Glycine receptor antibodies in PERM and related syndromes: characteristics, clinical features and outcomes

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The clinical associations of glycine receptor antibodies have not yet been described fully. We identified prospectively 52 antibody-positive patients and collated their clinical features, investigations and immunotherapy responses. Serum glycine receptor antibody endpoint titres ranged from 1:20 to 1:60000. In 11 paired samples, serum levels were higher than (n = 10) or equal to (n = 1) cerebrospinal fluid levels; there was intrathecal synthesis of glycine receptor antibodies in each of the six pairs available for detailed study. Four patients also had high glutamic acid decarboxylase antibodies (> 1000 U/ml), and one had high voltage-gated potassium channel-complex antibody (2442 pM). Seven patients with very low titres (< 1:50) and unknown or alternative diagnoses were excluded from further study. Three of the remaining 45 patients had newly-identified thymomas and one had a lymphoma. Thirty-three patients were classified as progressive encephalomyelitis with rigidity and myoclonus, and two as stiff person syndrome; five had a limbic encephalitis or epileptic encephalopathy, two had brainstem features mainly, two had demyelinating optic neuropathies and one had an unclear diagnosis. Four patients (9%) died during the acute disease, but most showed marked improvement with immunotherapies. At most recent follow-up, (2–7 years, median 3 years, since first antibody detection), the median modified Rankin scale scores (excluding the four deaths) decreased from 5 at maximal severity to 1 (P < 0.0001), but relapses have occurred in five patients and a proportion are on reducing steroids or other maintenance immunotherapies as well as symptomatic treatments. The glycine receptor antibodies activated complement on glycine receptor-transfected human embryonic kidney cells at room temperature, and caused internalization and lysosomal degradation of the glycine receptors at 37°C. Immunoglobulin G antibodies bound to rodent spinal cord and brainstem co-localizing with monoclonal antibodies to glycine receptor-α1. Ten glycine receptor antibodies...
positive samples were also identified in a retrospective cohort of 56 patients with stiff person syndrome and related syndromes. Glycine receptor antibodies are strongly associated with spinal and brainstem disorders, and the majority of patients have progressive encephalomyelitis with rigidity and myoclonus. The antibodies demonstrate in vitro evidence of pathogenicity and the patients respond well to immunotherapies, contrasting with earlier studies of this syndrome, which indicated a poor prognosis. The presence of glycine receptor antibodies should help to identify a disease that responds to immunotherapies, but these treatments may need to be sustained, relapses can occur and maintenance immunosuppression may be required.

**Keywords:** stiff person syndrome; progressive encephalomyelitis with rigidity and myoclonus; autoimmune encephalitis; glycine receptor; autoantibody

**Abbreviations:** GAD = glutamic acid decarboxylase; GlyR = glycine receptor; HEK = human embryonic kidney cells; NMDAR = N-methyl-D-aspartate receptor; PERM = progressive encephalomyelitis with rigidity and myoclonus; VGKC = voltage-gated potassium channel

**Introduction**

Antibodies to receptors, ion channels or related proteins have become important markers for diseases that often improve substantially with immunotherapies (Vincent et al., 2011; Lancaster and Dalmau, 2012; Zuliani et al., 2012). N-methyl-D-aspartate antibodies are associated with anti-NMDA receptor (NMDAR) encephalitis and antibodies to leucine-rich, glioma inactivated 1 protein and contactin-associated protein 2 (components of the voltage-gated potassium channel (VGKC)-complex), with limbic encephalitis, Morvan’s syndrome or peripheral nerve hyperexcitability. Antibodies to other CNS receptors have also been found in limbic encephalitis (Lancaster and Dalmau, 2012), and to dopamine receptors in children with a rare form of basal ganglia encephalitis (Dale et al., 2012). In addition, antibodies to the glial protein aquaporin 4 are an established cause of neuromyelitis optica (Jarius and Wildemann, 2010). Because each of these antibodies can bind to antigenic epitopes that are exposed on the surface of the cell they are likely to be pathogenic.

Stiff person syndrome is another antibody-associated syndrome, often with antibodies to glutamic acid decarboxylase (GAD); reviewed by Brown and Mardsen, 1999; Meinck and Thompson, 2002; Alexopoulos and Dalakas, 2010). This enzyme, however, is intracellular rather than on the cell surface. Many patients do not respond well to immunotherapies but there are reports of clinical improvement with intravenous immunoglobulin and cyclophosphamide. Retrospectively, the patient’s sera, at onset and peak of disease, contained antibodies that bound to GlyR α1 subunits (now known as GLRA1) expressed on the surface of transfected human embryonic kidney cells, and also immunoprecipitated GlyR α1 (Hutchinson et al., 2008). Since then, further patients with glycine receptor (GlyR) antibodies have been described (Clerinx et al., 2011; Mas et al., 2011; Turner et al., 2011; Iizuka et al., 2012; Peeters et al., 2013; Piotrowicz et al., 2011; Damasio et al., 2013; Stern et al., 2014; Bourke et al., 2014) with combinations of stiffness, rigidity, excessive stimulus-evoked startle, brainstem and autonomic signs. GlyR antibodies have also been found in retrospective cohorts of adults or children (Alexopoulos et al., 2013; Clardy et al., 2013; McKeon et al., 2013), with or without GAD antibodies. Here we describe the clinical spectrum of 52 GlyR antibody-positive patients (including those reported previously, see above), and the treatment responses, antibody characteristics and immunohistochemical localization of target antigens in rodent brain tissue.

**Materials and methods**

**Patients**

We identified 52 patients prospectively from 779 samples (including 55 serum/CSF pairs) referred to the Oxford Neuroimmunology service for GlyR antibody screening from 2008 to March 2012. Consent forms and questionnaires were sent to the neurologists (the glycine receptor antibody study group, see Appendix 1) who referred positive samples, and the data collated and analysed in Oxford. The study was performed with consent from the Regional Ethics Committee Ref: 07/Q1604/28. Sera positive for NMDAR or aquaporin 4 antibodies were used as serum controls, and six archived multiple sclerosis CSFs were used as CSF controls. We also examined GlyR antibodies in a retrospective cohort of 56 patients (35 females, 21 males) with stiff person syndrome, PERM or related disorders that had been collected in Heidelberg, Germany.
Detection of glycine receptor-bound antibodies for diagnosis

For screening we used immunofluorescence cell-based assays on human embryonic kidney (HEK) 293 cells transfected to express homo-pentamers of GlyRα1 tagged with enhanced green fluorescent protein, as previously described (Hutchinson et al., 2008) and used in the Oxford clinical diagnostic service (see Supplementary material for details). Serum was tested at 1:20 and CSF at 1:2. The intensity of the staining was assessed visually by two independent observers, using a semiquantitative score (0 = no binding; 1–2 = low level binding; 2–4 = increasing strength of binding) as described previously (Leite et al., 2008; Waters et al., 2008). All sera and CSFs with positive binding were retested and checked for negativity against a second antigen to confirm specificity. When sufficient sample was available, GlyR antibody-positive sera and CSFs were also tested at serial dilutions to identify the concentration at which binding was scored as 1 (‘endpoint’ dilution). All of the available first samples were also tested for NMDAR and GAD antibodies by routine tests; 28 had VGKC-complex antibodies requested previously by the referring neurologists.

The subclass of the GlyR antibodies was determined by use of specific anti-subclass antibodies to IgG1, IgG2, IgG3, IgG4 and IgM (Invitrogen). Their ability to fix complement was demonstrated on the transfected HEK293 cells. After the addition of the patient serum (1:20) and washing, fresh human complement was added at 37°C for 1 h. Surface-bound C3b was detected with a fluorescent commercial antibody (Dako) as previously described (Leite et al., 2008; Waters et al., 2008). To determine subunit specificity, all positive samples were also screened against the other α subunits of the GlyR, α2 and α3 (see Supplementary material for details).

Blood–brain barrier integrity and antibody index

Full details are given in the Supplementary material. The six serum/CSF pairs with sufficient volumes were used to determine the integrity of the blood–brain barrier and the intrathecal synthesis of specific GlyR antibodies. Western blotting was used to measure total levels of IgG in paired sera (1:300) and undiluted CSF and for the albumin content of the sera (1:300) and CSFs, comparing with IgG and albumin standard curves. The IgG index was calculated according to:

$$\frac{[\text{CSF IgG (mg/ml)/serum IgG (mg/ml)}]}{[\text{CSF albumin (mg/ml)/serum albumin (mg/ml)}]}$$

and the intrathecal synthesis of GlyR antibody by calculating:

$$\frac{[\text{CSF GlyR}α1 \text{ endpoint titre/CSF total IgG}]}{[\text{serum GlyR}α1 \text{ endpoint titre/serum total IgG}]}.$$

Effects of antibody binding to glycine receptor expressed on HEK cells

Full details are given in Supplementary material. To see whether GlyR antibodies were associated with internalization of the GlyRs, sera (1:80 to 1:160) were incubated with GlyRα1 expressing HEK cells for 1 h at 4°C and washed. Fixed or live cells were then incubated at either 4°C, or 37°C for 5 min to 16 h. After fixation of all cells, surface-bound human IgG was detected and the coverslips scored as for the diagnostic assay (see above). To see if the loss of surface GlyR antibody IgG was due to internalization, surface bound GlyR antibody was visualized (green) and scored at different times and compared with cells fixed and permeabilized in 0.3% Triton™ X-100 so that internal IgG could be visualized with Alexa Fluor® goat anti-human IgG (red). The results were quantified and data analysed with ImageJ software.

To examine the levels of surface GlyRα1, rather than bound IgG, the same procedure was carried out using GlyR-EGFP (enhanced green fluorescent protein) transfected cells, and cyclohexamide to prevent new GlyR-EGFP synthesis. The percentage of cells with GlyR-EGFP on the surface or internalized was analysed. Finally to look at the endosomal location of internalized GlyR-EGFP, mouse antibodies to the late endosomal antigen (1:250; SantaCruz) were used, detecting with Alexa Fluor® 568 goat anti-mouse IgG (Invitrogen).

Binding to rat brain tissue sections

Full details are given in the Supplementary material. Indirect single and double immunofluorescence staining was performed on 11-μm cryostat sections of fresh frozen adult rat brain. The sections were fixed, blocked and incubated overnight at 4°C with human sera (1:200 to 1:800) and mouse monoclonal antibodies to the GlyRα1 subunit (1:500; Synaptic Systems) or rabbit polyclonal antibody to GAD (1:500; Sigma). The sections were washed, and bound patient or commercial antibodies detected with the appropriate secondary antibodies. Slides were photographed under a Leica fluorescence microscope (DM 2500) with a digital camera (QImaging, Rolera XR, Fast 1394). Four sera were pre-adsorbed against HEK cells expressing GlyRα1, GlyRα2, GlyRα3 or untransfected HEK cells, or with recombinant GAD (12.5 μg/ml; RSR Ltd), overnight at 4°C before applying to the rat brain sections. To identify the neurons by confocal microscopy, sections were incubated with human sera (1:200 to 1:800), mouse monoclonal antibody to glycine receptor (as above) and either rabbit polyclonal antibody to microtubule-associated protein 2 (MAP2; 1:1000; Sigma) or rabbit polyclonal antibody to choline acetyltransferase (ChAT; 1:100; Millipore). Images were taken with a Zeiss confocal microscope (LSM 710).

Statistics

Kruskal-Wallis test and Dunn’s multiple comparisons were used to assess the immunotherapy responses. Two-way ANOVA and two-sided Students t-tests were used to analyse the internalization experiments.

Results

The prospective cohort was identified from samples referred for testing from 2008 to March 2012. Images of patient and healthy control sera binding to GlyRs expressed on HEK cells are shown in Fig. 1A and the initial scores for each patients’ serum and, when provided, CSF in Fig. 1B and C. For disease controls, we used samples identified as positive for NMDAR or aquaporin 4 antibodies, and multiple sclerosis CSFs. Only 1% of sera with aquaporin 4 antibodies, and none of those with NMDAR antibodies, bound to the GlyR cells at a score of >1 (Fig. 1B), and none of the control CSFs bound detectably (Fig. 1C). Therefore, we studied consecutive patients with GlyR antibody scores of >1 in serum and >0 in CSF. Forty-one sera and 11 serum/CSF pairs were positive. The remaining 727 patients, including 52 with
serum/CSF pairs and five unpaired CSF samples were negative (median 0).

The endpoint dilution titres in sera (1:40 to 1:60,000) and CSFs (1:5 to 1:640) varied widely (eg. Fig. 1D), but some patients had CSF levels equal to (n = 1) or very similar to serum levels suggesting marked intrathecal synthesis of the specific GlyR antibody. To assess this quantitatively, we measured serum and CSF IgG and albumin in each of six patients with sufficient remaining samples. The ratios of serum:CSF IgG (138–592) and albumin (187 to 403) were within normal limits with Q_hab of 0.6 to 1.4 (normal 0.7 to 1.3), indicating no general blood–brain barrier breakdown. By contrast, the antibody index for the GlyR antibody ranged from 8 to 400 (normal < 1.4). These values represent substantial intrathecal synthesis of the specific GlyR antibodies, particularly in the three patients with values > 50 (Fig. 1E).

**Clinical data of patients with glycine receptor antibody-positive referred samples**

For the 52 prospectively-referred GlyR antibody-positive patients, questionnaires were sent to the referring neurologists. Seven patients with very low GlyR antibodies (not titrating beyond 1:40) were excluded from the cohort because they had minimal symptoms and were lost to follow-up (n = 2), the clinician did not respond (n = 1) or the patients received a different diagnosis: one with Creutzfeldt-Jakob disease (Angus-Leppan et al., 2013), one with confirmed hereditary myoclonus dystonia (Patient DYT_11), one with a motor polyneuropathy and one with possible psychogenic movement disorder.

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**Figure 1** GlyR antibodies (Ab) in PERM and related disorders. (A) A patient's serum IgG binding to HEK293 cells expressing GlyRα1-EGFP (green); the IgG binding is detected with anti-human IgG (red). No IgG binding is observed with serum from a healthy individual. (B) Visual scores of sera (diluted 1:20) referred for routine GlyR antibody testing compared with scores in sera positive for NMDAR or aquaporin 4 (AQP4) antibodies studied as neurological controls. (C) Scores of referred CSF samples (diluted 1:1) compared with multiple sclerosis control CSFs. (D) Endpoint titrations for sera and paired CSFs; the CSF titres are generally lower than serum titres but in three patients are equal or only a dilution lower. (E) Calculation of intrathecal synthesis in six paired CSFs. GlyR antibody titres (from D) divided by IgG concentration for sera (serum/IgG) or for CSFs (CSF/IgG). Intrathecal synthesis rates are given as the ratio between these values as described above, and are raised in all patients, particularly in three. (F) Scores for each IgG subtype and for the deposition of complement C3 on the GlyRα1-expressing HEK cells. The antibodies are mainly IgG1 and IgG3 subtypes and activate complement on the surface of the GlyRα1-transfected cells.
Eighteen (40%) of the remaining 45 patients came from the UK and the rest from elsewhere (Table 1). There were 24 males and 21 females, aged 1 to 75 years (median 50 years). Four of the females were under 15 years of age at onset. At the time of first GlyR antibody-positive test, the duration of symptoms ranged from <1 to 96 months, but the majority (71%) of presentations were described as acute (20%), subacute (44%) or subacute with acute exacerbation (7%). In the others, the onset was more chronic or insidious and 18% presented with exacerbations of pre-existing disease.

Autoimmune and paraneoplastic associations

Comorbid autoimmune diseases were not uncommon (13/45, 29%, Table 1). Five patients had previous successfully-treated tumours (Table 1), and four tumours were first identified during the neurological illness (three thymomas, one B cell marginal zone lymphoma with IgM monoclonal gammapathy). In addition, one female patient with a previously treated breast cancer had metastases at presentation.

Clinical features

The presenting symptoms are summarized in Table 2. The commonest features (69%) were spasms, often painful, and stiffness and rigidity of the neck, trunk or limb muscles; these were associated with walking difficulties and frequent falls. Excessive startle (42%), and eye movement disorders (diplopia, ptosis, nystagmus, 40%) or difficulty opening the mouth, swallowing, or speech problems, were all frequent. Non-specific sensory symptoms (pruritus, dysesthesias, hyperaesthesias) in the limbs including pain unrelated to muscle spasms, were reported in 22% of patients. In addition, 29% of patients had cognitive disturbance or seizures (13%).

The clinical features were also documented at peak of illness in 30 of the patients. At this stage, spasms, stiffness and rigidity were prominent (80%), with eye movement disturbance and facial/bulbar motor disturbance in ~60%. Hyperekplexia and muscle weakness were more frequent by this stage, as were cognitive deficits, encephalopathy, and seizures. Autonomic disturbance was evident in 43%, with urinary retention particularly common, and respiratory failure was noted in 27%. Videos of representative patients are available with the published case reports (Hutchinson et al., 2008; Clerinx et al., 2011; Iizuka et al., 2012; Peeters et al., 2013; Damasio et al., 2013).

Investigations

Investigations performed were variable and the results are summarized in Table 3. In general MRI was uninformative, with no abnormality detected in 26/36, but in two patients there was evidence of inflammation in the temporal lobes, and two had other FLAIR lesions. Spinal cord MRI was uniformly negative in 18/23 patients but showed short or patchy lesions in four; one had longitudinally-extensive lesions. EMG was performed in 29; eight had continuous motor unit activity, six had spontaneous or stimulus-induced activity, two had evidence of sensory neuropathy and one had neuromyotonia, but 12 had no abnormality detected. EEG showed slow activity in 11/21, three with focal temporal lobe epileptiform activity and one with cortical disturbance; six patients
had no EEG changes. CSF examination in 30 patients showed a pleocytosis in 13, a raised protein in four and six with oligoclonal bands. Serum GAD antibodies were reported in nine, but were only confirmed on GlyR antibody-positive samples as high titre (\(\geq 1000\) U/ml) in four. One patient had high VGKC-complex antibodies and two patients had low VGKC-complex antibodies, three had NMDA receptor antibodies (one reported in Turner et al., 2011; two low positive) and six had thyroid antibodies. No onconeural antibodies were reported.

| Clinical features | Clinical features at onset n (%) | Clinical features at peak n (%) |
|------------------|---------------------------------|---------------------------------|
| Total number of patients | 45 | 30 |
| Spasms/stiffness/rigidity/myoclonus (neck, trunk or limb muscles) | 31 (69%) | 24 (80%) |
| Oculomotor disturbance: nerve or gaze palsy (eyelid ptosis, diplopia, nystagmus, slow/jerky movements) | 18 (40%) | 16 (53%) |
| Trigeminal, facial and bulbar disturbance (dysphagia, dysarthria, difficulty chewing, facial numbness, trismus) | 21 (47%) | 17 (57%) |
| Excessive startle (spontaneous or triggered by noise or touch) | 19 (42%) | 17 (57%) |
| Walking difficulties/falls, mostly related to stiffness/rigidity/spasms | 19 (42%) | 24 (80%) |
| Limb paresis/pyramidal signs | 10 (22%) | 18 (60%) |
| Limb or gait cerebellar ataxia | 6 (13%) | 6 (20%) |
| Autonomic disturbance (hyper/hypohidrosis, dry mouth, brady/tachycardia, hypo/hypertension, bladder, bowel or sexual dysfunction) | 13 (29%) | 13 (43%) |
| Cognitive impairment/encephalopathy/seizures | 16 (36%) | 15 (50%) |
| Sensory symptoms/pain | 10 (22%) | 14 (47%) |
| Respiratory failure (admission in ICU/ventilation) | 8 (18%) | 8 (27%) |

ICU = intensive care unit.

Table 3 Clinical investigations of GlyR antibody patients referred from 2008–12

| Total number of patients | Investigations (number performed) | 45 Results | Number of abnormal results/number tested |
|--------------------------|-----------------------------------|------------|-----------------------------------------|
| Brain MRI (n = 36)       | n = 3 with white matter lesions; n = 1 atrophy; n = 2 temporal lobe inflammation; n = 2 other FLAIR lesions; n = 2 small vessel disease; n = 26 normal | 10/36 |
| Spinal cord MRI (n = 23) | n = 4 short or patchy lesions; n = 1 longitudinally-extensive lesion; n = 18 normal | 5/23 |
| EMG (n = 29)             | n = 8 continuous motor unit activity; n = 6 spontaneous or stimulus-induced activity; n = 2 sensory neuropathy; n = 1 neuromyotonic discharges; n = 12 normal | 17/29 |
| EEG (n = 21)             | n = 11 slow activity; n = 3 focal epileptic; n = 1 cortical disturbance; 6 normal | 15/21 |
| CSF (n = 30)             | Any abnormality: n = 13 pleocytosis; n = 4 prot (>1 g/l); n = 6 OCB; normal n = 12 | 18/30 |
| GAD antibodies (n = 43)  | 4 >1000 U/ml (~25 000 IU/ml); 1 100 U/ml; 40 negative | 4/45 |
| Chest CT/whole body PET scan (n = 20) | n = 3 thymoma; n = 1 abdominal lymph nodes; n = 1 breast metastases; n = 15 normal | 5/20 |
| Other autoantibodies     | n = 4 high GAD (>1000 U/ml); n = 1 high VGKC (2248 pM); n = 2 low VGKC (<200 pM); n = 3 low NMDAR; n = 6 thyroid; n = 1 ANA; n = 1 Sjogrens Syndrome; 15 normal; no paraneoplastic antibodies reported | 13/28 |

Onconeural antibodies were reported negative by the referring neurologists and were not retested in this study.

ANA = antinuclear antibodies; OCB = oligoclonal bands.

Table 2 Clinical features of GlyR antibody-positive patients referred from 2008–12

Classification

We asked the referring neurologists for their diagnoses but our final classification was based on Meinck and Thompson (2002) defining stiff person syndrome as axial stiffness with or without hypereflexia and/or autonomic disturbance, but no other apparent brainstem involvement, and PERM as stiff person syndrome but with clear brainstem involvement, sometimes with additional ataxia or seizures. We classified those with only brainstem
features, epilepsy or limbic encephalitis as separate categories even though in some patients immunotherapies might have prevented them progressing to a diagnosis of stiff person syndrome or PERM.

The physicians’ diagnoses and our classification, with GlyR antibody titres in serum, and CSF when available, are shown for each patient in Table 4. Thirty-three patients were classified as PERM, two as stiff person syndrome (one with seizures), two with brainstem involvement (one respiratory, one auditory and balance problems), five with limbic or other encephalopathy without brainstem or spinal cord features, and three as ‘other’; the latter included two with probable demyelinating syndromes, and one with slowly progressive cognitive impairment and dizziness of unclear origin. In the majority of the patients the diagnosis of the referring neurologist was similar to our classification. However, there were two PERM patients who presented with myasthenia gravis-like weakness and stimulus-sensitive myoclonus, one with a history of Hodgkin’s lymphoma, the other with a thymoma; neither had acetylcholine receptor or MUSK antibodies. One PERM patient with co-existing high VGKC-complex antibodies was originally diagnosed as Morvan’s syndrome; another PERM patient had a diagnosis of post-encephalitic Parkinson’s disease but made a striking recovery with immunotherapies. Although the higher GlyR antibodies tended to be found in the patients with PERM or brainstem encephalitis, there was no clear relationship between GlyR antibody titre and clinical picture (Table 4).

The four children were female, and although two had PERM (Damasio et al., 2013; Chan et al., in press), one had an epileptic encephalopathy (Hacohen et al., 2013) and the other optic neuritis/acute demyelinating encephalomyelitis (ADEM).

Severity at onset, treatments and outcomes

Modified Rankin scores were provided by the neurologists on the questionnaires and by e-mail after subsequent follow-up. The scores and immunotherapies are given in Table 4. Although at maximum severity three patients had mild (score 2), and four had only moderate (score 3) disability, the remaining 38 patients were graded as 4 (n = 12) or 5 (n = 26). Twenty-four of thirty-three (72%) patients with PERM were graded as modified Rankin scale 5.

Most patients were given symptomatic treatments that were often helpful. The patients with neoplastic disease at presentation were treated with surgery (two removal of thymoma), or chemotherapy (one B cell lymphoma) and improved dramatically (immunotherapies also given). The remaining patient (myasthenia gravis-like presentation) with new radiological diagnosis of thymoma died before surgery as a result of a pulmonary embolism.

Immunotherapies were given in 37 patients (none given in seven, information unavailable in one; Table 4). Approaches to immunotherapy were variable but typically started with high dose (e.g. 1 mg/kg) prednisolone, often preceded by intravenous methyl-prednisolone, and followed by plasma exchange or intravenous immunoglobulin, or both; the latter were often repeated and the steroids generally weaned slowly. Three had additional cyclophosphamide, one received cyclosporine and two rituximab (one as part of his lymphoma treatment) before discharge (Table 4). A few are on azathioprine or mycophenolate.

Duration of follow-up in surviving patients was from 18 months to 7 years (median 3 years). The modified Rankin scale scores at latest follow-up are included in Table 4, and shown graphically stratified according to disease classification (Fig. 2A) or immunotherapies used (Fig. 2B). The outcomes were generally very good with the modified Rankin scale scores falling from a median of 5 at maximum severity to 1. However, four patients had died in hospital, two during the acute illness (one in Turner et al., 2011) and two from indirect causes (one pulmonary embolism while recovering from respiratory failure, one after a fall while on warfarin); these patients are included in Fig. 1A but not in Fig. 2B because long-term follow-up was not available. In addition, two who had made good responses, died subsequently of non-neurological conditions (one recurrence of breast cancer metastases, one systemic oedema with cardiac and renal failure of unknown cause). Another patient had a cardiac arrest during his illness (Mas et al., 2011) and was in a vegetative state when last known. Three case histories, not previously reported, describe some of the clinical features and the variable treatments required (Supplementary material).

Relapses

Relapses following successful treatment have occurred in the index case (Hutchinson et al., 2008) 9 years after good recovery, presenting with worse mobility, some limb stiffness but no recurrence of ophthalmoplegia or hyperekplexia. The patient is on mycophenolate and intravenous immunoglobulin and has a baclofen pump. Another male patient had had two episodes treated with steroids before the third, when the GlyR antibodies were first detected (Peeters et al., 2013). A year after discharge, while receiving steroids (8 mg ad) and plasma exchange every 3–4 months, he had a relapse (fourth event) with dysphagia, diplopia, bilateral ptosis, neck rigidity and severe slowing of vertical saccades. He was given intravenous methyl-prednisolone followed by tapering oral prednisolone, and azathioprine (100 mg daily) was commenced. Current modified Rankin scale is 1. Another patient with similar relapse history is described in the Supplementary material (Case 1). Three other patients have had relapses following discharge and good recovery. These and ongoing immunotherapies are summarized in Table 4.

Characterization of glycine receptor antibodies

Serum and CSF GlyR antibodies were found to be predominantly of the complement-fixing IgG1 subclass with some IgG3 and, as expected for this IgG subclass, the antibodies were able to activate complement on the cell surface of live GlyR-α1 expressing HEK cells, as shown by the deposition of C3b (Fig. 1F). We also looked for the ability of the antibodies to induce temperature-dependent internalization of the GlyRs. Incubation with PERM sera (1:80) at 37°C, but not at 4°C or when the cells were fixed before serum incubation at 37°C, resulted in a reduction in surface IgG binding.
| Sex, age | Physicians diagnosis | Our classification | Serum titre | CSF titre | Tumours newly identified or in past history | mRS maximum | Immuno-therapies | mRS final | Second line or ongoing treatments | Relapses after GlyR antibody identification and immuno-therapies |
|----------|----------------------|-------------------|-------------|-----------|--------------------------------------------|-------------|-------------------|-----------|-------------------------------------|-----------------------------------------------------|
| M,28     | PERM + seizures Turner et al. (2011) | PERM              | 1280        |           | No tumour identified                       | 5           | None              | 6         | Died                                |                                                      |
| M,68     | PERM                 | PERM              | 640         |           | No tumour identified                       | 5           | St, PEx, Ivlg     | 6         | Died                                |                                                      |
| M,56     | PERM                 | PERM              | Not titrated|           | No tumour identified                       | 5           | St, PEx, CyP      | 6         | Died                                |                                                      |
| M,34     | PERM/MG-like plus myoclonus | PERM              | 160, high GAD-Ab | 80       | Thymoma                                     | 5           | Not known         | 6         | Died after PE before thymectomy, died subsequently unclear cause | No relapse, died subsequently unclear cause |
| M,75     | PERM                 | PERM              | 1280        | 80        | Thymoma                                     | 5           | PEx               | 1         | Thymectomy                          |                                                      |
| M,49     | PERM Clerinx et al. (2011) | PERM              | 20 (post PEx) |           | Thymoma                                     | 5           | St, PEx           | 0         | Thymectomy                          | No relapse, died later from breast cancer metastases |
| M,72     | PERM/Morvan's syndrome | PERM              | 640         |           | Marginal zone B cell lymphoma                | 5           | St, PEx           | 1         | Rtx, CyP with lymphoma treatment    | No further relapses                                 |
| F,65     | PERM                 | PERM              | Not titrated|           | Metastases from PH breast cancer            | 5           | St, PEx           | 1         | No relapse                          |                                                      |
| F,50     | Brainstem syndrome (1999), seizures (2007), atypical SPS | PERM              | 80, high GAD-Ab |           | PH breast cancer 1999, thymoma 2007         | 4           | PEx               | 1         | Rtx, CyP                            | No further relapses                                 |
| M,51     | PERM/MG-like plus myoclonus | PERM              | 320         | 80        | PH Hodgkin’s lymphoma                       | 3           | St, Ivlg          | 4         | St                                  | Relapsing-remitting course One relapse No subsequent relapse |
| M,37     | PERM Peeters et al. (2013) | PERM              | 1280        | 640       | No tumour identified                       | 5           | St, PEx           | 1         | Aza                                  | One relapse                                           |
| M,29     | PERM Supplementary Case 1 | PERM              | 160         | 160       | No tumour identified                       | 5           | St               | 1         | St/Aza                              | No relapse                                           |
| M,58     | PERM Piotrowicz et al. (2011) | PERM              | 2560        |           | No tumour identified                       | 4           | St, PEx, Ivlg     | 1         | Aza then Myc                        | One relapse                                           |
| F,14     | PERM                 | PERM              | 640         | 80        | No tumour identified                       | 4           | St, PEx, Ivlg     | 1         | One relapse                         | One relapse                                           |
| M,54     | PERM Hutchinson et al. (2008), Sample from relapse | PERM              | 2560        | 5         | No tumour identified                       | 5           | St, PEx, Ivlg     | 2         | Myc, Ivlg post relapse              | One relapse                                           |
| M,40     | PERM Stern et al. (2014) | PERM              | 1280        |           | No tumour identified                       | 5           | St, PEx, Ivlg     | 3         | One relapse                         | One relapse                                           |
| M,48     | PERM Mas et al. (2011) | PERM              | 640         |           | No tumour identified                       | 4           | St, Ivlg          | 2         | CyP, reducing St                   | No relapse but still improving                        |
| M,54     | PERM/rhomboencephalitis Supplementary Case 2 | PERM              | 80          | 20        | No tumour identified                       | 5           | St, PEx, CyP      | 2         | CyP, reducing St                   | No relapse but not complete recovery                 |
| F,61     | Post-encephalitic parkinsonism | PERM              | 640         |           | No tumour identified                       | 5           | St, Ivlg          | 2         | No relapse                          |                                                      |
| F,61     | PERM                 | PERM              | 20          |           | No tumour identified                       | 5           | Ivlg              | 0         | St, Ivlg continuing                 | No                                                  |
| F,28     | Brainstem encephalomyelitis Supplementary Case 3 | PERM              | 1280        |           | No tumour identified                       | 4           | None              | 0         | No                                  | No                                                  |
| F,61     | PERM without startle lizuka et al. (2012) | PERM              | 1280        | 640       | No tumour identified                       | 5           | St, Ivlg, CySp    | 0         | No                                  | No                                                  |
| F,47     | PERM with seizures   | PERM              | 80          |           | No tumour identified                       | 5           | Ivlg              | 1         | No                                  | No                                                  |

(continued)
Table 4 Continued

| Sex, age | Physicians diagnosis | Our classification | Serum titre | CSF titre | Tumours newly identified or in past history | mRS maximum | Immuno-therapies | mRS final | Second line or ongoing treatments | Relapses after GlyR antibody identification and immuno-therapies |
|----------|----------------------|--------------------|-------------|-----------|------------------------------------------|-------------|-----------------|-----------|----------------------------------|---------------------------------------------------------------|
| M,69     | PERM                 | PERM               | 160         |           | No tumour                                | 5           | St, IvIg        | 1         |                                  | No                                                             |
| F,1      | PERM Damasio et al. (2013) | PERM         | 320         |           | No tumour                                | 5           | St, IvIg        | 1         | Myc + IvIg reducing IvIg         | No                                                             |
| F,40     | PERMS/Jerking SPS    | PERM               | 160         |           | No tumour                                | 3           | St             | 1         |                                  | No                                                             |
| M,39     | SPS                  | PERM               | 640         |           | No tumour                                | 3           | PEx, IvIg       | 1         |                                  | No                                                             |
| F,33     | PERM Mas et al. (2011) | PERM             | Not titrated |           | No tumour                                | 4           | St, IvIg        | 1         |                                  | No                                                             |
| M,50     | PERM                 | PERM               | 80          |           | No tumour                                | 5           | St, IvIg        | 2         | No                               | No                                                             |
| M,56     | PERM Bourke et al., (2014) | PERM           | 60000       | 80        | No tumour                                | 5           | St, IvIg        | 2         | Reducing St                       | No                                                             |
| M,49     | SPS atypical         | PERM               | 80          |           | No tumour                                | 4           | PEx             | 2         | Reducing St                       | No                                                             |
| M,53     | SPS                  | PERM               | 80          |           | No tumour                                | 5           | St             | 1         | LFU                              | No                                                             |
| M,60     | PERM plus Mas et al. (2011) | PERM         | 200         |           | No tumour                                | 5           | None            | 5         | LFU                              | No                                                             |
| F,22     | SPS                  | SPS                | 320         |           | PH thymoma, lymphoma                      | 4           | St             | 0         |                                  | No                                                             |
| F,53     | Epileptic encephalopathy | SPS             | 80, high GAD-Ab | 5        | No tumour                                | 4           | St, PEx        | 3         | Chronic course                   | No                                                             |
| F,5      | Epileptic encephalopathy | Epileptic     | 2560        |           | No tumour                                | 5           | St, PEx, IvIg   | 3         |                                  | No                                                             |
| M,25     | Limbic encephalitis with status epilepticus | Epileptic encephalopathy | 640         |           | No tumour                                | 5           | St, IvIg        | 1         |                                  | No                                                             |
| F,55     | Limbic encephalitis  | Epileptic encephalopathy | Not titrated |           | No tumour                                | 4           | IvIg            | LFU       |                                  | No                                                             |
| F,31     | Meningo-encephalitis | Epileptic encephalopathy | 40          |           | No tumour                                | 4           | None            | 3         | No known relapse                 | No                                                             |
| F,48     | Recurrent encephalopathy | Encephalopathy recurrent | 80          |           | No tumour                                | 3.0         | None            | 2         | No known relapse                 | No                                                             |
| F,58     | Mild PERM with hypoventilation episode Bourke et al. (2014) | Brainstem encephalitis with respiratory failure | 1280        |           | No tumour                                | 4           | None            | 0         |                                  | No                                                             |
| M,68     | Steroid-responsive deafness predominantly | Brainstem encephalitis with autoimmune deafness | 10240       |           | No tumour                                | 2.0         | St             | 1         | Aza                              | No                                                             |
| F,8      | ADEM with optic neuritis | Other          | 320         |           | No tumour                                | 5           | St, IvIg        | 1         |                                  | No                                                             |
| M,28     | Chronic relapsing inflammatory optic neuritis | Other          | 640         |           | No tumour                                | 2.0         | St             | 0         |                                  | No                                                             |
| F,70     | Slow cognitive decline, not Alzheimer’s disease | Other          | 100         |           | No tumour                                | 2.0         | None            | 1         |                                  | No                                                             |

ADEM = acute demyelinating encephalomyelitis; St = steroids; IvIg = intravenous immunoglobulins; PEx = plasma exchange; Myc = mycophenolate mofetil; Aza = azathioprine; CyP = cyclophosphamide; CySp = cyclosporine; PH = past history; mRS = modified Rankin scale; SPS = stiff person syndrome; MG = myasthenia gravis; PE = plasma exchange; Rtx = rituximab; LFU = lost to follow-up.

Some samples were insufficient for titration.
pre-adsorption of the GlyR antibodies against HEK cells expressing GlyRα1, GlyRα2 or GlyRα3 subunits, eliminated or substantially reduced binding to GlyRα1 indicating that the majority of the GlyR antibodies bound to an epitope common to all three α subunits (data not shown).

Binding of sera to rodent brain and spinal cord sections

GlyRα subunit expression in rodent brain was first examined using monoclonal antibodies to GlyRα1, GlyRα2 and GlyRα3 (Supplementary Table 1). The GlyRα1 was strongly expressed in the neuropil and on the cell surface of specific neurons in the brainstem and spinal cord, with no detectable expression in the hippocampus, cerebral or cerebellar cortex. GlyRα2 and GlyRα3 binding was mainly intracellular and in additional areas such as the hippocampus, striatum, cerebellar cortex and layers III–VI of the cerebral cortex. GlyRα3 was also found on dendrites in the hippocampus, granular cell layer of the cerebellum, brainstem and spinal cord (Supplementary Fig. 2).

Patient’s sera were then examined for binding to rat brain sections (at 1:200 to 1:800 dilution) and co-localization with the commercial antibodies. All the sera bound in a punctate manner to the cell bodies and neuropil of the brainstem and both ventral and dorsal horns of the spinal cord, and co-localized with monoclonal antibodies to GlyRα1 (Fig. 4A and B). This was clearly seen on large neurons, co-stained with antibodies to MAP2, in the pedunculo reticular complex in the brainstem (Fig. 4C), and on motor neurons co-stained with antibodies to choline acetyltransferase in the spinal cord (Fig. 4D). Dorsal horn neurons were also clearly stained (data not shown). The CSF staining patterns were similar to those observed with sera (Fig. 4E and F). In addition, the four AD antibody-positive sera bound in an uneven punctate distribution to the molecular and granular cell layer of the cerebellum and in the molecular and pyramidal striatum in the cornu ammonis and dentate gyrus of the hippocampus (Supplementary Fig. 3). This intracellular binding co-localized with GAD. Pre-adsorption experiments with mock-transfected or GlyRα1-expressing HEK cells, or with soluble GAD, confirmed the specificity of each antibody (Supplementary Fig. 3). Other sera, in addition to GlyRα1 co-localization, also bound to intracellular antigens that were widely distributed and were not adsorbed by GlyRα1-HEK cells or soluble GAD (data not shown). Representative results of seven sera are summarized in Supplementary Table 2.

Retrospective cohort of patients with stiff person syndrome and related syndromes

We also analysed sera and/or CSFs archived over many years from patients (17 males, 34 females; ages 13–72 years) with diagnoses of stiff person syndrome (n = 21, including two with stiff leg syndrome), PERM (n = 24) or suspected acquired hyperekplexia (n = 6). Thirty of the 47 tested were positive for GAD antibodies.
GlyR antibodies were found in six sera and four CSFs. Only one of the seven patients with GlyR/C11 antibodies also had raised GAD antibodies at a high level (>1000 U/ml). The clinical details of these patients and treatment responses are given in Supplementary Table 3.

**Discussion**

GlyR antibodies have only recently been recognized. Here we document the first prospectively-diagnosed cohort of 45 patients, classifying their clinical syndromes and characterizing...
their antibodies. Thirty-three patients were classified as PERM, but the disorders of eye movements, speech or swallowing required exclusion of myasthenia in two patients. Autonomic disturbance was marked in many patients and respiratory failure may have contributed to two of the four hospital deaths. A few of the patients had seizures or other supratentorial involvement but in addition there were five patients who only had encephalopathies with seizures and two with predominant brainstem involvement; three had unclear or other diagnoses. Despite the delay in a specific diagnosis in some cases, and the variable immunotherapies used, the majority of patients showed a good or very good response to these treatments, contrasting with previous studies of PERM, but relapses have occurred subsequently in six.

The patients were identified by the presence of a positive GlyR antibody but the final clinical classification was based principally on Meinck and Thompson (2002) and Espay and Chen (2006) defining PERM as brainstem involvement in addition to the axial or limb rigidity typical of stiff person syndrome in its varied forms (Brown and Marsden, 1999). Stiff person syndrome may evolve into PERM (Meinck and Thompson, 2002; Espay and Chen 2006) and we did find very high GAD antibody levels, typically associated with stiff person syndrome, in three patients with diagnoses of PERM (one in Iizuka et al., 2012), one of whom had a previous diagnosis of stiff person syndrome. The presence of both antibodies has also been reported in a proportion of archived adult and paediatric patients (Clardy et al., 2013; McKeon et al., 2013) similar to those in the historical cohort that we tested here.

Figure 4 GlyR antibody-positive patient serum from a typical PERM patient binds to CNS regions involved in motor regulation. (A) Top row: Double labelling of neurons in the pontine reticular nucleus (PnC) of the brainstem with patient serum (green) and monoclonal antibody to the GlyRa1 (red) show colocalization on the neuronal soma and in the neuropil (arrows); nuclei are stained with DAPI (blue). Middle row: Healthy control serum did not bind to the pontine reticular nucleus. Lower row: After pre-adsorption against HEK-GlyRa1 cells there was a marked decrease in pontine reticular nucleus staining. (B) Similar results are shown for binding to the ventral horn of the spinal cord. (C and D) Confocal images show the patient’s serum (green) colocalizing with GlyRa1 monoclonal antibody (red) on the surface of a large neuron in the pontine reticular nucleus, which is labelled with the cytoplasmic neuronal marker anti-MAP2 antibody (purple), or on a motor neuron in the ventral horn of the spinal cord labelled with motor neuron marker anti-choline acetyltransferase antibody [CHAT (purple)]. (E and F) CSF from the same patient binds in a similar pattern. Results of seven representative patients are summarized in Supplementary Table 2. HC = healthy control.
Table 3: Clardy et al detectable serum antibody in retrospective cohorts (Supplementary for detailed study (Fig. 1E). It seems that high CSF levels are thesis of the specific GlyR antibodies in three of the six available sera and CSFs and there was exceptionally high intrathecal syn-

Nevertheless, the original case (Hutchinson et al., 2008) examined at relapse in 2011 had low CSF GlyR antibodies (1:5) compared with very high serum levels (1:2560; Table 4). The relationship between serum and CSF levels of this antibody and clinical status is complex as for other cell surface antibodies.

Because the antibodies were predominantly IgG1 and deposited complement on GlyR-transfected cells, it is possible that complement-mediated mechanisms contribute to the disease pathology in vivo. However, NMDAR antibodies are predominantly IgG1 (Irani et al., 2010), but do not seem to cause complement-mediated damage (Dalmau et al., 2008; Tuzun et al., 2009). Another potential mechanism would be loss of GlyR by internalization following divalent antibody binding; this occurred in GlyR-transfected HEK cells, with a time course similar to that reported for antibodies to other cell surface antigens such as acetylcholine receptors (Drachman et al., 1978), although not muscle-specific kinase (Koneczny et al., 2013). Internalization has been shown for NMDAR antibodies in live neurons in culture (Dalmau et al., 2008; Hughes et al., 2010), but obtaining cultured inhibitory neurons in sufficient numbers for quantitative analysis of internalization of GlyRs is not easy. Regardless of the pathogenic mechanisms involved, which could include a direct inhibition of GlyR function, the normality in the MRI images and the substantial recovery after immunotherapies in the majority of patients argues against a de-

In conclusion, detection of GlyR antibodies may prove helpful in the diagnosis of patients with symptoms and signs that include ocular motor and other brainstem dysfunction, hyperekplexia,
stiffness, rigidity, myoclonus and spasms, and their detection will support the use of immunotherapies that are likely to be clinically effective. Investigations (e.g. CSF, MRI, EEG and EMG) may be normal, but neoplasia, particularly thymomas and lymphomas are not uncommon. Although in the majority of cases serum antibodies are sufficient, paired CSFs are valuable for comparison and can be helpful in rare cases when serum levels (after dilution to 1:20) are low (Supplementary material; Case 2). The detection of the antibodies in encephalopathic or demyelinating syndromes, or with brainstem features of auditory or vestibular dysfunction, suggests that there can be limited or more diverse phenotypes. Finally, involvement of autonomic and respiratory systems in many patients may be responsible for unexplained deaths, and relapses may occur following successful treatment, indicating that long-term follow-up and maintenance immunosuppression should be considered.

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Conflict of interest

The University of Oxford holds patents and receives royalties and payments for antibody tests, and AV, PW and BL receive a share of royalties for VGKC-complex antibodies. There is no patent on glycine receptor antibodies.

Supplementary material

Supplementary material is available at Brain online.

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Appendix 1

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