**SUPPLEMENTARY DATA**

**1. Questionnaire distributed to participating clinicians**

Patient’s code………………………….; Date of Birth…………………….; Sex: M / F

Clinician’s name…………………………Nationality of patient…………………………..

Tumour diagnosed? Y / N. If yes, which tumour?...............................................................

Days from neurological symptoms until tumour diagnosis……………………………………

Days from neurological symptoms until positive antibody result…………..............................

|  |  |  |
| --- | --- | --- |
|  | Days from  onset | Please provide further details/comments |
| First symptom(s) | 0 |  |
| Associated infection or other precipitant  (preceding or concurrent) |  |  |
| Headache |  |  |
| Fever or hypothermia |  |  |
| Higher cognitive dysfunction  – e.g. confusion/ disorientation / amnesia |  |  |
| Psychiatric symptoms - e.g hallucinations,  personality change, psychotic, depressive |  |  |
| Coma – occurring over what time period? |  |  |
| Did patient require sedation?  Were they on ITU? |  | Why? |
| Autonomic disturbance e.g. tachycardia,  sweating, constipation |  |  |
| Brainstem signs or deafness? |  |  |
| Seizures – please note  type/semiology and frequency |  |  |
| Movement disorders (incl. classification  and site e.g. orofacial choreiform) |  |  |
| Startle, rigidity or cerebellar disease? |  |  |
| Sleep disturbance e.g hypersomnia,  REM-SBD |  |  |
| Pain e.g. painful neuropathy |  |  |
| Peripheral nerve symptoms and  EMG/NCS results |  |  |
| Complications of disease / hospital stay  e.g. infection/ PE |  |  |
| Past medical and family history |  |  |
| Positive blood tests e.g. paraneoplastic or  anti-thyroid / infectious agent antibodies |  |  |
| Negative blood tests |  |  |
| EEG – please note timings if >1 available |  |  |
| MRI – please note timings if >1 available |  |  |
| Whole body imaging results |  |  |
| CSF: cells, protein, OCBs, PCR, serology.  Please include sequential data if available |  |  |
| Other clinical symptoms, signs or test  Results |  |  |

Days spent in hospital………………………………………………………….

Outcome – please choose 3 timepoints to record date, treatment, score\*\* and residual features

|  |  |  |  |
| --- | --- | --- | --- |
| Day from onset | D = | D = | D = |
| Treatments |  |  |  |
| Score\*\* |  |  |  |
| Residual features |  |  |  |

\*\*

0. Asymptomatic patient;

1. Symptoms do not interfere with lifestyle;

2. Symptoms lead to some restriction of lifestyle but do not prevent totally independent existence;

3. Symptoms significantly interfere with lifestyle or prevent totally independent existence;

4. Symptoms prevent independent existence, but patient does not need constant attention day & night;

5. Severe disability, with patient totally dependent and requiring constant attention day and night;

6. Death due to the encephalopathy

(Modified Rankin scale from Graus et al 2001, Brain 124;1138-48)

**2. Methods relating to neuropil antibody detection and hippocampal cultures**

**Antibodies to hippocampal neuropil**

This was performed as previously described (Niehusmann et al., 2009). Undiluted CSF or serum diluted 1:500 were incubated with cryoprotected brain sections of 4% paraformaldehyde-perfused rats for 3 hours at 37˚C. Bound antibodies were detected by biotinylated anti-human IgG (1:200, made in sheep, Amersham Pharma Biotech, Uppsala, Sweden). Labelling was visualized with 3,3 diaminobenzidine-tetrahydrochloride.

**Antibodies binding to primary hippocampal cultures**

Primary hippocampal neuronal cultures were prepared from P1 rat pups. Briefly, hippocampi were dissected and collected in chilled HBSS (Hanks’ Balanced Salt Solution) with penicillin/streptomycin/amphotericin (PSA), and incubated in 1% trypsin-EDTA solution at 37°C for 30 minutes. Hippocampi were triturated in complete MEM (Minimal Essential Medium, Sigma-Aldrich, UK) with 10% foetal calf serum (FCS) and PSA. After low speed centrifugation (1000 rpm, 4 minutes), the cells were resuspended in complete MEM and plated on poly-L-lysine-coated glass coverslips. Cultures were grown at 37°C in a humidified 5% CO2 atmosphere. Twice-weekly, half of the medium was replaced with Neurobasal culture medium (Invitrogen, UK), supplemented with glutamine, PSA and B27 (Invitrogen, UK).

After 10 days in vitro the unfixed cells were incubated with patients’ sera (diluted 1:250 in Neurobasal medium) for 1 hour at room temperature, followed by washing, 3% formaldehyde fixation and incubation with Alexa Fluor 488 anti-human IgG (Invitrogen-Molecular Probes, Paisley, UK) for 40 minutes. In a subsequent step, the cells were permeabilised with 0.3% Triton X-100 in PBS for 15 min at room temperature and incubated with mouse monoclonal anti-MAP2 (1:500, Sigma-Aldrich, UK) for 1 hour, followed by incubation with Alexa Fluor 568 anti-mouse IgG (Invitrogen-Molecular probes, Paisley, UK) for 40 minutes. Cells were mounted and visualized as for the cell based assay.

**3. Vignettes accompanying Figures 4A to F.**

4A. A 23 year-old male developed anxiety attacks and polymyoclonus with normal brain MRI and CSF examination. Initially thought to be of psychogenic origin, NMDAR-Abs were found to be positive and prompted treatment with plasma exchange, intravenous immunoglobulins and prednisolone. After a stormy course, this patient has shown a complete improvement.

4B. A 16 year-old female developed an abrupt onset of delusions and dysphasia. By day 18 she had developed dystonic arm posturing, generalised rigidity and bruxism. She developed cardiac asystole at this time. Her syndrome was recognised and serologically confirmed rapidly and she had prednisolone administered early. This was weaned as she improved. However, she had a persistent NMDAR-Ab titre once therapy was discontinued.

4C. A 25 year-old female presented with severe agitation, confusion and hypersomnolence. By day six she had spontaneously lost consciousness and by day ten she had orofacial and upper limb dyskinesias. She was treated with prednisolone, plasma exchange and oophorectomy but sadly died. Her antibody titres remained very high throughout the course of her disease.

4D. A 41 year-old male presented with psychosis, depression, obsessionality and seizures. Over the next three weeks he developed involuntary limb and orofacial movements and a reduced conscious level, necessitating ventilation. Cerebrospinal fluid demonstrated a lymphocytosis early in the disease and oligoclonal bands were not seen until day 100. After 120 days he received treatment with steroids, intravenous immunoglobulins and plasma exchange but unfortunately died after almost one year. He had no tumour detected after multiple attempts at whole body imaging.

4E. A 21 year-old female presented with a generalised tonic-clonic seizure and an acute psychosis with prominent visual hallucinations. She went on to suffer an oculogyric crisis, generalised dyskinesias and labile blood pressures. She received no immunotherapies and over three years recovered to return home, with significant support. However, one year later she relapsed with confusion and seizures. Her diagnosis was subsequently recognised and serologically confirmed in samples from her first and second episodes. Immunotherapy during this relapse produced a clinical improvement in concordance with a reduction in her NMDAR-Ab titres. She had no ovarian lesion detected by imaging on three occasions.

4F. A 23 year-old female developed an upper respiratory tract infection three days prior to the onset of personality change, hypersomnia and amnesia. She recovered to almost normality, albeit over five months, after receiving only three days of intravenous steroids. She subsequently relapsed with a psychosis. She was prospectively diagnosed with NMDAR-Abs during this relapse and a stored serum sample confirmed the presence of NMDAR-Abs during her initial presentation. She continues to improve after plasma exchange and now rituximab.

**4. Legends for supplementary data**

Figure 1. The cell-based assay used in diagnosis.

A. Binding to NMDAR-expressing HEK cells was scored from 0 – 4 by visual observation (two independent observers). Binding is scored as 0 (control serum), 1, 2 and 3 (NMDAR Ab positive patients). Red = anti-human IgG and green = enhanced green fluorescent protein expression (magnification x 200). B. The transfections were based on the increased ratio of NR1:NR2B cDNA that gave stronger binding for positive sera when we used a 3:1 NR1:NR2B cDNA ratio for transfection. C. From the first 450 referred sera (black bars), 50 were found to be NMDAR-Ab positive (score of ≥1) on this cell based assay (CBA). Inter-observer reliability (kappa statistic) = 1 (p<0.0001, n = 44 samples from these assays). 35 CSF samples paired with sera were also tested in parallel (white bars).

Figure 2. Evidence for NR1 as the main epitope

**A.** To determine the size of the EGFP-NR1/NR2B complexes solubilised from transfected cells, we performed **s**ucrose density gradient centrifugation and measured the green fluorsence in each of the fractions. Each column had a sucrose density gradient of 5-20 % sucrose, that examines protein complexes smaller than 1000 kDa. 250 ml of NR1-EGFP/NR2B/PSD95 extract was loaded on each column. The columns were spun for 21 h at 38 000 rpm (Rotor SW41 TI) and fractions of around 300 µl were then collected. The relative green fluorescence was determined (in duplicate runs) using 472 nm for excitation and 512 nm for emission. In previous runs, standard proteins (68 kDa - 957 kDa) were used to determine which sizes can be detected in the various fractions. The peaks observed in the graph in fractions 25 and 34 correspond to proteins/complexes of 280 and 40 kDa. This corresponds best with a dimer of NR1-EGFP (expected 274 kDa) and monomeric EGFP (27 kDa). A monomeric NR1-EGFP would be expected to give a peak in fraction 30 which was not seen. Below the plots shows that 8900 fluorescent units (FUs) equivalent to one microgram of EGFP (Alpha Diagnostic, EGFP16-R). The solubilised EGFP-NR1 extract was used for the FIPA B. To confirm the specificity of sera for the NR1 subunit, and to check for positivity in the sera negative using the FIPA, further assays were performed as illustrated here, and summarised in Supplementary Table 2. These sera also bound to EGFP-NR1 subunit expressed as the monomer in HEK cells (top panel, magnification X 600), as well as to NR1/2B transfected cells (cotransfected with EGFP, middle panel). Binding to NR1/2B receptor was lost after the serum was immunoabsorped against NR1-subunit expressing cells (lower panel). For this, each serum (1:20 dilution) was incubated with 5 million trypsinised EGFP-NR1-expressing HEK cells for 30 minutes at 4°C.

Figure 3.

Cortical to subcortical progression in individual patients. Data from all individuals showing progression from cortical features (psychiatric (Psy), cognitive (Cog) and seizures (Szs)) to subcortical features (movement disorder (MD), dysautonomia (ANS) and fall in consciousness (FIC)). The individual data for each clinical features are plotted against time for all patients in Figure 6. The numbers above each figure indicate the number of patients who showed a lag of ≥5 days between the clinical features : the number who showed a lag < 5 days. Single points are data from patients who did not develop both features.

Figure 4.

CSF data and clinical course in the six patients with >three CSF samples recorded. Cerebrospinal fluid (CSF) data and clinical correlations in individual patients. Mean time to onset of the clinical features within the first ‘cortical’ stage and the second ‘subcortical’ stage for each of the patients. 1st = time at onset of first ‘cortical’ disease stage; 2nd = time at onset of second ‘subcortical’ disease stage. Circled minus sign = no CSF-specific oligoclonal bands; circled plus sign = CSF-specific, unmatched oligoclonal bands.

**5. Tables**

Table 1. Serum and CSF titrations, ratios and calculation of intrathecal synthesis

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Serum  Microliters | | |  | CSF  Microliters | | | Ratio serum  /CSF  titres | Intrathecal  NMDAR-Ab synthesis\* |
| Patient | 8 | 4 | 2 |  | 50 | 25 | 12.5 |  |  |
| 1 | 3 | 2 | 1 |  | 2 | 2 | 1 | 6.25 | 64.00 |
| 2 | 4 | 3 | 3 |  | 3 | 2 | 1 | 18.75 | 21.33 |
| 3 | 4 | 4 | 3 |  | 3 | 2 | 2 | 9.38 | 42.67 |
| 4 | 2 | 1 | 1 |  | 2 | 1 | 1 | 6.25 | 64.00 |
| 5 | 2 | 2 | 1 |  | 2 | 2 | 1 | 6.25 | 64.00 |
| 6 | 2 | 1 | 1 |  | 0 | 0 | 0 | >50 | <8 |
| 7 | 2 | 1 | 1 |  | 1 | 1 | 0 | 12.50 | 32.00 |
| 8 | 2 | 2 | 1 |  | 2 | 1 | 1 | 6.25 | 64.00 |
| 9 | 2 | 2 | 1 |  | 1 | 1 | 0 | 12.50 | 32.00 |
| 10 | 3 | 3 | 2 |  | 3 | 2 | 2 | 6.25 | 64.00 |
| 11 | 3 | 3 | 2 |  | 1 | 0 | 0 | 50.00 | 8.00 |
| 12 | 3 | 2 | 2 |  | 2 | 2 | 1 | 12.50 | 32.00 |
| 13 | 4 | 3 | 3 |  | 2 | 2 | 1 | 18.75 | 21.33 |
| 14 | 4 | 3 | 3 |  | 3 | 3 | 2 | 9.38 | 42.67 |

Serum and CSF titrations using the cell based assay to determine intrathecal synthesis. Intrathecal synthesis of specific NMDAR Ab is calculated as the amount of (NMDAR Ab in CSF/total IgG in CSF)/ (NMDAR Ab in serum/total IgG in serum). \*Assuming that the total IgG in the serum is 400 times that in the CSF, the mean value calculated here is 46 (nvs <2). If the CSF total IgG is raised, the intrathecal synthesis would be correspondingly reduced.

Table 2. Antibody specificity and clinical features of patients with low CBA values, and patients with atypical syndromes

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Age, sex** | **Tumour** | **NR1/2B CBA** | **NR1-FIPA** | **NR1-only CBA** | **CBA result after absorption against NR1** | **Neuro-**  **psychiatric** | **Seizures** | **MD** | **ANS** | **FIC** | **Syn-drome** | **Treatment response** |
| 59, M | No | 1 | Negative | 1 | 0 | Y | N | Y (OF) | Y | Y | Typical | Y |
| 23, M | No | 1 | Negative | 1.5 | 0 | N | Y | N | N | N | Epilepsy | NA |
| 25, F | No | 1 | Negative | 1.5 | 0 | Y | Y | Y | Y | N | Typical | Y |
| 23, M | No | 1.5 | Negative | 1 | 0 | N | Y | N | N | N | Epilepsy | NA |
| 49, F | No | 1.5 | Negative | 1.5 | 0 | Y | Y | Y | Y | n | Typical | N |
| 26, F | OT | 1.5 | Positive | 1 | .5 | Y | Y | Y (OF) | Y | Y | Typical | NA |
| 17, M | No | 1.5 | Positive | 1.5 | .75 | Y | Y | Y (OF) | Y | Y | Typical | Y |
| 16, F | No | 1.5 | Positive | 1 | .25 | Y | N | Y (OF) | Y | Y | Typical | Y |
| 23, M | No | 2 | Positive | ND | ND | Y | N | Y | N | N | Bizzare MD | Y |
| 17, F | No | 2 | Positive | ND | ND | N | Y | N | N | N | Epilepsy | Y |
| 33, F | No | 2 | Positive | ND | ND | N | Y | N | N | N | Epilepsy | N |
| 49, M | HL | 3 | Positive | ND | ND | Y | Y | N | N | N | Limbic encephalitis | Y |

Clinical characteristics and NR1-antibody specificity of low-positive cases and those with atypical syndromes. CBA = cell-based assay; NR1-FIPA = NR1-EGFP subunit based fluorescent immunoprecipitation assay (see methods), MD = movement disorder, ANS = dysautonomia, FIC = fall in consciousness; OT = Ovarian teratoma; HL = Hodgkin’s lymphoma; ND = not done; OF = orofacial dyskinesias, Typical = typical NMDAR-antibody syndromes including both cortical and subcortical features. Treatment = tumour removal or immunotherapy or both (NA = not available as none were used).