected HEK293 cells incubated with IS1 or the serum of a healthy control (CS) and anti-human IgG-Alexa488. Nuclei were counterstained by incubation with TO-PRO-3 iodide (B-D). Scale bar B-D: 100 μm, Inserts B: 20 μm.

disorientation, followed by unsteady gait or gait ataxia (9/18) and visual dysfunction (8/18) with visual loss and double vision as well as oculomotor dysfunctions such as saccadic pursuit or nystagmus. Further symptoms involved headache (6/18), epileptic seizure/syncope (n = 2/18), psychiatric symptoms [n = 9/18, with depressive symptoms (7/18), delirium (4/18), hallucinations (2/18)], paresthesia/pain (5/18), paresis (6/18), hypesthesia (4/18), tremor (3/18), dysarthria (n = 2/18) and vertigo (4/18). The main clinical (suspected) diagnoses of the 18 index patients were heterogeneous and ranged from neurodegenerative disorders like Alzheimer's dementia and primary progressive aphasia to primary inflammatory diseases like limbic encephalitis, atypical Miller-Fisher syndrome, cerebellar syndrome, myelitis or cerebral vasculitis (Table 1).

A sensorimotor polyneuropathy was a concomitant or preexisting illness in 10/18 patients. Sensory and motor nerve conduction studies were available for 6/18 patients and revealed axonal damage (n = 4) as well as demyelinating features (n = 4). Further, chronic neurogenic changes were present in electromyographies in 4/5 examined patients. Partial or complete extinction of muscle reflexes of the lower extremities was documented in seven cases. In four patients the sensorimotor polyneuropathy was accompanied by damage of the optical nerve. For detailed descriptions of electrophysiological examinations refer to Supplementary Table 6.

As the electrophysiological data pointed to the possibility that also peripheral nervous system tissue was affected in anti-VGLUT2 positive patients, and VGLUT2 is known to be expressed in dorsal root ganglia (Scherrer et al., 2010), we analyzed whether patient sera bind to these structures. Interestingly, we observed a cytoplasmic staining on the soma of dorsal root ganglia neurons upon patient sera incubation, confirmed by a commercial anti-VGLUT2 antibody (Fig. 3). A control serum showed no reactivity against dorsal root ganglia neurons.

3.4. Brain MRI/CT findings

Brain MRI/CT was carried out for 17/18 index patients, and showed one or more of the following abnormalities in 14/17 cases: medullary,

Table 1
Clinical and paraclinical data of anti-VGLUT2 IgG positive patients.

patient	sex, age*	main diagnosis	symptoms*	previous/additional neurological illnesses*	previous/additional non-neurological illness*	tumor/ hemato-oncological diseases	MRI/CT	EEG	immunotherapy (response)	follow up*
1	M, 67	paraneoplastic encephalopathy	progressive psychomotor retardation, nestling, hallucinations, disorientation, delirium, unsteady gait, saccadic pursuit		diabetes mellitus type 2, atrial fibrillation, arterial hypertension, gastritis	lung carcinoma, monoclonal gammopathy suspected	normal	generalized slowing	4 cycles of plasmapheresis (*no effect*)	
2	M, 58	dementia with hippocampus sclerosis and previous HSV-encephalitis	memory impairment, disorientation, impairment of word fluency	delusions and hallucinations (*4 years ago, complete remission after therapy*), HSV-encephalitis after immunosuppressive therapy (*2 years ago*), critical-illness-polyneuropathy after immunosuppressive therapy (*2 years ago*)	ANCA-positive granulomatosis with polyangiitis and glomerulonephritis with suspected pulmonary involvement (*2 years ago*), iron overload caused by hemochromatosis (*current state*)	no tumor search	brain MRI: older hippocampus sclerosis, temporobasal parenchymal defects with flanking T2w hyperintensities including the lobus insularis, periventricular T2w hyperintensities of the white matter (microangiopathic etiology suspected)	generalized slowing	currently no immunosuppressive therapy	
3	M, 42	sensorimotor polyneuropathy with bilateral optic neuropathy	double vision and headache (*for 1 h, acute onset*), paresis right leg (*for 2 years*), hyp- and paresthesia fingers and fatigue (*for 1 year*)	visual impairments (*6 month ago*), chronic degenerative lumbar spine syndrome	hypercholesterolemia	no tumor search	periventricular and subcortical lesions	not conducted	steroid pulse therapy for 3 days (*subjective improvement*)	
4	F, 78	transitory cerebral ischemia with aphasia, demyelinating polyneuropathy	global aphasia (*acute onset*), paraphasias, disorientation	axonal demyelinating polyneuropathy, depression	diabetes mellitus type 2, arterial hypertension, hypercholesterolemia, hyperuricemia	not detected pleural effusions unknown etiology	brain MRI/CT: no correlate for ischemia, parietal cavernoma or microbleed, pronounced microangiopathic changes	normal	spontaneous remission of aphasia without immunosuppressive therapy	patient died after fall on intra-cerebral hemorrhage (*after 5 years*)
5	F, 55	bilateral optic neuropathy with severe visual impairment, axonal polyneuropathy	visual impairment on both sites with blurred vision and color saturation right and vision loss left (*for 9 months, acute onset*), unsteady gait with progressive weak foot dorsiflexion on both sides, paresthesias, hypesthesia of the toes, motor fatigue-syndrome, cognitive impairment	spinal canal stenosis L3/L4, L5-syndrome right, migraine (*for 30 years*)	diabetes mellitus type 2, chronic renal insufficiency, hyperuricemia, hypercholesterolemia	adrenal adenoma	brain MRI: single medullary lesions spinal MRI: normal	not conducted	steroid pulse therapy for 5 days (*no improvement*), 7 cycles of plasmapheresis (*minor improvement of vision impairment*)	pronounced thoracic and flank pain with suspected herpes zoster with central nervous system involvement but no detection of VZV in CSF (*after 9 months, acute onset*)

(*continued on next page*)

Table 1 (*continued*)

patient	sex, age*	main diagnosis	symptoms*	previous/additional neurological illnesses*	previous/additional non-neurological illness*	tumor/ hemato-oncological diseases	MRI/CT	EEG	immunotherapy (response)	follow up*
6	F, 29	autoimmune encephalitis	epileptic seizure, initial reduced cognition, impaired word fluency, mild depressive symptoms	delusion	vitamin B12 deficiency	no tumor search	brain MRI: signal increase of the gyrus parahippocampalis/ hippocampus	normal	tocilizumab and lamotrigine (*no further epileptic seizures, no significant improvement*)	memory performance improved and normal, word fluency worsened, depressive symptoms unchanged, new onset of headache after last tocilizumab treatment (*after 9 months*)
7	F, 46	cervical myelitis	cervicothoracic paresthesias with air shortage (*acute onset, similar episodes for 1 year)* resulting in syncope (without pulse and breathing), hypesthesia of both arms, impairment of fine motor skills	tetraparesis	diabetes mellitus type 2, chronic obstructive pulmonary disease	not detected	brain MRI: normal, spinal MRI: myelitis lesions (cervical vertebrae 2, 3)	normal	IVIG 2 g/kg body weight (*partial improvement*), 3x plasma separation (*stabilization, persisting neuropathic pain*)	
8	F, 51	mild cognitive impairment with suspected logopenic variant of primary progressive aphasia	increased forgetfulness (*for 10 years*), semantic paraphasias, word-finding disorders, writing with spelling mistakes in every word	depression	hypothyroidism	not detected	brain MRI: minor frontotemporal accentuated atrophy, minor expansions of the intrathecal space	normal	no immunosuppressive therapy as logopenic variant of primary progressive aphasia was suspected	no change (*after 2.5 years*)
9	M, 68	systemic and cerebral vasculitis with anterior ischemic optic neuropathy (AION), giant cell arteritis, multifactorial sensorimotor polyneuropathy	progressive pain, paresthesias and sensory disturbances in both legs (*for 10 years*), stand and gait ataxia, almost complete vision loss on right eye (*acute onset*)	depression (*for 3 years*), intracranial vascular affection	arterial hypertension, chronic renal insufficiency, giant cell arteritis suspected (no biopsy, no halo in duplex ultrasound), chronic obstructive pulmonary disease suspected, disturbed vitamin B12 metabolism	not detected pleural effusion unknown etiology	brain MRI: contrast-enhancement and change in caliber of the vertebral artery on both sides, cerebral vasculitis suspected	not conducted	prednisolone 70 mg over 7 days (*subjective improvement*), steroid pulse therapy with 2.5 g methylprednisolone in total (*minor improvement*), tocilizumab 640 mg (*good improvement*)	
10	M, 78	Alzheimer's dementia with multifactorial stand and gait ataxia	delirium, disorientation, hallucinations, anxiety, memory impairment	multifactorial stand and gait ataxia, dementia, depression	diabetes mellitus type 2, arterial hypertension, coronary heart disease, atrial fibrillation, chronic renal insufficiency, vitamin B12 deficiency	mesothelioma of unclear dignity	brain CT: clear expansions of the outer and inner intrathecal space with clear atrophy, pronounced microangiopathic medullary damage with	not conducted	no immunosuppressive therapy	patient died on pneumonia and acute kidney failure (*2 months later*)

(*continued on next page*)

Table 1 (*continued*)

patient	sex, age*	main diagnosis	symptoms*	previous/additional neurological illnesses*	previous/additional non-neurological illness*	tumor/ hemato-oncological diseases	MRI/CT	EEG	immunotherapy (response)	follow up*
							periventricular density reduction subcortical			
11	F, 72	suspected limbic encephalitis, glioblastoma	personality change, memory impairment, weight loss, unpleasant odor and taste perception, tremor, headache		arterial hypertension, hypercholesterolemia, gastritis, hepatic steatosis	glioblastoma	brain MRI: subcortical white-matter-lesions on both sides, left sided temporomesial hippocampal and parahippocampal lesion with contrast-enhancement (neuropathology: glioblastoma)	left sided focal slowing and sharp waves	IVIG 30 g/d for 5 days and 2x750 mg levetiracetam (*good improvement of smell and taste perception*)	diagnosis of glioblastoma (*6 months later*), postoperative hemiplegia, motoric and amnesic aphasia, psychomotor retardation, patient died from glioblastoma (*1 year later*)
12	F, 2	Miller-Fisher syndrome	facial nerve paresis (*acute onset*), fatigue, gait ataxia, loose limbs, no reaction on pain stimulus			no tumor search	brain MRI: neuritis of the facial nerve right	normal	2x IVIG 10 g (*almost complete improvement*)	
13	M, 47	subjective cognitive impairments	depressive symptoms, anxiety		intermittant arterial hypertension, interpreted as a result due to anxiety	no tumor search	brain MRI: normal, except chronic sinusitis	not conducted	subjective spontaneous improvement, therefore no immunosuppressive therapy wanted	remission of subjective cognitive impairment and depression
14	M, 78	chronic inflammatory sensorimotor polyneuropathy (CIDP)	unsteady gait, pallhypesthesia on both legs, tremor of the left hand, vertigo, headache, saccadic pursuit	cerebral amyloid angiopathy suspected	diabetes mellitus type 2, arterial hypertension, coronary heart disease, cardiomyopathy, atrial fibrillation, chronic renal insufficiency	no tumor search monoclonal gammopathy IgG lambda	brain MRI: peripheral microbleeds (cerebral amyloid angiopathy suspected), spinal MRI: fatty degeneration of the bone marrow, significantly thickened cauda fibers in T2w	not conducted	IVIG 0,4 g/kg body weight for 5 days (*no improvement*), methyl-prednisolone 1 g/d for 3 days (*no improvement*), plasmapheresis cumulative for 7 days (*minor improvement*), 4x rituximab 1000 mg (*no improvement*)	stable polyneuropathic syndrome with unchanged gait disturbances, progressive tremor of the left hand (*after 1.5 years*)
15	F, 57	cerebellar syndrome	gait ataxia with progressive tremor (*for 6 years*), vertigo, weakness of both arms, saccadic pursuit, mild dysarthria, mild dysphagia, dysdiadochokinesis on both sides, minimal amnestic aphasia		diabetes mellitus type 2, peripheral arterial disease, latent hypothyroidism, glaucoma	benign adrenal adenoma	brain MRI: minor cortical and subcortical atrophy, microangiopathic medullary lesions	not conducted	IVIG 185 g (*no improvement*), 4x rituximab (*no improvement*)	

(*continued on next page*)

Table 1 (*continued*)

patient	sex, age*	main diagnosis	symptoms*	previous/additional neurological illnesses*	previous/additional non-neurological illness*	tumor/ hemato-oncological diseases	MRI/CT	EEG	immunotherapy (response)	follow up*
16	F, 54	autoimmune encephalitis, differential diagnosiscerebral graft versus host disease	psychotic delusions after stem cell transplantation, graft versus host disease and prednisolone therapy	peroneus paresis with distal peroneus nerve damage, temporal pain and vertigo (*for 1.5 years*)	acute myeloid leukemia and monoclonal gammopathy (*for 1.5 years*), chronic obstructive pulmonary disease	acute myeloid leukemia monoclonal gammopathy IgG lambda	spinal MRI: spondylolysis L3, L5, ventrolosthesis L5	generalized slowing	immunotherapy as part of the acute myelogenous leukemia therapy and development of graft versus host disease	aphasia, disorientation, visual impairments after Jakavi therapy (*6.5 years later*)
17	F, 56	cerebral vasculitis	vertigo and headache (*for 3–4 weeks*), progressive aphasia, hemiparesis right and unsteady gait, emotional incontinence, apraxia, delirium, disorientation, anxious, psychomotor restless, memory impairment, nystagmus	depression	arterial hypertension		brain CT/MRI: hydrocephalus malresorptivus with expansions of the whole intrathecal space and the aquaeductus cerebri, lesion/ edematous swelling temporoparietal left, contrast-enhanced signal along the leptomeningeal area of the posterior fossa and adjacent areas of the medulla oblongata, pons, cerebellum and white matter (biopsy revealed vascular-accentuated inflammatory infiltrates)	subcortical irregularities	methylprednisolone steroid pulse therapy for 5 days (*improvement of aphasia*) cyclophosphamide 500 mg/m^2 (*no improvement*)	patient died from sepsis and the resulting multiple organ failure with pneumonia (*3 months later*)
18	F, 71	cerebellar ataxia	severe ataxic dysarthria, downbeat nystagmus, double vision, neurogenic dysphagia, rigor/ stiffness of the tongue, gait and stand ataxia, pallhypesthesia of both feet	double vision (*for 1–2 years*), dysphagia (*for several years*), dysarthria (*for 53 years*), genetic ataxia suspected but not confirmed	arterial hypertension, hypothyroidism, glaucoma	no tumor search	microangiopathic cerebral lesions	not conducted	no immunosuppressive therapy	

ANCA: anti-neutrophil cytoplasmatic antibody, CIDP: chronic inflammatory demyelinating polyneuropathy, CSF: cerebrospinal fluid, CT: computed tomography, EEG: electroencephalography, IVIG: intravenous immunoglobulin, HSV: herpes simplex virus 1, MRI: magnetic resonance imaging, VZV: varicella zoster virus.
*Age/time refer to first detection of anti-VGLUT2 autoantibodies.

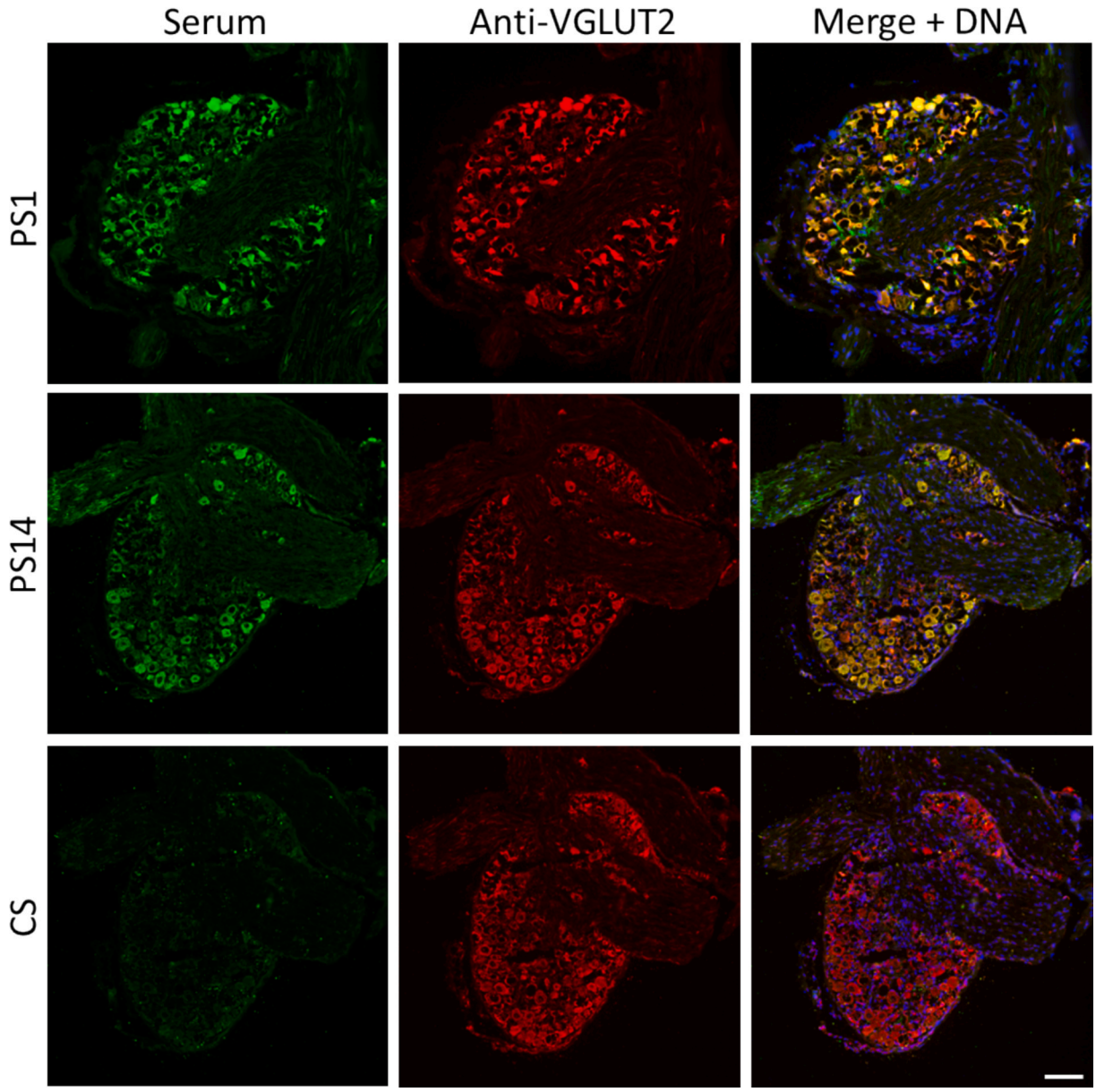

Fig. 3. Patient sera bind to VGLUT2 in dorsal root ganglia. Indirect immunofluorescence assays with formalin-fixed dorsal root ganglia and patient (PS1, PS14) and control sera (CS) and anti-human-IgG-Alexa488. Sections were stained subsequently with a commercial anti-VGLUT2 antibody and anti-rabbit-IgG-Alexa594 as secondary antibody. In the cytoplasm of dorsal root ganglia neurons, a colocalization of PS1 and PS14 with anti-VGLUT2 antibody was observed. Scale bar 100 μm.

periventricular, subcortical or temporoparietal lesions (n = 9, interpreted as microangiopathic for n = 5 or not specific for multiple sclerosis for n = 4), microbleeds (n = 2), atrophy (n = 3), signal increase in the gyrus parahippocampalis/hippocampus (n = 1), contrast-enhancement of a temporomesial lesion (n = 1, neuropathology revealed glioblastoma), contrast-enhancement of the arteria vertebralis (n = 1), hippocampal sclerosis and temporobasal parenchymal defects (n = 1, after HSV encephalitis) or neuritis of the facial nerve (n = 1). Spinal MRI was available for 4/18 patients and showed myelitis lesions (n = 1) or significantly thickened caudate fibers (n = 1) in 2/4 patients. For exemplary MRI images please refer to Supplementary Fig. 2.

3.5. CSF analysis

CSF samples of 13/18 index patients were analyzed by anti-VGLUT2 RC-IIFA, revealing positive reactivities in 10/13 cases (Supplementary Table 5, IgG CSF titers 1:1–1:320). 9/10 CSF positive patients suffered from one or more of the following CNS-related symptoms: visual dysfunction (n = 7), cognitive impairment (n = 6), headache (n = 5), depression (n = 3), vertigo (n = 3) or epileptic seizures (n = 1). Oligoclonal bands were detected in 9/18 CSF samples (type 4 n = 6, type 5 n = 2, type 3 n = 1). In 6/18 patients, a blood–brain barrier dysfunction was determined according to Reibeŕs diagram. 3/18 patients showed an elevated number of white blood cells in CSF. Only in one patient, an intrathecal IgG synthesis was detected (P17), suggesting that anti-VGLUT2 autoantibodies reached the CSF mainly by passive diffusion or a disturbed blood–brain barrier.

3.6. The majority of anti-VGLUT2 seropositive patients suffer from non-neurological comorbidities

82 % (14/17) of the adult index patients (mean age 59, range 29–78) suffered from additional non-neurological illnesses. Diabetes mellitus type 2 (n = 7/17, 41 %) and/or cardiovascular diseases [n = 12/17,

including microangiopathic changes n = 5/17 (29 %), arterial hypertension n = 7/17 (41 %), hypercholesterolemia n = 4/17 (24 %), atrial fibrillation n = 3/17 (18 %), coronary heart disease n = 2/17 (12 %), peripheral arterial disease n = 1/17 (6 %), cardiomyopathy n = 1/17 (6 %)] were observed in 13/17 patients. In addition, for 3/17 (18 %) patients, vascular inflammatory diseases were diagnosed (P2: granulomatosis with polyangiitis, P9: cerebral vasculitis/ suspected giant cell arteritis, P17: cerebral vasculitis). In 6/17 (35 %) patients, chronic kidney failure or hyperuricemia was found. Chronic obstructive pulmonary disease was present or suspected in 3/17 (18 %) patients. When comparing the frequencies of the observed comorbidities with literature data on the general prevalence of the respective diseases in age-related normal populations (Supplementary Table 7) diabetes mellitus, vasculitis, atrial fibrillation, chronic kidney failure and chronic obstructive pulmonary disease emerged as more common in the adult anti-VGLUT2 positive index patients.

Furthermore, a malignant or benign tumor was found in 5/18 index patients (lung carcinoma n = 1, mesothelioma n = 1, adrenal adenoma n = 2, glioblastoma n = 1) and hemato-oncological diseases were detected or strongly suspected in 3/18 patients (acute myeloid leukemia n = 1, monoclonal gammopathy n = 3). Altogether, an acute or preexisting tumor or hemato-oncological disease was observed in 7/18 patients.

One patient developed a herpes-simplex virus encephalitis and

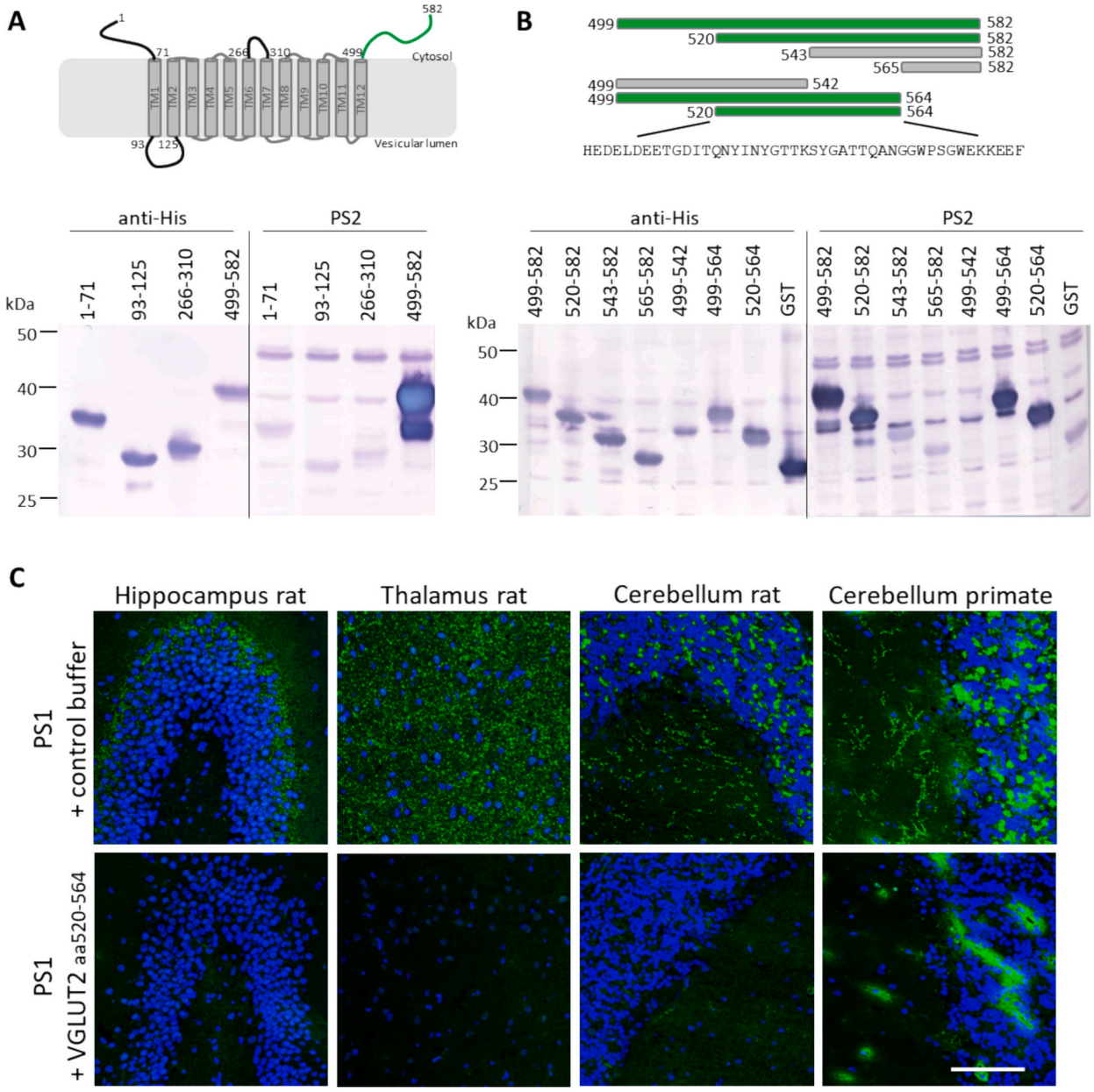

Fig. 4. VGLUT2 epitope characterization. A: Upper panel: Scheme of VGLUT2 with the four cytoplasmic or vesicular located fragments aa 1–71, aa 93–125, aa 266–310, aa 499–582. Lower panel: Immunoblots with E. coli lysates expressing His-GST-VGLUT2 fragments incubated with anti-His antibody or patient serum 2 (PS2). PS2 reacts with the C-terminal VGLUT2 aa 499–582 fragment. B: Upper panel: Scheme of His-GST-VGLUT2 aa 499–582, aa 520–582, aa 543–582, aa 565–582, aa 499–542, aa 499–564, aa 520–564 fragments and the amino acid sequence of fragment 520–564. Lower panel: Immunoblots with E. coli lysates expressing His-GST-VGLUT2 fragments incubated with anti-His antibody or patient serum (PS2). The shortest fragment which is still recognized by PS2 is VGLUT2 aa 520–564. C: IIFA competitive inhibition experiments with patient serum 1 (PS1) pre-incubated with purified His-GST-VGLUT2 aa 520–564 or control buffer and Alexa488-labelled goat anti-human IgG as secondary antibody. His-GST-VGLUT2 aa 520–564 abolished the immune reaction of PS1 on hippocampus, thalamus and cerebellum. Nuclei were counterstained by incubation with TO-PRO-3 iodide. Scale bar 100 μm.

critical illness polyneuropathy after immunosuppressive treatment of a preexisting vasculitis (P2). Elevated levels of the general inflammatory marker CRP were detected in 8/17 analyzed patients.

3.7. Anti-VGLUT2 positive patients received immunosuppressive treatments with different responses

12/18 patients received different types of immunotherapies (steroid pulse therapy, IVIG, plasmapheresis, tocilizumab, rituximab, cyclophosphamide), emphasizing the assumption that an autoimmune pathology was suspected in most of the patients. Steroid pulse therapy led to minor or partial improvements in 3/5 cases (P3, P9, P17). IVIG led to almost complete remission in 1/5 patients (P12) and to partial remission in 2/5 patients (P7, P11), while no improvement was observed in 2/5 cases (P15, P14). Plasmapheresis showed slight improvement (P5, P14) or no effects (P1, P7). Treatment with tocilizumab resulted in good response in 1/2 patients (P9). Altogether, immunotherapy had beneficial effects to different extents in 8/12 patients. In 3/12 patients, immunotherapy had no effect (P1, P6, P15). However, in one of these cases, therapy was interrupted due to the poor general condition of the patient (P1). P16 received immunotherapy as part of the acute myelogenous leukemia therapy which included chemotherapy and stem cell transplantation.

In 6/18 cases no immunotherapy was initiated because other reasons were suspected to cause the neurological symptoms (P2, P8, P10, P18) or a spontaneous remission occurred (P4, P13).

3.8. Patient anti-VGLUT2 autoantibodies recognize a cytoplasmic epitope between aa 520–564

To assess whether VGLUT2 autoantibodies bind to an intra- or extracellular epitope, we performed immunoblot assays with four VGLUT2 fragments (aa 1–71, 93–125, 266–310, 499–582). Here, 17/17 analyzed index patient sera recognized only the C-terminal cytoplasmic VGLUT2 aa 499–582 fragment (Fig. 4A). The epitope was narrowed down further by analysis of shorter C-terminal VGLUT2 fragments (aa 520–582, 543–582, 565–582, 499–542, 499–564, 520–564). VGLUT2 aa 520–564 was the shortest VGLUT2 fragment which was recognized by 17/17 patient sera (Fig. 4B). In competitive inhibition experiments the reaction of PS1 on neuronal tissue sections could be abolished by preincubation with VGLUT2 aa 520–564 (Fig. 4C). Serum of P12 could not be used for epitope characterization due to limited volume.

Next to VGLUT2, two additional VGLUT isoforms are known (VGLUT1 and 3), in which the determined epitope is only poorly conserved. In agreement with this, anti-VGLUT2 positive sera do not cross-react with VGLUT1 or VGLUT3, as confirmed by RC-IIFA with HEK293 cells recombinantly expressing VGLUT1 or VGLUT3 (Supplementary Fig. 3).

4. Discussion

We describe the identification of VGLUT2 as novel autoantibody target of sera of 285 patients with a very distinct staining pattern in neuronal tissue IIFA. The initial suspected diagnoses of 87 patients and the detailed clinical data of 18 index patients (age 2–78, 61% female, RC-IIFA IgG titers 1:320–1:3200) revealed that anti-VGLUT2-IgG-associated neurological diseases affect both the central and peripheral nervous system, with encephalitis, dementia or cognitive impairment and polyneuropathy representing the three primary diagnoses. The main symptoms of the 18 index patients were cognitive changes including memory impairment, aphasia and disorientation as well as gait abnormalities and sensorimotor symptoms including neuropathic pain, occasionally accompanied by visual obstructions. Many of the anti-VGLUT2 positive index patients had a chronic course with preexisting neurological impairments and progressive symptoms. In addition, the majority of the 18 index patients had non-neurological comorbidities with cardiovascular and inflammatory involvement (e.g. diabetes mellitus type 2 or vasculitis). Epitope characterization revealed that autoantibodies of 17/17 anti-VGLUT2 positive sera investigated target a linear epitope located at the cytoplasmic C-terminus of VGLUT2 between amino acid 520–564.

The manifestations of central and peripheral nervous system symptoms in VGLUT2 seropositive patients is in line with the expression of the protein (alternative names DPNI, SLC17A6) in the vesicular membrane of glutamatergic excitatory neurons throughout the entire brain and spinal cord (Moriyama and Yamamoto, 2004; Varoqui et al., 2002) in the horizontal and ganglion cells of the retina (Gong et al., 2006; Mimura et al., 2002) and in neurons of trigeminal ganglion and dorsal root ganglia (Li et al., 2003; Oliveira et al., 2003; Scherrer et al., 2010). In the CNS, VGLUT2 has been shown to be responsible for glutamate uptake into presynaptic vesicles potentially regulating glutamatergic synaptic transmission. Interestingly, in dorsal root ganglia neurons, VGLUT2 is likely involved in the transduction of nociceptive signals from the periphery to the spinal cord (Brumovsky et al., 2007).

However, since this is the first comprehensive report on VGLUT2 autoantibodies and direct pathogenicity was not studied in our work, we can only hypothesize on potential effects of anti-VGLUT2 autoimmunity. In analogy to mouse conditional knock out models inhibiting VGLUT2 expression in the cortex and amygdala leading to reduced memory function and schizophrenia-like behavior (Wallen-Mackenzie et al., 2010), autoantibodies could similarly relate to cognitive deficits which was indeed the predominant complaint of our cohort. Further, neuropathic pain and other neuropathy symptoms such as paraesthesia, numbness and peripheral paresis were present in 9/18 patients, aligning with the essential role of VGLUT2 in physiological and pathological pain response, as shown in conditional knockout animal slacking VGLUT2 in nociceptors of dorsal root ganglia (Leo et al., 2009; Scherrer et al., 2010).

The variety of symptoms could also be attributed to other factors, such as disease duration and different time periods of anti-VGLUT2 autoantibody exposures. As some patients had preexisting neurological impairments years before anti-VGLUT2 autoantibody detection, e.g., psychosis (P2), paresis and paresthesia (P3), polyneuropathy and depression (P4 and P9), migraine (P5), memory impairment (P8 and P10), or dysarthria (P18), it is tempting to speculate that these patients harbored anti-VGLUT2 autoantibodies already for many years before an autoimmune etiology was suspected. This might have led to an accumulation of anti-VGLUT2-mediated effects in some patients, leading to chronic neurological impairments. These might become accompanied by acute or subacute symptoms, like visual impairments (P3, P5, P9), aphasia (P4, P8) or epileptic seizures (P6) after certain circumstances, like transient or permanent blood–brain barrier disfunction.

Additionally, several patients presented with multimorbidity, so that most likely a combination of multiple factors influenced the observed phenotypes. Particularly, 7/10 index patients with polyneuropathy, included in the present study, additionally suffered from diabetes type 2 and/or kidney diseases, which might be alternative causes of neuropathic symptoms. However, cognitive impairments, which are observed in some of those patients are less likely to be related to diabetic or hyperuricemic nerve injury and might rather hint towards potential autoantibody-associated aggravation of symptoms.

Altogether, 82 % (14/17) of anti-VGLUT2 positive adult index patients had non-neurological comorbidities. Among those, diabetes mellitus, vasculitis, atrial fibrillation, chronic kidney failure and chronic obstructive pulmonary disease emerged as more common in the adult anti-VGLUT2 positive index patients compared to literature data about age-related normal populations. Although this is highly speculative, it might be possible that a link between these conditions and anti-VGLUT2 autoantibodies exists and that they might be even consequence or cause of anti-VGLUT2 autoantibody development.

A role of VGLUT2 in the regulation of the vascular system or blood glucose levels has been previously described (Bai et al., 2003; Hayashi

et al., 2003a,b; Stornetta et al., 2002; Uehara et al., 2006). Interestingly, it was observed that rat meningeal and brain microvasculature pericytes, which regulate vascular functions as well as maintenance of the blood–brain barrier, express VGLUT2 (Bhattacharya et al., 2020; Dabravolski et al., 2022; Mathur and Deutch, 2008). For future studies, it would be interesting to analyze VGLUT2 expression in peripheral pericytes and to investigate possible anti-VGLUT2 mediated effects on pericyte function.

On the other hand, it would also be possible that diabetes, vasculitis and other diseases causing damage to VGLUT2-expressing cells might be involved in the induction of anti-VGLUT2 autoimmunity.

It has been reported that VGLUT2 expression in dorsal root ganglia is upregulated following peripheral nerve or tissue injury (Crosby et al., 2015; Izumi et al., 2015; Wang et al., 2016) and that increased spinal expression of VGLUT2 was detected during chronic peripheral inflammation or after streptozotocin-induced diabetic peripheral neuropathy (Leiguarda et al., 2020; Zhang et al., 2024). Interestingly, an increase in VGLUT2 expression was also observed in rodent demyelinating lesions and human multiple sclerosis lesions (Gautier et al., 2015). One could speculate that an upregulation of VGLUT2 expression combined with an increased accessibility of VGLUT2 in damaged VGLUT2-expressing cells might trigger anti-VGLUT2 autoantibody production. Known factors which can cause damage to peripheral nerves include diabetes mellitus, vitamin B12 deficiency, kidney or liver diseases, hypothyroidism, monoclonal gammopathy, vascular disorders, or infections, e.g., with herpes zoster viruses (Hammi and Yeung, 2024). In 15/18 anti-VGLUT2 positive index patients, at least one of these conditions was documented. Remarkably, diabetes was discussed previously as a possible risk factor for developing peripheral neuropathies with anti-paranodal autoantibodies (Appeltshauser et al., 2022).

However, it cannot be ruled out, that anti-VGLUT2 autoantibodies represent only bystanders of neurodegeneration. Alternatively, it might be possible that an induction of anti-VGLUT2 autoimmunity might further facilitate inflammatory processes that reinforce neurodegenerative cascades and additionally induce symptoms exceeding the ones typical for the underlying disease.

A remarkable observation was the restriction of the recognized epitope to amino acid 520–564 located at the cytoplasmic C-terminus of VGLUT2. One could raise the hypothesis that mimicry of this sequence by infectious agents might also have played a role in the induction of cross reactive anti-VGLUT2 autoimmune responses.

In a subgroup (5/18) of anti-VGLUT2 autoantibody-positive index patients, a malignancy was detected. However, our data did not indicate an association to a certain cancer type. Of note is that hemato-oncological diseases (acute myeloid leukemia n = 1, monoclonal gammopathy n = 3) were observed in 3/18 patients. It might be possible that anti-VGLUT2 autoantibodies are paraneoplastic in some cases. Therefore, it would be interesting to study whether VGLUT2 expression might be induced in hemato-oncological diseases or other malignancies and if this might trigger anti-VGLUT2 autoimmunity.

More than half of the index patients (8/12) who received immunotherapy had beneficial effects, although only slight or partial improvements were observed in most of the cases. However, 2/12 patients showed strong improvements with almost complete remission (P9, P12). Treatment response overall seems not to depend on anti-VGLUT2 autoantibody titers in serum or the detection of anti-VGLUT2 IgG in CSF. The limited immunotherapy response in most of the patients might be due to several factors. Some patients had been suffering already for many years from different neurological symptoms before autoantibody testing and immunotherapy was initiated, and it is unclear for how long they have already been exposed to anti-VGLUT2 autoantibodies in serum or CSF. One could speculate that an early therapy start after symptom onset might have been favorable as previously suggested in other autoantibody-associated neurological diseases (Ayzenberg et al., 2019; Nissen et al., 2020; Pruss, 2021). Moreover, due to the multifactorial disease spectrum, in many patients, a beneficial effect of immunosuppressive therapy might be covered by additional non-autoimmune symptoms. Poorer treatment responses could also be attributed to the intracellular epitope of the autoantibodies, which are generally associated with reduced immunotherapy success rates compared to diseases with autoantibodies targeting extracellular epitopes.

During the preparation of the manuscript, one anti-VGLUT2 case report was published, describing a patient with mixed dementia and anti-VGLUT2 autoantibodies (Hansen et al., 2023). In this report, anti-VGLUT2 autoantibodies were detected by the reference laboratory, which used our prototypic anti-VGLUT2 RC-IIFA. Similar to the observations we describe in the present study, the patient suffered from impaired memory, disorientation, reduced semantic word fluency, psychomotor slowing, depression and visual impairments. In addition, brain MRI showed cerebral microangiopathy and isolated cerebral lesions in the periventricular white matter.

Limitations of this study are the relatively low number of patients (18/285) with detailed clinical data available and their retrospective collection. Due to this limited number of well characterized patients and the variety of different symptoms observed, it is difficult to define a clear and recognizable phenotype of anti-VGLUT2-associated neurological diseases yet. In addition, the frequencies of observed comorbidities need to be confirmed with lager patient numbers. Not all patients, of which clinical data could be reported, were analyzed for cognitive impairments or peripheral nerve affection and different diagnostic tests were carried out at the participating hospitals. Furthermore, in some patients anti-VGLUT2 autoantibody determination was performed years after onset of the first neurological symptoms. Here, it is unclear for how long anti-VGLUT2 autoantibodies have been present. Similarly, in most of the cases, we were not able to determine the exact time between symptom onset and start of immunotherapy. Only in 2/12 patients a strong improvement after immunosuppressive therapy was observed, while most of the patients showed only partial responses. Hence, the effect of immunotherapy in anti-VGLUT2 positive patients is so far unpredictable. A prospective analysis of further patients with longer follow up times is mandatory to develop a complete picture of the clinical correlations and to allow the deduction of treatment recommendations.

Another limitation are missing analyses unraveling functional effects of anti-VGLUT2 autoantibody-mediated effects and their pathogenic role. It is unclear whether anti-VGLUT2 autoantibodies inhibit VGLUT2 directly by reaching their target *in vivo* or if primarily T cell-mediated processes play a role.

5. Conclusions

We propose that anti-VGLUT2-associated disorders represent a novel group of autoimmune-mediated diseases. The high frequency of coexisting non-neuronal illnesses could emphasize the relevance of anti-VGLUT2 autoantibodies as markers for systemic autoimmune processes with central and peripheral nervous system involvement. Thereby, detection of anti-VGLUT2 autoantibodies might indicate the existence of neuropathic changes. In all cases a tumor search should be initiated. Immunotherapy might be beneficial for patients, especially with acute or subacute onset of symptoms.

CRediT authorship contribution statement

Merle Corty: Writing – review & editing, Visualization, Resources, Investigation, Conceptualization. **Friederike A. Arlt:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Conceptualization. **Kathrin Borowski:** Writing – review & editing, Visualization, Investigation. **Stephanie Wernick:** Writing – review & editing, Visualization, Investigation. **Madeleine Scharf:** Writing – review & editing, Methodology, Investigation. **Fabian A. Piecha:** Writing – review & editing, Investigation. **Claudia Steen:** Writing – review & editing, Investigation. **Philipp Oehler:** Writing – review & editing,

Investigation. **Maria Buthut:** Writing – review & editing, Investigation. **Moritz Krill:** Writing – review & editing, Investigation. **Johann-Philipp Zöllner:** Writing – review & editing, Investigation. **Tammo Krafft:** Writing – review & editing, Investigation. **Robert Markewitz:** Writing – review & editing, Investigation. **Christiane Radzimski:** Writing – review & editing, Methodology, Investigation. **Yvonne Denno:** Writing – review & editing, Methodology, Investigation. **Christian Probst:** Writing – review & editing, Investigation. **Bianca Teegen:** Writing – review & editing, Resources, Investigation. **Christian Grefkes:** Writing – review & editing, Investigation. **Susanne Knake:** Writing – review & editing, Investigation. **Karsten Witt:** Writing – review & editing, Investigation. **Carsten Finke:** Writing – review & editing, Investigation. **Georg Hagemann:** Writing – review & editing, Investigation. **Jana Becker:** Writing – review & editing, Investigation. **Markus Kraemer:** Writing – review & editing, Investigation. **Torsten Witte:** Writing – review & editing, Investigation. **Lars Komorowski:** Writing – review & editing, Supervision. **Klaus-Peter Wandinger:** Writing – review & editing, Investigation. **Harald Prüss:** Writing – review & editing, Investigation. **Nico Melzer:** Writing – review & editing, Investigation. **Ramona Miske:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: MS, CR, YD, CP, LK and RMi are employees of EUROIMMUN, a company that manufactures diagnostic tests and instruments. RMi, YD, CR and KB have patent applications, concerning the detection of an autoantibody against VGLUT2 issued and pending. KW has received funding from the Deutsche Forschungsgemeinschaft (German Research Association) and STADAPHARM GmbH outside the present study. He has received honoraria for presentations/advisory boards/consultations from BIAL, Indorsia, Boston Scientific and STADAPHARM GmbH, outside the present study. He has received royalties from Thieme Press and Elsevier Press. He serves as an editorial board member of Wileys "Parkinson's Disease", "Behavioural Neurology" and PLOSone. NM has received honoraria for lecturing and travel expenses for attending meetings from Biogen Idec, GlaxoSmith Kline, Teva, Novartis Pharma, Bayer Healthcare, Genzyme, Alexion Pharmaceuticals, Fresenius Medical Care, Diamed, UCB Pharma, AngeliniPharma, BIAL and Sanofi-Aventis, has received royalties for consulting from UCB Pharma, Alexion Pharmaceuticals and Sanofi-Aventis and has received financial research support from Euroimmun, Fresenius Medical Care, Diamed, Alexion Pharmaceuticals and Novartis Pharma. MC, FAA, SW, FP, CS, PO, MB, MK, JPZ, TK, RMa, BT, CG, HP, SK, CF, GH, JB, MK, TW, KPW report no competing interests to the work described.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2025.06.014.

Data availability

Data will be made available on request.

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