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CENTRAL NERVOUS SYSTEM NEURONAL SURFACE ANTIBODY ASSOCIATED SYNDROMES
Clinical and Laboratory Characterization

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Riassunto dell'attività svolta

Il concetto di sindromi del sistema nervoso centrale (SNC) associate ad anticorpi è relativamente recente. Si ritiene che le classiche sindromi paraneoplastiche neurologiche abbiano una patogenesi immunitaria prevalentemente cellulo-mediata e che gli anticorpi onconeurali costituiscano semplicemente dei biomarker. Sulla base di questo assunto si è ritenuto a lungo improbabile che autoanticorpi potessero determinare malattie del SNC.

Recentemente si è resa sempre più evidente l'esistenza di sindromi del SNC associate ad anticorpi diretti contro antigeni presenti sulla superficie neuronale e pertanto potenzialmente patogenetici. L'identificazione negli ultimi dieci anni di forme di encefaliti autoimmuni con anticorpi diretti contro antigeni della superficie neuronale, ed in particolare le proteine del complesso dei canali del potassio voltaggio-dipendenti (VGKC-complex) e i recettori del glutammato di tipo NMDA, ha dimostrato l'esistenza di sindromi del SNC, generalmente non associate alla presenza di neoplasie, che possono essere anticorpo-mediate e pertanto potenzialmente trattabili. Lo spettro clinico di queste malattie non è stato ancora pienamente esplorato, vi sono altre malattie che devono ancora essere scoperte, e vi sono forme di epilessia o sindromi psichiatriche che potrebbero avere una base autoimmune.

Definiamo questi anticorpi neuronal surface antibodies (NSAbs) e le malattie ad essi associate NSAS (Neuronal Surface Antibody Syndrome).

Lo scopo della ricerca effettuata in questo percorso di tre anni di dottorato è stato la caratterizzazione clinica e laboratoristica di alcune NSAS.

Il primo anno di dottorato è stato effettuato presso l'Università di Oxford sotto la supervisione della Prof. Angela Vincent. Durante questo periodo ho contribuito alla caratterizzazione delle due più importanti forme di NSAS, le encefaliti associate ad anticorpi anti-VGKC-complex e ad anti-NMDAR. I risultati di questi studi sono stati pubblicati (Irani et al., 2010b; Irani et al., 2010c). Il mio personale contributo è consistito nello sviluppo e ottimizzazione di tecniche di immunofluorescenza per l'identificazione di NSAbs, utilizzando sia colture primarie di neuroni ippocampali che cellule di mammifero transfettate con l'antigene di interesse.

Inoltre durante il soggiorno presso il laboratorio di Oxford ho cercato di mettere a punto un approccio di proteomica per l'identificazione di nuovi NSAbs mediante

immunoprecipitazione e spettrometria di massa. Tale approccio è stato successivamente applicato nello screening di pazienti con sindromi cerebellari autoimmuni. I risultati di questo studio sono descritti nella terza parte della tesi.

La tesi di dottorato è divisa in tre parti.
Nella prima parte ho esaminato lo stato dell'arte delle sindromi associate ad anticorpi contro antigeni di superficie iniziando con un confronto tra disordini associati ad anticorpi contro antigeni intracellulari e antigeni di superficie. Ho quindi riassunto i maggiori aspetti clinici e paraclinici delle sindromi che sono state già identificate, e da queste osservazioni ho disegnato delle linee guida per il futuro riconoscimento di queste e altre condizioni potenzialmente mediate da NSAbs. Il lavoro di stesura di queste linee guida è stato supervisionato dalla Prof. Angela Vincent e riesaminato criticamente dai Prof. Francesc Graus (Barcelona, Spain) e Prof. Christian Bien (Bielefeld, Germany) e dal Dr. Bruno Giometto (Treviso, Italy).

La seconda parte della tesi descrive la caratterizzazione clinica e immunologica di una coorte di pazienti con sospetta encefalite autoimmune e anticorpi anti-VGKC-complex che ho raccolto ed esaminato a Padova nei 3 anni di dottorato. Recentemente è stato dimostrato dal gruppo di lavoro di Oxford che la maggior parte degli anticorpi precedentemente attribuiti ai canali del potassio VGKC (mediante immunoprecipitazione) in realtà hanno come bersaglio due proteine canale complessate con i VGKC, Lgi1 e Caspr2; anticorpi diretti contro queste due proteine tendono ad associarsi rispettivamente con encefalite limbica (EL) e sindrome di Morvan (vedi Irani et al 2010). Ho quindi deciso di focalizzare la mia indagine su pazienti con encefaliti autoimmuni associate ad anticorpi anti-VGKC-complex con l'obiettivo di riportare il profilo clinico e immunologico di questi pazienti e possibilmente dimostrare una correlazione tra antigene e fenotipo. Durante i tre anni di dottorato ho analizzato 503 campioni da 366 pazienti con sospetta encefalite autoimmune inviati al nostro laboratorio tra gennaio 2005 e dicembre 2011: 279 campioni di siero (e/o di liquido cerebrospinale) da 232 pazienti sono stati testati per anti-VGKC-complex; inoltre 226 campioni sono stati testati per anticorpi anti-NMDAR, 59 per anti-AMPAR1 e 2 e GABAbR, 91 per anti-GAD; 21 campioni inoltre sono stati testati su neuroni ippocampali. Le metodiche di screening hanno incluso: radioimmunoprecipitazione

(RIA) per anti-VGKC e -GAD65; immunofluorescenza indiretta (IFI) su cellule transfettate con NMDAR, AMPAR1, AMPAR2, GABAbR, Lgi1 and Caspr2; IFI su neuroni ippocampali. I dati raccolti dai pazienti risultati positivi per anti-VGKC-complex sono quindi stati analizzati. Trentadue pazienti su 232 testati sono risultati positivi per anti-VGKC-complex. Informazioni cliniche sono risultate disponibili per 18 pazienti: 10 positivi per anti-Lgi1, 3 per anti-Caspr2 mentre 5 sono risultati positivi su RIA ma non su IFI. Due pazienti hanno presentato un quadro sindromico compatibile con la sindrome di Morvan, in un caso in associazione ad anti-Lgi1 e nell'altro ad anti-Caspr2 e timoma. In tutti i pazienti in cui è stato somministrato un trattamento immunomodulante si è assistito ad un miglioramento clinico con l'eccezione di una paziente deceduta a causa di un microcitoma polmonare. Un titolo anticorpale persistentemente elevato nonostante la remissione clinica in una paziente con EL e anticorpi anti-Lgi1 ha condotto all'individuazione e alla successiva rimozione di un timo iperplastico; un paziente con anti-Caspr2 ha manifestato un'encefalite di grado lieve in concomitanza di una sindrome da anticorpi anti-fosfolipidi; un altro paziente con una forma di encefalomielite responsiva al trattamento è risultato negativo in IFI ma ha presentato un alto titolo anticorpale per anti-VGKC su RIA. Un ipermetabolismo dei gangli della base è stato individuato in 4 pazienti con anti-Lgi1, in un caso associato alla presenza di crisi epilettiche distoniche facio-brachiali. In conclusione i risultati di questo studio hanno esteso lo spettro delle manifestazioni cliniche note associate ad anticorpi anti-VGKC-complex. E' stato dimostrato che le EL con anti-VGKC possono associare non solo a timomi ma anche ad iperplasia del timo, in maniera analoga a quanto avviene nella miastenia gravis. La persistenza di un alto titolo anticorpale dovrebbe pertanto indurre un accurato imaging del timo.

La terza parte della tesi descrive uno studio sperimentale che ha avuto lo scopo di identificare nuovi autoanticorpi nelle sindromi cerebellari di possibile origine autoimmune. La Dr.ssa Ester Becker dell'Università di Oxford ha contribuito in egual misura al sottoscritto in questo studio. Pochi studi in precedenza avevano ricercato anticorpi potenzialmente patogenetici in pazienti non paraneoplastici con atassia cerebellare. Inizialmente 52 pazienti con atassia cerebellare idiopatica sono stati sottoposti a screening mediante immunofluorescenza su neuroni cerebellari. Un siero che ha dimostrato un intensa reattività sui neuroni è stato selezionato per gli esperimenti

di immunoprecipitazione e spettrometria di massa. Tale approccio ha portato all'identificazione di Caspr2 (contactin-associated protein 2) come maggiore antigene. Anticorpi anti-Caspr2 sono stati poi identificati mediante immunofluorescenza su cellule transfettate in 9 pazienti su 88 con atassia (10%), in confronto a 3 pazienti su 144 di controllo affetti da sclerosi multipla o demenza. Caspr2 è altamente espresso nel cervelletto, e solo in parte in associazione ai canali del potassio voltaggio-dipendenti. Studi prospettici saranno necessari per valutare se l'identificazione di anticorpi anti-Caspr2 abbia valore nella diagnosi e trattamento delle sindromi cerebellari.

Summary

The concept of antibody–associated central nervous system (CNS) disorders is relatively recent. The classical CNS paraneoplastic neurological syndromes are thought to be T-cell mediated, and the onconeural antibodies merely biomarkers for the presence of the tumour. Accordingly it was thought that antibodies rarely, if ever, caused CNS disease. Recently, however, it has become increasingly clear that there are CNS syndromes associated with antibodies that bind to cell surface determinants on neuronal cells and are likely to be pathogenic. Over the last ten years identification of autoimmune forms of encephalitis with antibodies against neuronal surface (NS) antigens, particularly the VGKC-complex proteins or the glutamate NMDA receptor, have shown that CNS disorders, often without associated tumours, can be antibody-mediated and benefit from immunomodulatory therapies. The clinical spectrum of these diseases is not yet fully explored, there may be others yet to be discovered, and some types of more common disorders, as epilepsy or psychosis, may prove to have an autoimmune basis. Here, we call these antibodies neuronal surface antibodies (NSAbs), and the diseases associated with them NSAb syndromes or NSAS.

The aim of the research performed in the three years of PhD study has been to characterize NSAS from both a laboratory and a clinical point of view.

The first year of the PhD was spent at the Oxford laboratory under the supervision of Prof. Angela Vincent. During this period I contributed to the laboratory characterization of the two most important groups of NSAS: encephalitis associated with VGKC-complex-Abs and NMDAR-Abs. The results of these studies have been published (Irani et al., 2010b; Irani et al., 2010c). My personal contribution in these studies included the development and optimization of immunofluorescence techniques for the detection of autoantibodies against neuronal surface antigens (NSAbs) using both primary cultures of rat hippocampal neurons and mammalian cells transfected with the antigen of interest.

In addition, during my time at Oxford I investigated a possible novel proteomics approach to identify new NSAb by means of immunoprecipitation and mass spectrometry. These experiments have subsequently been applied to screen samples of patients with autoimmune cerebellar syndromes. The results of this study are described in the third part of the thesis.

The doctoral thesis is divided into three parts.
In the first part I have reviewed the state of the art of syndromes associated with neuronal surface antibodies, starting with a comparison of the conditions associated with antibodies to intracellular antigens and those associated with antibodies to cell surface antigens. I have then summarised the main clinical and paraclinical features of the syndromes that have already been identified. On the basis of these observations, I have designed guidelines for recognizing these and other immune-mediated conditions in the future. The guidelines work has been supervised by Prof. Angela Vincent (Oxford, UK) and critically reviewed by Prof. Francesc Graus (Barcelona, Spain), Prof. Christian Bien (Bielefeld, Germany) and Dr. Bruno Giometto (Treviso, Italy).

The second part of the thesis describes the clinical and immunological characterization of a cohort of patients with suspected autoimmune encephalitis and VGKC-complex-Abs that I collected and reviewed in Padua during the 3-year period of the PhD. Recently the Oxford group showed that most antibodies previously attributed to VGKC (by radioimmunoprecipitation) target two proteins complexed with the channels Lgi1 and Caspr2, which also tend to associate with LE and Morvan's syndrome phenotypes, respectively (see (Irani et al., 2010b)). I therefore decided to focus my research on patients with autoimmune encephalitis associated with 'VGKC'-complex-Abs in order to report their clinical and immunological profile, with a view to showing a possible antigen-phenotype correlation. During the 3-year period of my PhD, I screened 503 samples for NSAbs from 366 patients with suspected autoimmune encephalitis sent to our laboratory between January 2005 and December 2011: 279 serum (and cerebrospinal fluid) samples from 232 patients were tested for VGKC-complex-Abs; in addition 226 samples were tested for NMDAR-Abs, 59 for AMPAR1,2- and GABAbR-Abs, 91 for GAD-Abs; 21 samples were also tested on live hippocampal neurons. Screening methods included: radioimmunoprecipitation (RIA) assay for VGKC-Abs and GAD65-Abs; cell-based assay for antibodies binding human NMDAR, AMPAR1, AMPAR2, GABAbR, Lgi1 and Caspr2; indirect immunofluorescence on live hippocampal cell cultures. Thirty-two of the 232 patients tested for VGKC-complex-Abs were positive. Clinical data were available for 18 patients: 10 patients harboured Lgi1-Abs in their sera, 3 Caspr2-Abs, 5 patients were positive on RIA but not on IIF. Two patients had Morvan's syndrome, one associated with Lgi1-ab and the other with Caspr2-Ab and a thymoma. Response to

immune modulation was reported in all patients with the exception of a patient who died from lung cancer. A consistently high VGKC-complex-Abs titre despite clinical remission prompted the discovery and subsequent removal of an enlarged hyperplastic thymus in a patient with Lgi1-Abs associated LE; one Caspr2-Abs positive patient had mild encephalitis overlapping with an anti-phospholipid syndrome; another patient with an immunotherapy-responsive encephalomyelitis tested negative on IIF but had high-titre VGKC-complex-Abs on RIA. Striatal hypermetabolism was found in 4 patients with Lgi1-Abs in one case associated with faciobrachial dystonic seizures. In conclusion, these results have expanded the clinical spectrum of autoimmune encephalopathy associated with VGKC-complex antibody. It has been shown that VGKC-LE can associate not only with thymomas, but also with a hyperplastic thymus, similarly to the well-known finding in myasthenia gravis and therefore that the persistence of high VGKC-Abs titres should prompt the search for an enlarged thymus.

The third part of the thesis describes a study whose aim was to identify new autoantibodies in cerebellar syndromes of possible autoimmune origin. Dr. Esther Becker from Oxford University contributed equally to me in this work. Relatively few studies have searched for potentially pathogenic antibodies in non-paraneoplastic patients with cerebellar ataxia. We first screened sera from 52 idiopathic ataxia patients for the binding of serum IgG antibodies to cerebellar neurons. One strong-binding serum was selected for immunoprecipitation and mass spectrometry, which resulted in the identification of contactin-associated protein 2 (Caspr2) as a major antigen. Caspr2 antibodies were then found by a cell-based assay in a total of 9/88 (10%) ataxia patients, compared to 3/144 (2%) multiple sclerosis or dementia controls. Caspr2 is strongly expressed in the cerebellum, only partly in association with voltage-gated potassium channels. Prospective studies are now needed to see whether identification of Caspr2-antibodies has relevance for the diagnosis and treatment of idiopathic cerebellar ataxia.

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1. CENTRAL NERVOUS SYSTEM NEURONAL SURFACE ANTIBODY-ASSOCIATED SYNDROMES

1.1 Introduction

Well recognised conditions such as myasthenia gravis (MG) and the Lambert-Eaton myasthenic syndrome (LEMS) have been shown by rigorous experimental approaches to be antibody-mediated. The antibodies are directed against essential membrane receptors or ion channels involved in transmission at the neuromuscular junction; the antibodies bind to extracellular epitopes on the membrane proteins; plasma exchange leads to clear clinical benefit; and both in vitro and passive transfer experiments show that the IgG antibodies are pathogenic (Vincent et al., 2006).

Several antibodies to "onconeural" antigens are found in central nervous system (CNS) disorders associated with cancers (paraneoplastic neurological syndromes)(Dalmau and Rosenfeld, 2008; Giometto et al., 2010; Graus et al., 2004), including antibodies to Hu (Hu-Abs), and many others (Graus et al., 2001). However, as the target of these antibodies are intracellular proteins, and patients do not usually improve with immunotherapy, the pathogenic role is not clear. Rather, it is thought that T-cell cytotoxicity is a more likely mechanism to account for the neuronal cell loss that occurs in these rare but serious conditions. T-cell cytotoxicity could also contribute in patients with antibodies to glutamic acid decarboxylase (GAD-Abs), since these are also directed against an intracellular antigen, but at very high levels are associated with non-paraneoplastic forms of stiff person syndrome (SPS) and other CNS disorders (Graus et al., 2009; Saiz et al., 2008).

Over the last few years it has become increasingly clear that there are CNS syndromes associated with antibodies that bind to cell surface determinants on neuronal cells and are likely to be pathogenic (Lancaster et al., 2011b; Vincent et al., 2011). Here, we call these antibodies neuronal surface antibodies (NSAbs), and the diseases associated with them NSAb syndromes or NSAS. These syndromes can be indistinguishable at presentation from classical paraneoplastic syndromes, such as limbic encephalitis (LE), but one is a newly defined entity, NMDAR-Ab encephalitis (Dalmau et al., 2011). The patients can be

diagnosed by serum/CSF antibody tests, are not so rare, are frequently non-paraneoplastic, and they respond to immunotherapy with a good chance of substantial recovery (Dalmau et al., 2011; Irani et al., 2010c; Irani et al.).

Although these syndromes are beginning to be widely recognised, there are likely to be others for which no NSAb has yet been defined, and in which immunotherapies have not yet been tested. There is a need, therefore, to define guidelines for their recognition so that an immune-mediated basis can be explored. In this review, we start by comparing conditions that are associated with antibodies to intracellular antigens and those that are associated with antibodies to cell surface antigens. We then summarise the main clinical and paraclinical features of the syndromes that have already been identified, and, largely from these observations, suggest guidelines for recognizing these and other immune-mediated conditions in the future. We concentrate on the diseases predominantly affecting the "grey" matter, and will not include those diseases such as neuromyelitis optica and acute disseminated encephalomyelitis in which antibodies to "white" matter glial or myelin antigens have also recently been discovered (Brilot et al., 2009; Lennon et al., 2005).

1.2 General features of diseases associated with antibodies to intracellular versus those with NSAbs

Table 1.1 summarises some features of the CNS autoimmune syndromes according to the presence of onconeural antibodies or NSAbs. Patients with onconeural Abs present at ages which are typical of the tumours, but those with NSAbs can occur at any age. Limbic encephalitis (LE) or a more complex encephalopathy are, to date, the most frequent presentations in the NSAS and more common than either cerebellar degeneration or encephalomyelitis with onconeural/intracellular-Abs. In both categories tumours can be present: SCLC, ovarian and breast cancers with onconeural Abs, and ovarian teratomas and thymomas in the NSAS; but many of the NSAS patients do not have tumours. Evidence of CSF inflammation including oligoclonal bands can be present in both groups but a normal CSF is more common with some of the NSAS (Giometto et al., 2010; Graus et al., 2004; Lancaster et al., 2011b; Vincent et al., 2011).

The most important distinctions relate to the course and treatment responses. Patients with onconeural antibodies usually present subacutely and have a relentlessly progressive course, despite immunotherapies, although there may be stabilisation of the neurological syndrome if tumour treatment is effective (Dalmau and Rosenfeld, 2008). By contrast, patients with NSAbs may have an acute or subacute onset, usually with short duration to nadir, and can make a very good response to immunotherapies, with tumour treatment if required; in many cases immunotherapies can be weaned over a year or two suggesting that the condition is monophasic (Vincent et al., 2011).

It is generally accepted that the onconeural Abs are markers for the immune-mediated process but not pathogenic; T-cell cytotoxicity towards the same or other antigens are thought to be causative mostly based on the presence in the postmortem studies of abundant T-cell infiltrates in the brain parenchyma in close apposition with neurons. The NSAS are not well studied yet, but T-cell infiltration is less conspicuous in the few postmortem studies and plasma cells predominate in the few reports of patients with anti-NMDAR encephalitis (Dalmau and Rosenfeld, 2008; Graus et al., 2009; Vincent et al., 2011).

1.3 Well-defined CNS syndromes associated with NSAbs

Features of the main syndromes recognised so far are summarised in Table 1.2. NSABS were described in subacute cases of cerebellar degeneration associated with small-cell lung cancer (SCLC) and voltage-gated calcium channels (VGCC) antibodies, although the patients did not respond to immunotherapies (Graus et al., 2002), and in a patient with a remote history of Hodgkin disease and mGluR1 antibodies (Sillevis Smitt et al., 2000). Voltage gated potassium channel (VGKC) Abs were identified by immunoprecipitation in Morvans' syndromes and then in non-paraneoplastic LE (Buckley et al., 2001). Thereafter these and other antibodies, designated "Neuropil" antibodies, were also found to label rodent hippocampus by indirect immunohistochemistry or immunofluorescence, using patients' serum or CSF (Ances et al., 2005). By far, the most common of these antibodies are against glutamate receptors of NMDA (Dalmau et al., 2007), while other neuropil antibodies subsequently characterised are against AMPA (Lai et al., 2009),

GABA-B (Lancaster et al., 2010) and mGluR5 receptors (Lancaster et al., 2011c). Recently, it has been shown that antibodies immunoprecipitating VGKCs extracted from mammalian brain do not target the potassium channel, as originally thought (Buckley et al., 2001; Hart et al., 1997; Kleopa et al., 2006), but proteins that are tightly complexed with potassium channels in situ, mainly leucine-rich glioma inactivated 1 protein (LGI1) or contactin-associated protein-2 (CASPR2) (Irani et al., 2010b; Lai et al., 2010; Lancaster et al., 2011a). We now call these antibodies VGKC-complex antibodies generically, or LGI1 and CASPR2 specifically. Contactin-2 is another component of the complex but antibodies to this protein are not so common (Irani et al., 2010b).

1.3.1 Limbic encephalitis

LE is a well-recognised condition characterized by subacute development of short-term memory loss, behavioural change and seizures involving the temporomedial lobes and sometimes the amygdale, with variable evidence of CSF inflammation and neuronal antibodies (Graus et al., 2004; Gultekin et al., 2000). For years it was considered a rare paraneoplastic disorder with a poor prognosis, but it is now recognised that patients with LE are non-paraneoplastic (Ances et al., 2005; Lancaster et al., 2011b; Vincent et al., 2004). In a few cases reported, a single clinical feature (e.g. delirium, psychosis, seizures, amnesia) can be prominent or isolated; therefore, the concepts of autoimmune forms of encephalopathy, psychiatric disorders, epilepsy or dementia are beginning to be explored (Flanagan et al., 2010; Kayser et al., 2010; Vernino et al., 2007; Vincent et al., 2010; Zandi et al., 2010).

1.3.1.1 LE associated with VGKC-complex antibodies

VGKC-Abs-associated LE was the first immunotherapy-responsive NSAb-associated CNS syndrome to be well characterized (Thieben et al., 2004; Vincent et al., 2004) and since then it has become widely recognized. A high proportion of patients with LE have LGI1-Abs, and a few CASPR2-Abs, but there are other VGKC-complex proteins to be defined and the antibodies are best identified by the established radioimmunoprecipitation assay. Around 60% of the patients have MRI evidence of medial temporal lobe inflammation, but pleocytosis or other CSF changes are uncommon, and oligoclonal bands are rare (Irani et al., 2010b). The patients respond within a few weeks to intense

immunotherapies with good or very good outcomes, but even without treatment a few patients have shown spontaneous improvement (Irani et al., 2010b; Irani et al., 2011). Interestingly a distinctive seizure semiology, termed faciobrachial dystonic seizures, can be identified before manifestation of LE (Irani et al., 2011), and these seizures respond rapidly to immunotherapies, which might prevent the onset of cognitive dysfunction and more widespread seizures in future cases (Irani et al., 2011).

A rarer condition associated with VGKC-complex-Abs is Morvan's syndrome, characterized by sleep disorders and psychosis, peripheral nerve hyperexcitability (including neuromyotonia and pain) and dysautonomic features (Josephs et al., 2004; Liguori et al., 2001; Spinazzi et al., 2008). CASPR2-Abs are more common than LGI1-Abs in Morvan's syndrome but some patients have both specificities or neither. Around 40% of Morvan's syndrome patients have tumours, often recurrent or malignant thymomas, sometimes associated with previous myasthenia gravis, and these patients have a poor prognosis. However, those patients without tumours do well with immunotherapies (Irani et al., 2010b).

1.3.1.2 Other NSAbs associated with LE

LE can also associate with antibodies against AMPA- and $GABA_B$ – receptors (Lai et al., 2009; Lancaster et al., 2010). These often have a classical LE phenotype and many have tumours, including small cell lung cancer, thyroid and breast tumours. There may be prominent psychiatric features with AMPAR-Abs and prominent seizures with $GABA_B$R-Abs, but only small cases series have been reported so far (Bataller et al., 2010; Boronat et al., 2011; Lai et al., 2009; Lancaster et al., 2010). $GABA_B$R-Abs are probably the most common antibodies found in LE in association with SCLC previously thought to be "seronegative" for onconeural antibodies (Alamowitch et al., 1997; Boronat et al., 2011). Most of the patients with $GABA_B$R-Abs or AMPAR-Abs who receive immunotherapy and cancer treatment show neurological improvement although relapses have been observed with AMPAR-Abs (Lai et al., 2009; Lancaster et al., 2010).

Another novel NSAb has been recently described against type 5 metabotropic glutamate receptor in two patients with prominent limbic encephalopathy and Hodgkin lymphoma (namely Ophelia syndrome) (Lancaster et al., 2011c).

Although not NSAbs, GAD-Abs have been identified in younger females with a form of LE, presenting mainly with temporal lobe epilepsy and MRI evidence of temporal lobe

inflammation; these patients did not usually respond to immunotherapies but were not treated aggressively at onset (Malter et al., 2010).

1.3.2 NMDAR-encephalitis

The encephalitis associated with NMDAR-Ab is a well-characterized and easily recognisable condition, distinct from the forms of LE described above and probably much more common (Dalmau et al., 2011). However, in distinction to most patients with LE, a high proportion of patients are children or young women who may initially be seen or admitted to psychiatric wards for acute anxiety, behavioural change, or psychosis. Within a few days the presence of seizures is recognised, defining an organic condition, and within days or weeks reduced consciousness, movement disorders, hypoventilation and autonomic imbalance often require admission to intensive care units (Dalmau et al., 2011). Up to 50% of young adult female patients have an ovarian teratoma, but these are less common in children (Dalmau et al., 2011; Irani et al., 2010c). Importantly, MRI is frequently not informative but pleocytosis at onset is very common (Dalmau et al., 2011; Irani et al., 2010c). In children, the disease can present with behavioural disturbance and dyskinesias (Florance et al., 2009) and in the past such patients have often been diagnosed as encephalitis lethargic (Dale et al., 2009). Relapses can occur in 20-25% of non-paraneoplastic patients and they can be separated by months or years (Dalmau et al., 2011; Irani et al., 2010c).

1.3.3 PERM and GlyR-Ab associated conditions

A few patients with a well-recognised but rare condition, progressive encephalomyelitis with rigidity and myoclonus (PERM), which is part of the spectrum of stiff person syndrome (Meinck and Thompson, 2002), have GAD-Abs, but some are now being identified with antibodies against glycine receptors (GlyR-Abs) (Clerinx et al., 2011; Hutchinson et al., 2008; Mas et al., 2010). PERM was initially described as a subacute disorder characterized by muscle rigidity, stimulus-sensitive spasms, brainstem dysfunction, and pathological findings (often post-mortem) of perivascular lymphocyte cuffing and neuronal loss in the brainstem and spinal cord. Generalized myoclonus,

hyperekplexia, cerebellar ataxia, and autonomic dysfunction were later described in several reports (Meinck and Thompson, 2002). A few reports of GlyR-Abs in single cases and a series of three patients show a wide spectrum of features on presentation with often prominent brainstem dysfunction and little MRI or CSF evidence of inflammation (Hutchinson et al., 2008; Mas et al., 2010); one patient presented with an immunotherapy responsive isolated medio-temporal lobe status epilepticus (Zuliani et al., 2011). One had a thymoma, with dramatic improvement after surgery and immunotherapy (Clerinx et al., 2011). Although there are only a few reports to date, patients with GlyR-Abs, in distinction to most of those with GAD-Abs do well on immunotherapies; however, one patient who also had NMDAR-Abs died before testing for either antibody was available (Turner et al., 2011).

1.3.4 Cerebellar ataxia associated with NSAbs

Antibodies against VGCCs were demonstrated to be present in some cases of cerebellar ataxia in association with lung tumours (Graus et al., 2002). However the lack of response to immunotherapies, despite improvement of coexistent Lambert-Eaton myasthenic syndrome, suggests that the antibodies are unlikely to be contributing to the cerebellar pathology or alternatively they cause permanent Purkinje cell damage before treatment can be initiated (Graus et al., 2002) (Fukuda et al., 2003). Antibodies to mGluR1 were initially reported in two patients with subacute cerebellar ataxia and a past history of Hodgkin disease and were shown in passive transfer to lead to ataxia in experimental animals.(Sillevis Smitt et al., 2000) One other patient with this antibody without a tumour and with a partial treatment response has been reported (Marignier et al., 2010).

1.3.5 Proof of the pathogenicity of NSAbs

Despite the very good clinical evidence that the syndromes described above are antibody-mediated, there is little direct experimental evidence to prove this concept. Although there are studies on the effects of the serum or CSF IgG antibodies on neuronal function in cultured cells (Dalmau et al., 2008; Hughes et al., 2010; Lai et al., 2009) or on brain slices (Lalic et al., 2010), the transfer of clinical or electrophysiological evidence of

disease to experimental animals by either systemic or intrathecal injection has not yet been reported, with the exception of mGluR1-Ab in cerebellar ataxia (Sillevis Smitt et al., 2000). Unexpectedly, interesting results have been obtained with the transfer of IgG from patients with antibodies targeting intracellular antigens (as reported below) (Geis et al., 2010; Manto et al., 2007).

1.4 Well-defined CNS syndromes without identified NSAbs

There are several syndromes which are well recognized and generally thought to be immune-mediated but in which a potentially pathogenic antibody has not been defined. Below we remind the reader of these syndromes and recent work that may in the future result in discovery of relevant NSAbs.

1.4.1 Stiff-person syndrome and related disorders

The autoimmune basis of stiff-person syndrome (SPS) (reviewed by Meinck and Thompson (Meinck and Thompson, 2002)) is supported by response to immunomodulation (Dalakas et al., 2001; Meinck and Thompson, 2002), association with organ-specific autoimmune diseases, high titer GAD-Abs (often intrathecally synthesized) (Saiz et al., 2008), or amphiphysin-Abs in rarer paraneoplastic cases (Folli et al., 1993; Murinson and Guarnaccia, 2008). A direct pathogenic role for antibodies against GAD and amphiphysin, both intracellular antigens, is controversial but successful passive transfer to rodents from patients both with GAD-Abs (Manto et al., 2007) and amphiphysin-Abs (Geis et al., 2010) are encouraging, in the latter case with evidence of internalisation of antibodies into the neurons. These experiments suggest that there are pathogenic antibodies that can access the presynaptic nerve terminal but more work needs to be done to define more clearly how this occurs, and the possibility of NSAbs co-existing with GAD-Abs needs to be explored.

1.4.2 Opsoclonus-Myoclonus syndrome

Opsoclonus–myoclonus syndrome (OMS) is a rare disorder characterized by chaotic saccadic eye movements, myoclonus, ataxia, and encephalopathy (Dalmau and Rosenfeld, 2008). It is best characterised in infants who may have neuroblastomas, but can be non-paraneoplastic; the acute disease remits but the children are often left with cognitive and other problems. Immunotherapies appear to be of benefit but no systematic studies have been reported. There is also an idiopathic adult-onset OMS, frequently in females, who have a monophasic course with a good response to IvIg or corticosteroids (Bataller et al., 2001). By contrast, a paraneoplastic form of OMS is more common in older females and associated with breast and SCLC (Bataller et al., 2003). Some evidence of possible NSAbs that are able to induce apoptosis of neuroblastoma cell lines has been shown in children (Blaes et al., 2005; Korfei et al., 2005) but not in adults (Sabater et al., 2008) (Blaes et al., 2008).

1.4.3 Autoimmune cerebellar ataxia

A possible role for autoantibodies in cerebellar ataxia has been hypothesized in other settings, although without identified NSAb.

Post-infectious cerebellitis. These conditions are well-known in children (Connolly et al., 1994) but also adult cases have been reported (Klockgether et al., 1993). Most cases are not associated with any identified antibody although autoantibodies cross-reacting with EBV have been reported (Uchibori et al., 2005).

Cerebellar ataxia associated with antibodies against intracellular antigens. A subacute onset in adults, once other causes are excluded, is suggestive of a paraneoplastic process which generally leads to cerebellar degeneration, and in which onconeural antibodies (eg. Yo-Abs) are merely biomarkers for the immune response against the tumour (Graus et al., 2004). A more insidious course is described in non-paraneoplastic GAD-Ab associated cerebellar syndromes in which an autoimmune mechanism is further supported by CSF inflammation and polyendocrine autoimmunity (Honnorat et al., 2001; Saiz et al., 2008). Other antibodies against intracellular antigens have been reported in patients with non-paraneoplastic cerebellar ataxia (eg. Homer3 (Zuliani et al., 2007a))(Hadjivassiliou et al.,

2008) and also with coexisting coeliac disease/gluten sensitivity (ie. anti-gliadin antibodies cross-reacting with cerebellar antigens) although the latter hypothesis remains controversial (Hadjivassiliou et al., 2003).

1.5 Other possible NSAS

There are many reports of patients in whom a NCS-Ab mediated mechanism may be present even though they do not fit within one of the conditions described above. For example, there are patients reported with epilepsy (Giometto et al., 1998; Irani et al., 2008; McKnight et al., 2005) or psychosis (Graus et al., 2010; Zandi et al., 2010) with GAD-Abs, VGKC-complex or other NSAb; others with GAD-Ab associated nystagmus (Antonini et al., 2003) or palatal tremor (Marnane et al., 2008; Nemni et al., 1994; Vianello et al., 2003). Moreover there are CNS disorders for which a role for autoantibodies has been hypothesized but is still controversial. These include post-streptococcal neurological and psychiatric syndromes (PANDAS), Sydenham's chorea with antibodies targerting "basal ganglia antigens", and also encephalopathies associated with systemic autoimmunity (i.e. antiphospholipid syndrome and neuropsychiatric lupus) or organ-specific conditions for which a vasculitic or ischemic mechanism can be excluded (Lang et al., 2003). Hashimoto's encephalopathy (HE), also called steroid-responsive encephalopathy associated with autoimmune thyroiditis (SREAT), is an example of the latter group and is only defined by the presence of serum thyroperoxidase or thyroglobulin antibodies, often without evidence of thyroid dysfunction (Chong et al., 2003). Given the high frequency of thyroid antibodies in the normal population, it is likely that in some cases they are incidental and that NSAbs are the real pathogenic agent (LZ observations); indeed, thyroid antibodies were found coexisting with NMDAR- or VGKC-complex-Abs in a recent study of LE (Tuzun et al., 2011). Finally, there are many forms of childhood encephalitis and epilepsy which are often treated with steroids but are not yet recognised as antibody-mediated although a few cases with VGKC-complex, GAD or NMDAR-Abs are beginning to be reported (Haberlandt et al., 2011; Suleiman et al., 2011). Below, we consider how one might go about recognising these conditions and defining NSAS for future diagnosis and management.

1.6 Antibody screening

Indirect immunohistochemisty or immunofluorescence on fixed frozed rat brain tissue is commonly used as a preliminary screening to identify recognizable staining patterns that represent intracellular and/or surface (e.g. neuropil) antibodies, although for the latter, sensitivity depends on laboratory expertise. The target of the antibodies may be strongly suspected from these results, but should be confirmed by more specific techniques. Commercial kits for immunoblotting with recombinant proteins for the most common/well-characterized onconeural antibodies (Hu, Ma2, CV2/CRMP5, Ri, amphiphisin) are widely available. GAD-Abs and VGKC-complex antibodies are often detected by radioimmunoassay but a GAD-Ab ELISA is also available.

The gold standard for NSAb detection (and for other antibodies against cell surface antigens, eg. AQP4) is an assay based on mammalian cells (generally human embryonic kidney cells) transiently transfected with the antigen of interest and incubated with the patient's serum, diluted 1:10 or greater (or CSF, usually diluted from 1:1 – 1:10). Positive samples are visually identified at the cell surface or throughout the cell using an anti-human IgG tagged with a fluorescent dye. This technique, commonly named a cell-based assay (CBA), is by definition more specific and sensitive, as only one antigenic target is over-expressed in these cells. In addition commercial kits are now available (Wandinger et al., 2011). Given the plethora of antibodies that have been reported so far, the clinician faced with the dilemma of which antibody to test first, especially if IHC/IIF gave inconsistent results, should bear in mind that most NSAb-related CNS disorders are covered by NMDAR-Abs and VGKC-complex Abs. If the sample proves negative for both NMDAR- and VGKC-complex Abs it may be worth referring to a lab with research experience in this area. However, multiple antibody testing (for NMDAR, LGI1, CASPR2, AMPAR, GABAbR, GlyR) may be the way forward since there are now kits with mosaics of cells displaying different NSAbs. If no antibodies are positive with these specific tests, immunostaining of live hippocampal or other neurons can be done to look for NSAb in patients who are negative on specific tests. This approach, not yet available

commercially, would allow detection of potentially pathogenic NSAbs, justify immunotherapies, and could lead to identification of new antigens in the future.

1.7 Immunotherapy

There is no consensus or evidence-base to indicate which kind of immunotherapies should be tried in these patients, but in well-defined syndromes it is thought important to start early without waiting for the results of the antibody determinations, and while the screening of the tumour is conducted. First line treatments are intravenous followed by oral high-dose corticosteroids, intravenous immunoglobulins, or plasma exchange, and frequently a combination of these (plasma exchange preceding IvIg). Most patients with encephalitis associated with NSAbs respond to first line treatments but responses can be slow in patients with NMDAR-Ab encephalitis. For non-responders, if the tumour screen is negative, a second-line immunotherapy, with rituximab, cyclophosphamide or both, is suggested (Dalmau et al., 2011). There are no data on the value of chronic long-term immunotherapy to prevent relapses in those syndromes that do so, i.e. NMDAR-Ab encephalitis, although patients who are not treated with immunotherapy at the first event seem to have a higher risk for relapses (Gabilondo et al., 2011). Based on the authors' experience weaning should be very careful. Serial estimations of antibody levels, in serum and CSF if available, can be helpful.

1.8 Approach for the recognition of neuronal surface antibody syndromes (NSAS)

1.8.1 Well-defined syndromes

If the clinical features are typical of a well-defined syndrome, such as LE or OMS, after exclusion of other potential causes (infective, trauma, toxic, metabolic, tumours or histories of previous CNS disease), the priority is to rule out a paraneoplastic syndrome as previously established (Graus et al., 2004). A search for a tumour should be undertaken and testing of serum, and CSF if possible, performed for onconeural Abs, for those

NSAbs that are currently available (NMDAR, VGKC-complex proteins), and also for GAD-Abs. If a tumour is found or if onconeural antibodies are positive, the syndrome will be a Definite Paraneoplastic Neurological Syndrome (Graus et al., 2004), and tumour therapy and limited immunotherapy can be performed. Irrespective of the presence of a tumour, a positive NSAb would justify the diagnosis of NSAS and more intensive immunotherapy; a screen for specific tumours should be intensified (eg teratoma for NMDAR-Abs or thymoma for VGKC-Abs).

1.8.2 Suspected NSAS

In case of other neurological syndromes, the following three criteria can be used to suggest a possible immune mediated cause associated with an NSAb. Supportive features are not mandatory but their presence would strengthen the diagnostic suspicion and help the subsequent diagnostic classification.

Criteria

- Acute or subacute onset of symptoms (< 12 weeks)
- Evidence of CNS inflammation (at least one):
    - CSF (lymphocytic pleocytosis, CSF specific oligoclonal bands or elevated IgG index);
    - MRI (eg. mediotemporal lobes FLAIR/T2 hyperintensities in case of a LE – like syndrome - otherwise unexplained [eg post-seizure]; or enhancement of cerebellar sulci; etc.) or functional imaging (hypermetabolism on FDG-PET or hyperperfusion on SPECT in the acute-subacute phase); Inflammatory neuropathology
- Exclusion of other causes (infective, trauma, toxic, metabolic, tumours, demyelinating or histories of previous CNS disease).

Supportive features

- History of other antibody-mediated disorders (MG, NMT, LE, etc.) or organ-specific autoimmunity;

- Preceding infectious, febrile illness or viral disease – like prodroms; this feature is based on the evidence that (1) many cases of autoimmune encephalitis (eg. NMDAR) are preceded by prodroms and that (2) a CNS (as well as a PNS) disorder with an acute or subacute onset following a viral disease can be generated by a parainfectious autoimmune mechanism.

As all these conditions can associate with tumours, a screening for onconeural antibodies should be performed (Graus et al., 2004). If a tumour is found or onconeural antibodies are positive, the syndrome will be a Paraneoplastic Neurological Syndrome, definite or possible according to the Graus criteria (Graus et al., 2004). A search for NSAbs and GAD-Abs should not be delayed, and while waiting for the results a trial of immunotherapy can be started. Even in patients who are negative for the known NSAbs, a trial with steroids and IVIG/PE can be considered if there are no contraindications, particularly if infectious diseases have been ruled out.

Ultimately a diagnostic classification as definite, probable or possible NSAS will depend on the clinical presentation, antibody testing and response to immunotherapies (Figure). Antibody titres may sometimes be misleading, as very low titres (eg. <1:50) may sometimes be found in patients with unrelated conditions, but at present there are no standards for assessing the NSAbs. To define a syndrome as immunotherapy-responsive, a sustained improvement in the modified Rankin score of at least 1 point would be appropriate (Graus et al., 2001).

The following diagnostic definitions do not have merely a "classificatory" purpose but are aimed at giving further clues to justify improvements in detection of antibodies, more intense immunotherapies, and search for novel NSABs.

Classification

- A diagnosis of definite NSAS can be made if known NSAb are present in the serum or CSF AND there is a response to immunotherapies.
- A diagnosis of probable autoimmune NSAS can be made if:
    - known NSAb are present

    - OR there are other neuronal antibody markers of an immune process (GAD-Ab, unknown neuronal surface/neuropil Abs) or at least one of the above-mentioned clinical supportive features
    - AND there is a response to immunotherapies.
- If clinical and paraclinical criteria suggest a possible NSAS, but no known NSAb are found a diagnosis of possible autoimmune NSAS can still be made if:
    - Other neuronal antibody markers of an immune process (GAD-Ab, unknown neuronal surface/neuropil Abs) or at least one of the above-mentioned clinical supportive features are present.
    - OR there is a response to immunotherapies.

A diagnosis of probable or possible NSAS will prompt search for novel unknown antibodies or a second line immunotherapy (with or without tumour screening).
An alternative diagnosis will be warranted in any other case (see the figure).

1.9 Conclusions

This new field of immune-mediated CNS diseases offers considerable satisfaction but also challenges. There is a need for more intense research into those conditions that are shown to be immunotherapy responsive, and thereby can be defined as Possible NSAS. The presence of other NSAbs in patients with more common conditions such as epilepsy, psychosis and dementia should be systematically examined. Ideally antibody testing should be performed locally so that the diagnosis can be made and treatments started with confidence as soon as possible in the hope of restoring health, limiting hospitalisation and optimising outcomes. Systematic studies of the treatments are needed in order to establish best practice.

Table 1.1. Central nervous system syndromes associated with anti-neuronal antibodies

	Classical paraneoplastic CNS syndromes associated with onconeural antibodies	**CNS syndromes associated with Neuronal Surface Antibodies**
Main syndromes	Cerebellar degeneration (PCD) Encephalomyelitis Limbic encephalitis (LE) Brain stem encephalitis	Limbic encephalitis Morvan's syndrome NMDAR-Ab encephalitis Progressive encephalomyelitis with rigidity and myoclonus Cerebellar ataxia
Age range and sex	Mainly adults (40-70); both genders (PCD more frequent in women).	NMDAR-ab encephalitis common in children and young women
Antibodies commonly detected or recently reported	Antibodies against intracellular antigens or PNS-related onconeural antibodies (Hu, Yo, Ri, Ma2, Cv2, Amphiphysin, Sox1/2,);	Antibodies to VGKC-complex antigens (LGI1 or CASPR2), NMDAR, AMPAR, GABA(B)R, GlyR, VGCC-ab, mGluR1, mGluR5
Tumours	SCLC, breast, ovary, testicular	Teratoma, thymoma, SCLC; breast No tumour found is some cases, particularly LE-associated with LGI1 –ab
Relationship between antibody and tumour	Antibody presence indicates the presence of a particular tumour type,	Antibody presence does not indicate if a case is paraneoplastic
Immunotherapy	Not usually effective	Generally effective
Outcome	Poor; improvement or stabilization related mainly to tumour treatment	Variable but generally good; possible spontaneous remission
Neuropathology	Loss of neurons, gliosis, T-cell infiltrates in close aposition to neurons, some with immunophenotype of cytotoxic T-cells	Sata basically limited to anti-MDR encephalitis. B- and plasma cell infiltrates, deposits of IgG, but no evidence of complement deposition
Prevalent pathogenic mechanism	Antibodies are markers for the tumour and are not likely to be pathogenic. T cell cytotoxicity is the proposed pathogenic mechanism	Autoantibody-mediated, probably down-regulation of target antigen,

Table 1.2. Neuronal Surface antibody-associated syndromes

Syndrome	**Antibodies**	**Particular clinical features**	**Tumour**	**Immunotherapy-response**	**In vitro evidence of Ab pathogenicity**	**Frequency or # cases reported**
NMDAR-ab encephalitis	NMDAR	Psychiatric presentation in young women. Epilepsy and abnormal movements more frequent at onset in children.	Ovarian teratoma. Rare in children. 58% after age 18	Yes	In vitro and in vivo reduction of NMDA receptors	More than 500 cases reported
Limbic encephalitis	LGI1 CASPR2 (<10%)	Male predominance Hyponatremia, facio-brachial dystonic seizures, myoclonus	None	Yes	In vitro production of epileptogenic activity in brain slices	More than 500 cases reported
	AMPAR	Possible isolates psychiatric symptoms	70% (lung, breast, thymus)	Yes, frequent relapses	Downregulation of AMPA receptors	14
	GABA(B)R	Prominent seizures	60% (SCLC)	Yes	None	25
	mGluR5	Ophelia syndrome	Hodgkin lymphoma	Unknown	None	2
Morvan's syndrome	CASPR2	Encephalopathy, peripheral nerve hyperexcitability, dysautonomia	Thymoma	Yes	Not tested	9
PERM	GlyR	Encephalomyetis with myoclonus, rigidity and brainstem signs	Thymoma	Yes	Not tested	6
Cerebellar ataxia	VGCC	Possible co-existence of LEMS	SCLC	Poor	Not tested	16
	mGluR1	- Remote history of Hodgkin lymphoma	no	Yes	In vivo	3

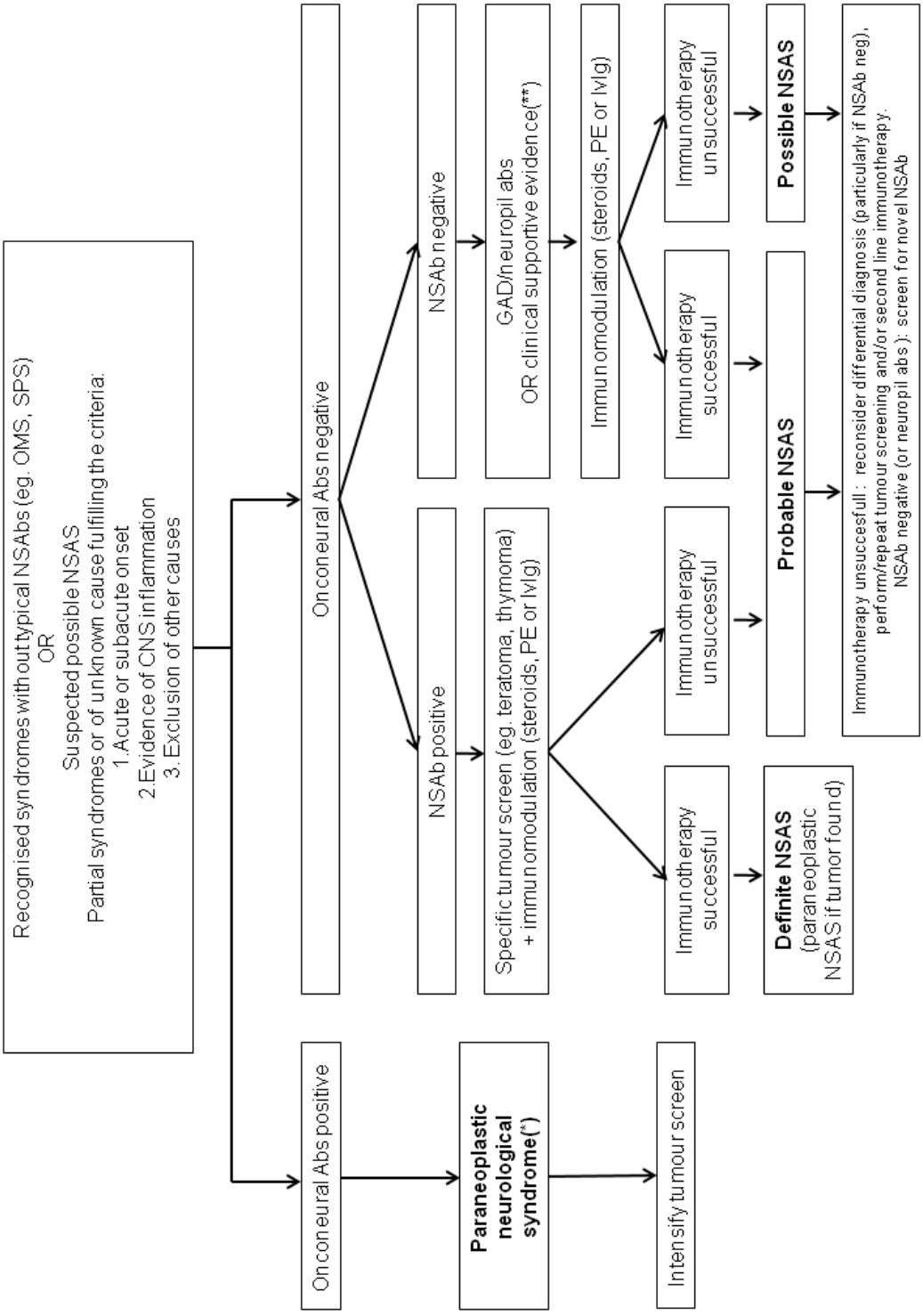

Figure 1.

Flow chart showing our suggestion for approaches to the recognition and diagnostic criteria for the NAMS. T is important to recognise that the field is developing and the scheme is intended to help identify further NSAS. *for details see Graus et al. JNNP 2004. ** History of other antibody-mediated disorders or organ-specific autoimmunity; or previous infectious/febrile illness.

2. CLINICAL SPECTRUM OF VGKC-COMPLEX ANTIBODY ENCEPHALITIS

2.1 Introduction

The first reports of voltage-gated potassium channel antibody (VGKC-Abs) associated-encephalopathy appeared in the English literature about ten years ago (Buckley et al., 2001; Liguori et al., 2001; Thieben et al., 2004; Vincent et al., 2004). From then on VGKC-encephalitis has become widely recognized and now represents one the most frequent causes of autoimmune encephalitis, together with NMDAR-encephalitis (Granerod et al., 2010; Lancaster et al., 2011b; Vincent et al., 2011). Recent evidence has shown that the targets of Abs immunoprecipitating VGKC extracted from mammalian brain are not the channel subunits but other associated neuronal surface proteins, mainly leucine-rich glioma inactivated 1 protein (Lgi1) and contactin-associated protein-2 (Caspr2) (Irani et al., 2010b; Lai et al., 2010). In addition Irani and colleagues suggested that Lgi1-Abs were more frequently associated with limbic encephalitis (LE) and a novel type of seizure referred to as faciobrachial dystonic seizures (Irani et al., 2011) and Caspr2-ab with Morvan's syndrome and thymoma (Irani et al., 2010b).

Here we describe the clinical profile of 18 consecutive patients with encephalitis associated with VGKC-complex antibody, detected by radioimmunoprecipitation (RIA) and immunofluorescence (IIF) for Lgi1 and Caspr2 Abs, expanding the known clinical spectrum of this condition.

2.2 Patients and Methods

2.2.1 Methods

Between January 2005 and December 2011, 503 samples from 366 patients with suspected autoimmune encephalitis were sent to our laboratory. 279 serum (and CSF) samples from 232 patients were tested for VGKC-complex-Abs. In addition, 226 samples

were tested for NMDAR-Abs, 59 for AMPAR1,2- and GABAbR-Abs, 91 for GAD-Abs; 21 samples were also tested by indirect immunofluorescence on live hippocampal neurons.

The screening was performed in Oxford (Neuroscience Group - Weatherall Institute of Molecular Medicine and West Wing, John Radcliffe Hospital, University of Oxford, United Kingdom) and Padova (Laboratorio di Neuroimmunologia – Clinica Neurologica 2a, Ospedale Sant'Antonio, Università di Padova).

The term VGKC-complex-Abs includes both VGKC-Abs detected by radioimmunoprecipitation using ^{125}I-α-dendrotoxin-VGKC performed in the laboratory in Oxford and/or VGKC-complex proteins Lgi1- and Caspr2-Abs detected by indirect immunofluorescence. IIF was performed at Oxford with a live cell-based assay as previously reported (Irani et al., 2010b) or a commercial kit combining fixed cells transfected with Lgi and Caspr2 proteins according to the manufacturer's instructions (Euroimmun, Luebeck, Germany).

Immunoprecipitation assay for VGKCs. This was performed as described earlier (Hart et al., 1997). VGKC complexes were extracted from rabbit brain membranes solubilized in 2% digitonin (Calbiochem, USA) in DTX-buffer (100mM NaCl, 20mM Tris, 5mM KCl adjusted to pH 7.12) at 37°C for 20 min. The supernatant was diluted 1:2 with PTX (0.02M phosphate buffer and 0.1% Triton-X-100, pH 7.2) and incubated with 106 cpm/ml of 125 I-DTX (Perkin Elmer, USA). An amount of 50 ml volumes of the 125I-DTX-labelled extracts were incubated with 5 and 1 ml of each serum, the IgG-VGKC complexes were precipitated with anti-human IgG (The Binding Site, UK), and the pellets were washed and counted on a gamma counter (Cobra2, Perkin Elmer, USA).

Live cell-based assay for antibodies binding human NMDAR, Lgi1 and Caspr2. Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum (TCS Cellworks Ltd, Buckingham, UK) and 100 units/ml each of penicillin G and streptomycin (Invitrogen) at 37°C in a 5% CO2 atmosphere. Cells were grown on six-well culture plates for immunoadsorption and toxin binding experiments, on 13mm glass coverslips placed in six-well cell culture plates for microscopy, and in 175 cm2 flasks for protein extraction. Using polyethylenimine, cells were transiently co-transfected with plasmids containing NMDAR, EGFP, Caspr2, Lgi1. Forty-eight hours after transfection, the immunofluorescent staining of the cells was performed. The coverslips were transferred to individual wells in 24-well culture plates

and incubated at room temperature for 45 min with patient serum (1:20–1:100, as specified); these were diluted in Dulbecco's modified Eagle's medium buffered with HEPES [N-(2-hydroxyethyl)piperazine-N0-(2-ethanesulphonic acid)] with added 1% bovine serum albumin to block non-specific binding. The cells were subsequently washed three times in Dulbecco's modified Eagle's medium/HEPES buffer and fixed with 3% formaldehyde in phosphate buffered saline at room temperature for 15 min, followed by further washing. They were then labelled for 45 min at room temperature with anti-human IgG Alexa Fluor 568-conjugated secondary antibody (Invitrogen-Molecular probes, Paisley, UK) at 1:750 in 1% bovine serum albumin/Dulbecco's modified Eagle's medium/HEPES buffer. All coverslips were subsequently washed three times in phosphate buffered saline and mounted on slides in fluorescent mounting medium (DakoCytomation, Cambridge, UK) with DAPI (40,60-diamidino-2-phenlindoledichloride, 1:1000). They were visualized using a fluorescence microscope with a MacProbe v4.3 digital imaging system.

Cell-based assay for antibodies binding human NMDAR, AMPAR1, AMPAR2, GABAbR, Lgi1 and Caspr2 (Euroimmun, Luebeck). Slides with mosaic of transfected and non-transfected cells were incubated with patient samples at a starting dilution of 1:10 (serum) or undiluted (CSF). After incubation for 30 min at room temperature, the slides were rinsed with a flush of PBS-Tween and incubated in PBS-Tween for at least 5 min. Bound IgG was labelled using Fluorescein-conjugated goat anti-human IgG antibody for 30 min and washed as described before. Samples were classified as positive or negative based on the intensity of surface immunofluorescence of transfected cells in direct comparison with non-transfected cells and control samples.

Hippocampal cell cultures and immunofluorescence staining. Primary cultures of hippocampal neurons were prepared from E18 or P1 rat pups. Rat pups were killed by decapitation. Hippocampi were isolated from the brain and collected in chilled HBSS (Hanks' Balanced Salt Solution), with antibiotic-antimytotic, and incubated in1% trypsin-EDTA solution at 37°C for 30 minutes. The solution was aspirated and the hippocampi triturated using a 1 ml and 200 µL pipette in 2-3 ml of complete MEM (Minimal Essential Medium) with 10% foetal calf serum and penicillin/streptomycin. After low speed centrifugation (1000 rpm, 4 minutes) the supernatant was discarded and the cells were resuspended in complete MEM and plated onto 13mm diameter glass coverslips coated with poly-L-lysine in 6-well plates. Cultures were grown at 37°C in a humidified 95% O_2 5% CO_2 atmosphere. Twenty four hours after plating, and then twice weekly, half of the

medium was replaced with neurobasal culture medium with added glutamine, antibiotic-antimytotic and B27 (Invitrogen). For immunofluorescence experiments, after at least 7 days in vitro, the coverslips were transferred into 24-well plates and incubated with patients' sera diluted 1:250 in 1% BSA-Hepes-Neurobasal for 1 hour at room temperature, followed by fixation (3% formaldehyde for 15 minutes) and by incubation with Alexafluor anti-human IgG-488 conjugated antibody (Invitrogen, UK) for 40 minutes. Subsequently the cells were permeabilised with 0.3% PBST (0.3% Triton X-100 in PBS) for 15 min at RT and incubated with mouse monoclonal anti-MAP2 (Sigma-Aldrich, UK) diluted 1:500 in 1% BSA 5% normal goat serum for 1 h at RT, followed by incubation with Alexafluor anti-mouse IgG-568 conjugated antibody (Invitrogen, UK) for 45 minutes. Cells were mounted and visualized as for the HEK293 cells based assay.

Radioimmunoassay (RIA) with recombinant GAD65. GAD antibody were assayed by a commercially available kit following the manufacturer's instructions. Briefly, 20 Al of serum samples were incubated with 50 Al of 125I labelled human recombinant GAD65. Then, 50 Al of protein A-sepharose was added and the mixture incubated for 1 h at room temperature. After centrifugation at 1500g for 30 min the precipitates were counted for 125I with a gamma scintillation counter. The results were interpolated in the standard curve constructed using the dilution of a positive control serum.

2.2.2 Patients

Patients who tested positive for VGKC-complex-Abs, with available clinical data and a follow-up of at least 3 months were included in the study. VGKC-Abs had to be raised above 100 pM by RIA or positivity for Lgi1- or Caspr2-Abs on indirect immunofluorescence confirmed by two independent observers.

Clinical information was obtained retrospectively or prospectively, where possible, by means of structured patient and family interviews, medical record review, telephone interviews or questionnaires sent to neurologists.

Clinical features suggestive of autoimmune encephalopathy or LE as proposed by Gultekin and colleagues in 2000 (Gultekin et al., 2000) were investigated: 1. A subacute onset (less than 12 weeks); 2. the presence of one or more of the following clinical features: a. short-term memory loss or diffuse cognitive decline; b. seizure (temporal

lobes or generalized); c. psychiatric abnormalities or behavioural changes; 3. at least one of the following: a. CSF with inflammatory signs (pleocytosis, oligoclonal bands, increased immunoglobulin content or increased protein content); b. brain MRI showing unilateral or bilateral temporal lobe abnormalities (hyperintensity) on T_2-weighted images (and FLAIR) or atrophy on T_1-weighted images; c. EEG showing slow or sharp-wave activity in one or both temporal lobes; 4. exclusion of other possible causes (viral, toxic, metabolic, thyroid-autoimmunity- related, vasculitis). The neurological disability and outcome were evaluated by a modified Rankin score (Graus et al., 2001).

2.3 Results

Among 232 patients with a presumptive diagnosis of autoimmune encephalopathy / limbic encephalitis, 32 had raised VGKC-complex-Abs in their sera. Clinical data were available on 18 out of 32 cases, which were included in the study. Seven of the 18 patients were women and the median patient age was 57 years (range 31-77). The mean follow-up period was 18 months, with a range of 3-70 months. Clinical data are summarized in Table 2.1 and 2.2.
Below is a report on 3 illustrative cases with specific clinical features.

2.3.1 Case 9

This 46-year-old woman presented with a one-month history of diffuse asthenia followed by subacute onset of episodic memory deficits and mild confusional states. Subsequently, daily episodes characterized by head deviations, oral automatisms, and vocalizations were referred and her mood began to fluctuate, with behavioural changes and paranoid ideation. The patient was admitted to hospital after a generalized seizure. Electroencephalography (EEG) demonstrated, left frontotemporal epileptic discharges. Brain MRI revealed mild bilateral medial temporal lobe T_2-weighted hyperintensity (Fig. 2.1 B). Seizures were controlled by carbamazepine. Neuropsychological tests revealed moderate impairment of memory and attention, and mild impairment of visual-spatial and executive functions. MMSE was 29/30. Laboratory tests were negative except for

hyponatraemia (131 mmol/L). CSF analysis was negative including CSF PCR for herpetic viruses. A screen for onconeural antibodies proved negative while a total body FDG-PET failed to disclose any occult tumours. During hospitalization the patient's conditions subsequently worsened and she began to manifest delirium and suicidal ideation. Intravenous (IV) methylprednisone (0.5 gr for 5 days) followed by oral prednisone and IV immunoglobulin were administered (0.4 gr/kg for 5 days) together with treatment with quetiapine and continuous IV midazolam infusion. A patient's serum sample tested in Oxford was strongly positive for VGKC-Abs (raised to 5056 pM [nv <100]). The patient received an additional course of high dose IV steroids and plasma-exchange (PE) was started in association with oral cyclophosphamide (CFX). The VGKC-Abs titre decreased to 2993 over the next two weeks. Cognitive functions and behaviour improved. Midazolam was stopped and neuroleptics were reduced. Monthly PE cycles were maintained. CFX was substituted by azathioprine (3mg/kg) associated with 25 mg prednisone every other day. An MRI obtained 5 months after the clinical onset revealed very mild hippocampal atrophy while an ^{18}F-FDG brain PET months showed low frontotemporal glucose metabolism. Seven months after onset she returned to work. She had amnesia about the onset of and the period 6 months prior to the disease and complained of occasional dysthymia and feelings of persecution towards her relatives. Pilomotor seizures on her right hemibody were also reported requiring treatment with levetiracetam. Nine months after onset the VGKC-Abs titre declined to 1481 pM. Azathioprine (100mg) and monthly PE were continued. Surprisingly, despite persisting clinical remission, a serum sample obtained 17 months after onset revealed a marked increase in the VGKC-ab titre (4089 pM) (Fig. 2.1 A), which decreased only transitorily after courses of PE. In order to rule out an occult tumour, a further screening with a CT-thorax was performed revealing an enlarged thymus (Fig. 2.1 C). Thirty-one months after onset she underwent thymectomy and the pathology revealed thymic hyperplasia (Fig. 2.1 D) with B-cell-containing germinal centres (Fig.3 X). Meanwhile the real target of VGKC-Abs was identified as leucine-rich glioma inactivated protein 1 (Lgi1) by indirect IIF on transfected HEK cells. The VGKC-Abs titre did not consistently decrease at the follow-up controls. One year after thymectomy, the titre decreased to 721 and azathioprine and PE were stopped. Three months later, the patient's behaviour changed and she started complaining of delusions, mainly persecution and jealousy, and ideas of reference. Brain MRI was unchanged. The VGKC-Abs titre was 1150. PE was repeated

and azathioprine restarted. Valproic acid was also administered. Her ideation had greatly improved at the latest follow-up 3 months later.

2.3.2 Case 8

A 77-year-old male was hospitalized with a 3–month history of apathy and cognitive decline associated with imbalance, gait difficulties and urinary incontinence which had required bladder catheterism one month before admission. Neurological examination showed short-term memory deterioration, mild to severe confusion, bradyphrenia, slowed speech, bradykinesia, severe imbalance and ataxic gait possible only with aid, mild limb ataxia, bilateral Babinski sign, reduced vibratory sensation and statokinesthesia of the lower limbs. A brain CT scan, performed to rule out a normal pressure hydrocephalus, revealed mild diffuse cerebral atrophy. Neuropsychological tests indicated mild diffuse cognitive impairment with more severe deficits in executive, instrumental and mnesic functions. MMSE was 20/30. Laboratory exams were normal except for ANA 1:40 and mild hyponatraemia (134 mmol/L). CSF showed increased proteins (75 mg/dl, nv<45), CSF-specific IgG oligoclonal bands and positive 14.3.3 protein. The search for known onconeural Abs and total body CT scan were negative. EEG showed diffuse slowing. A brain MRI revealed diffuse mild atrophy and diffuse non-specific T_2-weighted hypertintensities in the periventricular and subcortical white matter. Evoked motor and somatosensory potentials were centrally slowed bilaterally. A spinal cord MRI was also performed showing a T_2-weighted hyperintense lesion in the left posterior white matter of the dorsal spine suggestive of myelitis. Treatment with IV ceftriaxone and aciclovir was initiated with no benefit. IV methylprednisone (1 gr for 5 days) was subsequently administered followed by oral prednisone. A marked improvement of cognitive deficits was observed, with disappearance of confusion and dysexecutive deficits, bladder catheterism was stopped and the patient started to deambulate autonomously. A sample of serum obtained before steroid treatment showed a VGKC-Abs titre of 751 pM (nv <100) which declined to 470 pM in a week. Brain perfusory single-photon-emission-tomography (99mTc-ECD SPET) performed one month later revealed mild hypoperfusion of the temporal, parietal and frontal lobes bilaterally and of the right thalamus and left putamen. MMSE score was 24/30. Azathioprine was started. Three months later the patient presented a relapse characterized by severe confusion which

responded to a new course of steroids followed by plasma-exchange. Azathioprine was maintained. One year after onset the VGKC-Ab titre was below 100pM and PE was stopped. Ten months later he presented subacute worsening of cognitive performance and gait disturbances. The VGKC-Ab titre was found to be 935pM and PE was restarted. After 8 sessions of PE a moderate improvement was observed paralleling a mild decrease in titre (708 pM). However, he subsequently accidentally broke his femur and his clinical conditions progressively deteriorated. He died of pneumonia one year later. A subsequent screen for VGKC-complex antigen by IIF (Lgi1, CASPR2 and Contactin-2) proved negative.

2.3.3 Case 15

This 68-year old man started complaining of sporadic episodes of disorientation (about one a month). He experienced a feeling of unfamiliarity for his home town or amnesia that lasted a few seconds while consciousness was maintained, as testified by his wife. His past medical history included arterial hypertension and chronic atrial fibrillation. He also referred experiencing leg cramps lasting for several months about one year previously. An EEG showed slow waves in the left temporal lobe and brain MRI disclosed swelling and T2-weighted hyperintensity in the mesial temporal lobes bilaterally (more prominent on the left), interpreted to be a possible low grade glial lesion (Fig. 2.2 A). Brain MRI was repeated some months later confirming the hippocampal lesions. More than one year after onset the patient was still experiencing monthly episodes of simple partial temporal lobe seizures (jamais vu). Screening for classical onconeural antibodies and neuronal surface antibodies (including NMDAR, GABAbR, AMPAR 1 and 2, LGI1 and CASPR2) was performed showing strong positivity for Caspr2-Abs and faint positivity for Lgi1-Abs. The patient was hospitalized. On admission neurological examination was unremarkable, MMSE score was 30/30 and FAB 14/18. However, after discontinuation of warfarin to permit a lumbar puncture, he had a myocardial infarction requiring observation at the coronary unit. Extensive immunological screening showed serum positivity for anti-streptolysin, anti-thyroperoxidase, anti-nuclear (titre 1:640), anti-DNA, and anti-phospholipid antibodies (lupus anticoagulant, cardiolipin and beta2-microglobulin). Warfarin was restored with an INR ratio range of 3 to 4. An anti-phospholipid-Abs syndrome was confirmed 12 weeks

later. Positivity for Caspr2-Ab was also confirmed. At the latest follow-up, 2 years after seizure onset , he reported two episodes of possible complex partial seizures (oral automatisms followed by confusion). Treatment with valproic acid was started.

2.3.4 Clinical features, investigations, treatment and outcome

All patients had a subacute onset. In one case (no. 14) the encephalitis appeared with rapid worsening of cognitive performance in a patient with a previous diagnosis of dementia.

All patients presented one or more of the following clinical features: a. short-term memory loss or diffuse cognitive decline [16 out of 18]; b. seizure (temporal lobes or generalized) [13/18]; c. psychiatric abnormalities or behavioural changes [11/18]. Previously reported psychiatric abnormalities in patient 3, (Spinazzi et al., 2008) were prominent and characterized by a change in personality, hypomania and an obsessive-compulsive-like disorder. He was unable to refrain from buying unreasonable amounts of food on a daily basis and from stealing useless things in shops and at the hospital (sheep from the nativity crib). He lost his critical appraisal, becoming prone to rage and aggressiveness.

Additional clinical features included hyponatraemia present in 4 out of 8 cases with available data; sleep disorders in 5 cases, including insomnia, hypersomnia and REM sleep suppression; dysautonomia (3 patients: hyperhydrosis, tachycardia with inversion of blood pressure circadian rhythm, hypertension and bladder dysfunction); peripheral nerve hyperexcitability (3); gait disturbances (3).

Seventeen out of 18 patients had at least one of the following features suggestive of encephalitis: a. CSF with inflammatory signs (pleocytosis, oligoclonal bands, increased immunoglobulin content or increased protein content) [6 out 13 with available data]; b. brain MRI showing unilateral or bilateral temporal lobe abnormalities (hyperintensity) on T_2-weighted images (and FLAIR) or atrophy on T_1-weighted images [11/18]; c. EEG showing slow or sharp-wave activity in one or both temporal lobes [16/18]. None of these 3 features was observed in one case (no. 13), who had Morvan's syndrome. He presented with a syndrome of peripheral nerve hyperexcitability prompting the search for a thymoma; three months after thymectomy he presented with sleep disorders, mood deflection and cognitive decline which spontaneously resolved in 3 months.

Other MRI findings included: not-specific hemispheric white matter abnormalities in 2 cases and unilateral basal ganglia hyperintensity in one case on brain MRI.

18-FDG brain PET or perfusion SPECT were performed in 9 patients revealing abnormalities in 8: low frontal and temporal metabolism in 7 cases, 2 of them with a negative MRI, and basal ganglia hypermetabolism in 4.

Fifteen patients received one or more immunomodulatory treatments (14 steroids, 7 plasma-exchange and IV immunoglobulins); 4 cases received additional immunosuppressants (azathioprine or cyclophospamide).

Fifteen patients improved, in 9 cases markedly (defined by a decrease in the MRS of at least 3), 6 mildly (MRS decrement = 1-2). In 2 cases improvement was spontaneous. Relapses were observed in 3 patients who had initially responded to treatment. One patient did not show a substantial improvement at the time of the latest follow-up (3 months) despite treatment with immunosuppressive drugs (no. 18); the other previously reported case (case no. 1) died from small-cell lung cancer (Zuliani et al., 2007b).

RIA for VGKC-Abs was performed in 13 patients, the titre ranging between 132 and 5056 pM; 9 patients had a high titre (> 400pM). The titre declined in the five cases with available data, paralleling the clinical improvement (data not shown). IIF for CASPR2 and Lgi1 was performed in all patients: 10 cases proved positive for Lgi1-Abs and 3 for Caspr2-Abs; 5 patients' sera did not react with Lgi1 or Caspr2 transfected cells. Nine out of 10 Lgi-Abs positive cases had a final diagnosis of LE, and in one of these cases faciobrachial dystonic seizures were retrospectively recognized at the onset of the syndrome (no. 12). One Lgi1-Abs positive patient had Morvan's syndrome (no. 3) (Spinazzi et al., 2008). The thymoma-associated Morvan's syndrome was positive for Caspr2-Abs while the other two Caspr2-Abs positive cases had LE. No statistically significant differences were observed among the 3 groups (data not shown). However, all four cases (out of 9) with striatal hypermetabolism on FDG-PET were positive for Lg1-Abs.

2.4 Discussion

Our study confirmed some important, well-known features of VGCK-complex-Abs encephalitis (Vincent et al., 2004): the generally remarkable response to immune-therapy

and the low incidence of tumours (~10%). Only one patient with positivity for anti-glial nuclear antibodies (SOX1; marker of SCLC-related autoimmunity (Graus et al., 2005)) died from small cell lung cancer (previously reported (Zuliani et al., 2007b). In addition, Lgi1 was confirmed to be the main antigenic target (56% versus 18% of Caspr2) of antibodies previously attributed to VGKC (Irani et al., 2010b; Lai et al., 2010).

Nevertheless our unselected cohort of patients also expands the clinical spectrum of VGKC-complex-encephalitis. Case 9 presented a typical LE which remarkably improved after immunotherapy associated with reductions in Abs levels as previously described (Thieben et al., 2004; Vincent et al., 2004), and which subsequently proved to harbour antibodies against Lgi1 (Irani et al., 2010b). VGKC-complex-associated neurological syndromes can associate with thymoma (Evoli et al., 2007; Vincent and Irani, 2010), explaining why the anomalous persistence of high serum Abs after remission prompted the discovery of a mediastinal mass, which fortunately turned out to be a hyperplastic thymus. As far as we know, this is the first reported case of VGKC-LE associated with thymic hyperplasia. However, we cannot make assertions on the role of our patient's thymus as its removal did not lead to a fall in Ab titre nor prevented a subsequent relapse. It may be that B-cell activation and homing to germinal centres in our patient's thymus (see fig. 2.1 D) contributed to the initiation but not the maintenance of Ab production, suggesting another site of Ab production, as observed in myasthenia gravis (Fujii et al., 1985). We can further speculate that the integrity of the blood-barrier combined with the immunotherapy prevented pathological Ab access to the CNS, although we cannot exclude the existence of CSN germinal centres and Ab intrathecal synthesis. Thymectomy was performed partly because of the risk of a malignant evolution. However, only one out of over 100 reported cases of Lgi1-Abs-LE (Irani et al., 2010b; Lai et al., 2010) was associated with thymoma (Lai et al., 2010), a tumour more commonly reported in association with Caspr2-Abs (Irani et al., 2010b; Vincent and Irani, 2010).

Caspr2-Abs have also been suggested to associate more frequently than Lgi1-Abs with Morvan's syndrome or neuromyotonia in association with encephalitis (Irani et al., 2010b; Lancaster et al., 2011a). Of the two patients with a final diagnosis of Morvan's syndrome, one proved positive for Lgi1-Abs and the other for Caspr2-Abs. In the latter a thymoma was found after the initial development of a peripheral nerve hyperexcitability syndrome. Interestingly, comparable to another reported case (Cottrell et al., 2004), he

developed an encephalopathy months after thymectomy and some months later spontaneously remitted, confirming the clinical and immunological heterogeneity of Morvan's syndrome (Liguori et al., 2001; Spinazzi et al., 2008). Indeed one of the other two Caspr2-positive patients had a history of persistent cramps and very mild LE with only infrequent partial temporal lobe seizures (dreamy states); moreover the coexistence of a primary anti-phospholipid syndrome (PAPS) in this patient suggests an interesting, previously unreported overlapping syndrome.

Findings in Lgi1- and Caspr2-encephalitis did not significantly differ from the remarkable exception of striatal hypermatabolism observed in Lgi1 positive patients only. Basal ganglia hypermetabolism has previously been reported in autoimmune chorea (Furie et al., 1994; Guttman et al., 1987; Weindl et al., 1993), in a case of steroids-responsive frontotemporal dementia associated with anti-basal ganglia Abs (Leger et al., 2004) and in Morvan's syndrome by our group (case no. 5) (Spinazzi et al., 2008). Irani et al. recently described a new seizure type, termed faciobrachial dystonic seizures (FBDS), which generally precedes the overt manifestation of Lgi1-Abs encephalitis (Irani et al., 2011). Striatal hypermetabolism has been described in some of these patients suggesting that the ictal dystonia reflects basal ganglia involvement (Irani et al., 2011). FBDS were retrospectively documented in only one of our four patients although we cannot rule out that this symptom could have been missed. An alternative explanation is that in our Lgi1-patients, striatal hypermetabolism reflects some of their psychiatric features caused by hyperstimulation of the central dopaminergic pathways. Basal ganglia dysfunction is in fact associated with psychiatric abnormalities (Ring and Serra-Mestres, 2002) and hypermetabolism has been reported in obsessive-compulsive disorders (Schwartz et al., 1996).

About one third of our patients did not harbour Abs that reacted with Lgi1 or Caspr2 and had low-titre VGKC on RIA (> 100pM). A cut-off value of 400 pmol/l has been proposed to avoid low specificity in CNS involvement (Vincent et al., 2004). However, unlike reports of epileptic encephalopathy with low-titre VGKC-Abs in which the role of Ab was uncertain (Majoie et al., 2006; McKnight et al., 2005), in our experience low titres were an unequivocal marker of autoimmune encephalopathy. Ongoing studies will clarify the real antigenic target of these Abs (e.g. contactin-2 (Irani et al., 2011)).

One case that tested negative on IIF (no. 8) had encephalomyelitis whose diagnosis challenged us until it was supported by evidence of CSF inflammation, high-titre VGKC-

Abs and response to immunotherapy. Indeed the insidious onset with gait disturbances, urinary incontinence and cognitive disorders initially led to suspect a normal pressure hydrocephalus. Spinal cord involvement associated with VGKC-Abs has been reported in one out of 72 VGKC-Abs positive patients (Tan et al., 2008) but the occurrence of a myelopathy associated with encephalopathy has not previously been reported with VGKC-Abs. The myelopathy was unlikely to have been caused by VGKC-Abs but, interestingly, it had the same time course as the encephalopathy. It is possible to speculate that the myelitis had a parainfectious aetiology which could have triggered a bystander activation of VGKC-autoreactive lymphocytes and disrupting the blood-brain barrier and generating the encephalopathy.

In conclusion, our unselected cohort of patients recapitulates and further expands the wide range of manifestations of "VGKC-complex-Abs encephalitis". Clinical phenotypes have been only partially explained by different, recently defined VGKC-complex antigenic targets and multiple Abs may coexist to determine peripheral and central nervous system involvement as recently suggested (Loukaides et al., 2011). Interestingly, striatal hypermetabolism proved to be a specific feature of Lgi1-Abs LE, correlating with dystonic seizures, as suggested (Irani et al., 2011), but possibly also with the intriguing neuropsychiatry of basal ganglia. The pathogenic mechanisms of Abs against Lgi1, Caspr2, and other VGKC-complex antigens has not yet been elucidated. Likewise, the function of thymus remains to be clarified in these conditions, where it seems to play a similar role to the one in AchR-Abs-associated MG. Further studies are required to understand the incidence of thymic hyperplasia and the appropriateness of thymectomy. Nevertheless we suggest it is important to carefully image the thymus especially in the case of persistent high titres and even if the initial screening for tumours has proved negative.

Although international consensus is necessary to establish the best screening approach, VGKC-complex-Abs detected by both RIA and IIF are specific markers of an immunotherapy-responsive encephalopathy.

Table 2.1. Clinical features of VGKC-complex-Abs encephalitis patients

Pt	Sex/Age	Presentation (chronological order)	Other clinical and immunological features
1	F/60	Subacute behavioural changes, C and M; complex partial and generalized S	AGNA and SCLC
2	M/56	Subacute complex partial S and M	
3	F/43	Subacute behavioural changes, M, anosognosia	GAD-Abs (27,4 U/ml)
4	M/71	Subacute partial S, depression, hallucinations; M; dysexecutive syndrome	Hyperhydrosis
5	M/66	Subacute severe P (maniacality, irritability, physical violence, compulsive stealing and shopping; paranoid ideation); complex-partial S, M (and paramnesia), sleep disorder	neuromyotonia; polineuropathy; dysautonomia; neuroormonal abnormalities; circadian rythm disruption
6	F/70	Acute complex partial S; C, M, confabulation; perseveration	Thyiroiditis
7	M/64	Subacute C, complex partial S, M	Mild ataxic gait
8	M/77	3-months history of C, M, behavioural changes and dysexecutive syndrome	Ataxic gait and leg pain; reduced vibratory sensation and statokinestesia at lower limbs; urinary incontinence (myelopathy)
9	F/46	Subacute M, C and behavioural changes; complex partial and generalised S; psychosis	
10	M/47	Subacute M, behavioural changes, S	sleep disorder (narcolepsy); headache, dizzyness and nausea, weakness; weight loss (due to compulsion to exercise); nystagmus
11	F/55	Acute S (partial status epilepticus)	ANA 1:320
12	M/64	Subacute M, C, sleep disorder, behavioural changes and dysexecutive syndrome, tonic/facio-brachial dystonic S	influenza vaccination one month before onset; falls; weight loss; cognitive deficits: executive functions, apraxia.
13	M/31	Weight loss, weakness, fasciculations; thymectomy; 3 months later subacute onset of headache, sleep disorders (enacted dreams, hypersonnia), depression and dysexecutive syndrome	Motor neuron disease initially suspected; EGM signs of motor neuropathy. Dysautonomia: hypertension and excessive sweating
14	F/72	Rapid worsening of alzheimer type dementia; acute generalized S, status epilepticus	
15	M/68	Subacute sporadic partial S (dysmnesic)	Past history of cramps (2 years); anti-phospolipid syndrome
16	F/37	Subacute C and M	
17	M/51	Subacute insomnia and behavioural changes; psychosis and complex partial S	
18	M/56	Acute C (delirium), M, partial S	Mild ataxia

Table 2.2. Clinical features of VGKC-complex-Abs encephalitis patients

Pt	Brain MRI – [*SPET/PET*]	RIA VGKC-Ab (pM) - IIF for LGI1 and CASPR2	Treatments (chronological order)	Outcome (follow-up months) [differential MRS]
1	Neg – [*n.p.*]	220 – neg	Steroids	Death for pneumonia (8) [+1]
2	BT; right nucleus caudatus and putamen hyperintensity, contrast-enhanced caudatus lesion – [*n.p.*]	1392 – Lgi1	Steroids	mI; MRI: reduced BT hyperintensity (11) [-2]
3	BT and hippocampal atrophy – [*PET: fronto-temporal lobes hypometabolism*]	185 – neg	Steroids	mI (6) [-1]
4	BT, frontal and parietal hyperintensity; periventricular WM hyperintensities – [*n.p.*]	1496 – Lgi1	Steroids	mI (6) [-1]
5	Supratentorial WM lesions – [*PET: basal ganglia hypermetabolism*]	2100 – Lgi1	Steroids; PE and CFX, Aza	MI (70) [-4]
6	BT – [*PET: bilateral fronto-temporal hypometabolism; mild bilateral basal ganglia and cerebellar vermis hypermetabolism*]	706 – Lgi1	PE, IvIg, steroids	MI (11) [-4]
7	UT, left hippocampal atrophy; non specific WM lesions – [*n.p.*]	132 – neg	Steroids	mI on cognitve functions and control of seizures; left hippocampal sclerosis (17) [-1]
8	Diffuse mild atrophy – [*SPET: hypoperfusion of bilateral temporal and parietal lobes and mild hypoperfusion of frontal cortex, right thalamus and left putamen*]	751 – neg	Steroids, Aza; PE	initial MI; 2 relapses; subsequent death due to pneumonia (14) [-3]
9	BT – [*PET: fronto-temporal hypometabolism*]	5056 – Lgi1	Steroids, IvIg, PE; CFX, Aza	MI; MRI: mild hippocampal atrophy; 1 relapse (53) [-3]
10	UT – [*PET: neg*]	150 – neg	Steroids	MI (4) [-3]
11	BT – [*n.p.*]	146 – Caspr2	-	MI; MRI: hippocampal atrophy. Subsequent right parietal lobe metastatis from lung cancer (18) [-4]
12	Neg – [*PET: fronto-termporal hypoperfusion; relative striatal hyperperfusion*]	3006 – Lgi1	PE	Mild residual congnitive impairment (19) [-2]
13	Neg – [*PET: right temporal hypometabolism*]	447 – Caspr2	-	Spontaneous improvement (21) [-4]
14	MT – [*n.p.*]	Lgi1	Steroids, IvIg	mI with persistence of seizures; 1 relapse (10) [-2]
15	MT – [*n.p.*]	Caspr2	-	Stable (24) [=]
16	BT – [*n.p.*]	Lgi1	Steroids	MI (3) [-4]
17	Neg – [*n.p.*]	Lgi1	Steroids, PE	MI (5) [-5]
18	MT – [*PET: relative striatal hypermetabolism*]	Lgi1	Steroids, IvIG, PE, CFX	very mI (3) [-0,5]

Table 2.1 – 2-2 legend. M = Memory disturbances; S = Seizures; C = Confusion. BT-UT = bilateral-unilateral medial temporal lobe T2-weighted hyperintensity; WM = white matter. MI – mI = Marked – mild improvement. Modified Rankin scale: 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 (from Graus et al 2001, Brain 124;1138-48).

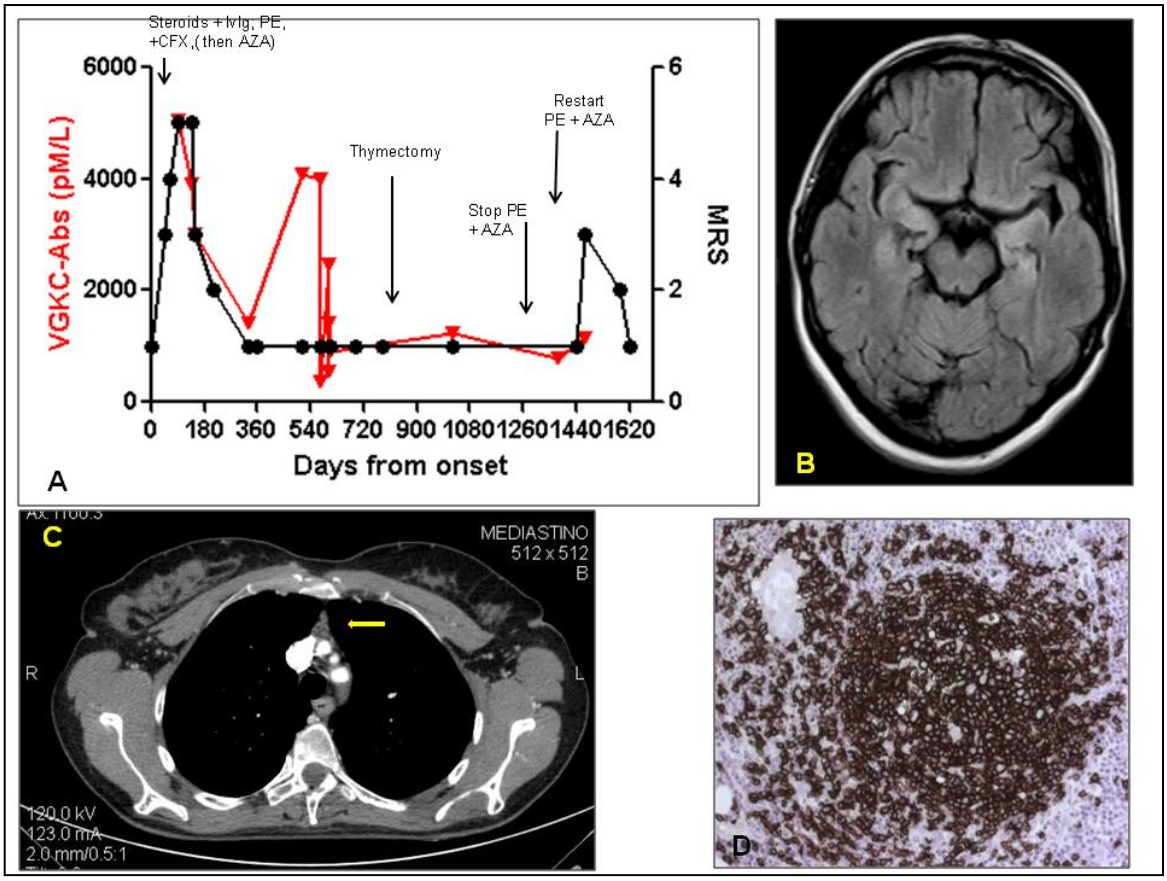

Figure 2.1. Patient 9: clinical and serological clinical course (A); axial brain MRI FLAIR images at the onset showing T2-weighted hyperintense hippocampi (B); CT scan of the thorax showing an enlarged thymus (arrow) (C); thymus: follicular hyperplasia and a germinal centre after immunostaining for B-cell marker CD20 (200x) (D).

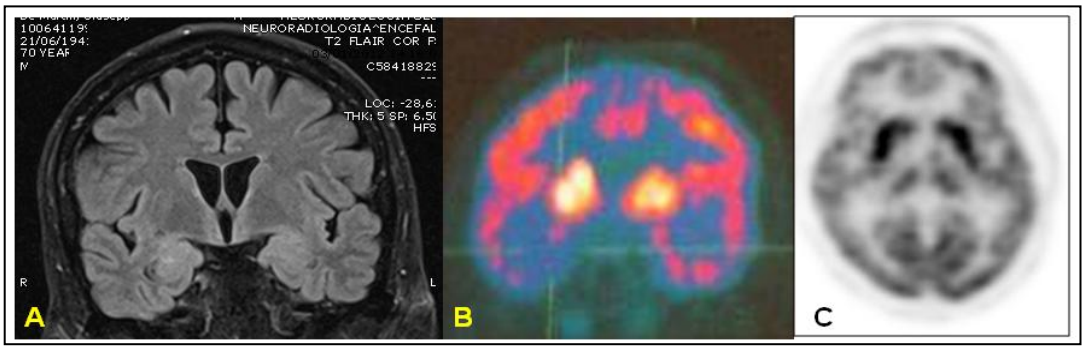

Figure 2.2**.** Patient 12's coronal brain MRI FLAIR images showing hippocampal swelling (A); Patient 2's 18-FDG brain PET scan showing increased bilateral striatal captation of radioligand 4 months after onset and during a phase of relative remission (B); Patient 3's 18F-FDG brain PET showing pathologically increased metabolism in basal ganglia (C).

3. CONTACTIN-ASSOCIATED PROTEIN-2 ANTIBODIES IN NON-PARANEOPLASTIC CEREBELLAR ATAXIA

3.1 Introduction

Cerebellar ataxia is a relatively common syndrome with diverse causes. Some patients have a paraneoplastic aetiology associated with autoantibodies to intracellular antigens, such as Yo (PCA-1), but these antibodies are unlikely to be directly pathogenic, and the patients seldom respond well to immunotherapies. In the last few years, antibodies to neuronal surface antigens have been demonstrated in patients with immunotherapy-responsive forms of limbic encephalitis and related disorders (see (Graus et al., 2009; Vincent et al., 2011)), raising the possibility that other CNS disorders may also result from autoantibodies to cell-surface proteins. There have been some previous reports of potentially pathogenic antibodies in cerebellar ataxia, such as voltage-gated calcium channel (VGCC) antibodies (Graus et al., 2002), glutamic acid decarboxylase (GAD) antibodies mainly in patients with polyendocrine syndromes(Honnorat et al., 2001), a small number with mGluR1 antibodies(Sillevis Smitt et al., 2000), and associations of cerebellar ataxia with gluten sensitivity and gliadin antibodies(Hadjivassiliou et al., 2008), but there have been few systematic cohort studies to identify new antigens. Here we have identified a potentially pathogenic antibody against the neuronal membrane protein CASPR2 in a total of nine of 88 (10%) patients with unexplained ataxia.

3.2 Subjects and Methods

3.2.1 Clinical Material

Twenty-five Spanish sera (Valencia, 10; Barcelona, 15) were from patients with acute or subacute cerebellar ataxia (< 3 months duration at testing) referred for onconeural antibody-testing, with some evidence of an autoimmune mechanism (CSF raised cells or

oligoclonal bands, partial response to immunotherapy and/or spontaneous remission) but with no serological or imaging evidence of tumours, and exclusion of other causes including infectious disorders. We also studied 27 and subsequently a further 36 sera from Welsh patients with idiopathic late onset cerebellar ataxia who had been recruited to a population-based study in south Wales between 1999 and 2008 (Wardle et al., 2009). All three centres had ethical approval for the study of these patients' sera. Control sera included 101 from patients with multiple sclerosis and 43 from patients with dementia. Radioimmunoprecipitation assays were used to look for antibodies to VGCC, GAD and voltage-gated potassium channel complexes (VGKC-complex) as previously described (Graus et al., 2002; Honnorat et al., 2001; Irani et al., 2010b).

3.2.2 Antibody-Binding Assays

Cerebellar organotypic slice cultures were prepared from 9-day-old mice and dissociated cultures of cerebellar granule neurons (CGNs) were prepared from 5-day-old mice as previously described (Becker et al., 2009). Antibody-binding assays were performed on organotypic slices after 12 days (P9+DIV12) in culture and on CGNs after 10 days (P5+DIV10). Unfixed slices or neurons were incubated with patient sera (1:125) in serum-free culture media supplemented with 25 mM Hepes and 1% bovine serum albumin (BSA) for 1 hr at room temperature (RT), washed three times and fixed with 3% formaldehyde in phosphate-buffered saline (PBS) for 30 min (slices) or 15 min (CGNs) at RT. Subsequently, slices were permeabilized with methanol for 5 min at -20^{o}C. After 3 washes, slices and CGNs were incubated with anti-human IgG Alexa Fluor 568-conjugated secondary antibody (Invitrogen) for 45 min at RT. Slices were counterstained with an anti-calbindin antibody (Swant) to label Purkinje neurons. Slices and CGNs were washed and mounted with mounting medium containing DAPI (Vectashield, Vector Laboratories).

The cell-based assay was performed as described[(Irani et al., 2010a)]. Briefly, human embryonic kidney cells (HEK293T) (American Type Culture Collection) were transfected with EGFP-tagged CASPR2. 48 hrs post-transfection, live cells were incubated with patient sera (1:50) for 1 hr at RT and subsequently fixed and stained as described above. Antibody binding was visualised using an immunofluorescence microscope and scored by

at least two independent blinded observers from 0 - 4 as in previous studies (Leite et al., 2008; Waters et al., 2008). The final score was the median of scores of 2-3 independent assays for each serum (variance <1).

3.2.3 Immunoprecipitation and Mass Spectrometry

Live CGNs in culture (P5+DIV10) were incubated with patient sera (1:40) in serum-free culture media buffered with 25 mM Hepes for 30 min at RT. Subsequently, neurons were washed twice and solubilised with 10 mM Tris pH7.5, 100 mM NaCl, 1 mM EDTA, 1% TritonX-100 and protease inhibitors for 45 min rotating at 4°C. Samples were centrifuged for 15 min at maximum speed at 4°C, and the supernatants were incubated with protein A-agarose for 5 hr at 4°C. The beads were washed five times and resuspended in SDS sample buffer, boiled and analysed by gradient SDS-PAGE (Nupage, Invitrogen) with Coomassie staining (Imperial protein stain, Thermo Scientific). Coomassie-stained bands were extracted as previously described (Shevchenko et al., 2006). Digested protein material was analysed by liquid chromatography using an Ultimate 3000 nano-HPLC system interfaced with a 3D high capacity ion trap mass spectrometer (AmaZon, Bruker Daltonics). Raw LC-MS/MS data were processed using DataAnalysis 3.4 (Bruker Daltonics) and Mascot software with the SwissProt database.

3.3 Results

To identify surface antigens that might mediate autoimmune cerebellar ataxia, we first screened the 25 Spanish and 27 of the Welsh sera. None had onconeural antibodies, evidence of a tumour, or an identifiable genetic or other cause. One patient had very high antibodies to GAD (4000 U/ml), four patients had low levels of antibodies against VGCC (range 84-122 pM, normal values (nv) <45pM), and two patients had antibodies to VGKC-complexes (757 and 165 pM, nv <100 pM).

We tested these 52 patient serum IgGs for binding to live, unpermeabilized cerebellar neurons using organotypic cerebellar slice cultures and dissociated primary cerebellar granule neurons. We identified 14 sera that showed positive staining on cerebellar neurons and no significant binding with seven healthy sera, all observed in a blinded

manner. We focussed on the serum that bound most strongly in a punctate manner to the axons of the cerebellar granule neurons in the molecular layer of the slices and to cerebellar neurons (Fig. 3 A, B). To identify the neuronal surface proteins recognized by the patient serum IgG, we performed an immunoprecipitation from the granule neuron cultures using this patient's and one healthy control serum. A 180-kDa polyprotein was selectively precipitated by the patient serum (Fig. 3 C), and identified as contactin-associated protein like-2 (CASPR2) by mass spectroscopy. The other distinctive bands yielded no significant hits (data not shown).

To confirm the presence of CASPR2 antibodies in this patient's serum, and to test the whole cohort, we employed a cell-based assay using full-length GFP-tagged human CASPR2 expressed in HEK293T cells (Irani et al., 2010b). We scored the binding of the serum antibodies with a visual scoring system on coded samples that has been well established and validated for other antibodies (Leite et al., 2008; Waters et al., 2008) and is used for routine diagnosis. The index patient serum bound strongly to the CASPR2 cells (score 4; normal values <1; Figure 3 D), but not to untransfected cells or control-transfected cells (not shown). An additional six sera (three of those positive on slices) also bound to CASPR2, with scores between 1 and 3 (total 7/52). We then tested another 36 ataxia patients and found a further two CASPR2-antibody positives, making a total of 9/88 (10%); one additional patient had VGKC-complex antibodies by immunoprecipitation (186 pM) but none were positive for GAD antibodies. Only 3/144 (2%) disease control sera tested in parallel were positive for CASPR2 antibodies (scores 1-2). All results are shown in Fig. 4 E.

Information on the CASPR2-Ab positive patients is given in Table 1. Most had a subacute or insidious onset mainly with a progressive course, but one with the lowest CASPR2 antibody had an acute onset, fully reversible cerebellar ataxia. Most samples were not taken early in the course of the disease. The index patient, who had the high VGKC-complex antibody titre of 757 pM, was a man of 59 years whose predominant presenting features were slurred speech, unstable gait and personality changes, followed by a nocturnal generalized tonic-clonic seizure. MRI, CSF and EMG were reported as normal. Although a heavy smoker all tumour screens were negative. He progressively deteriorated with difficulties in phonation, swallowing, and walking, as well as psychiatric disturbance, and probable temporal lobe seizures. CSF was normal, and viral screen negative. By that time, there was mild FLAIR hyperintensities in both hippocampi, with mild cerebellar atrophy, and interictal EEG showed bilateral temporal epileptiform

discharges. VGKC-complex antibodies were first found to be positive at this stage, and he was treated with high dose intravenous immunoglobulins with partial temporary amelioraton of symptoms including the ataxia; cyclosporine was introduced a year later and used for three years. When last seen, he was stable on lamotrigine and sertraline only, with scanning dysarthria and mild ataxia but able to walk in open spaces. He had monthly temporal partial seizures but his mood, behaviour and memory were normal. All the patients were negative for LGI antibodies (data not shown).

3.4 Discussion

We identified CASPR2 as an antigenic target in 10% of patients with idiopathic, cerebellar ataxia. CASPR2 is a transmembrane protein with a large extracellular domain and is highly expressed in the axons of the granule neurons of the cerebellum (Savvaki et al., 2008), thus representing an excellent candidate antigen for cerebellar ataxia. CASPR2 is essential for the clustering of the VGKC subunits Kv1 and Kv2 at juxtaparanodal regions of myelinated axons and at the axon hillock (Poliak et al., 2003) and recently has been described as one of the components of the VGKC-complex that is the target for antibodies in patients with limbic encephalitis, Morvan's syndrome and acquired neuromyotonia(Irani et al., 2010b; Lancaster et al., 2011a). However, in this study, only one of nine CASPR2-antibody positive patients was positive by radioimmunoprecipitation for VGKC-complex antibodies. This patient presented with ataxia and also had some limbic involvement but none of the other CASPR2-antibody positive patients had evidence of limbic encephalitis. The lack of VGKC-complex antibodies in the remaining patients suggests that CASPR2 antibodies in ataxia may recognize CASPR2 epitopes that are independent of the VGKC-complex. Indeed, immunostaining of rodent cerebellar tissue shows strong CASPR2 staining in the cerebellar granule and molecular layers but not in the strongly Kv1.1-positive cerebellar pinceau (Irani et al., 2010b). Thus, CASPR2 may have additional functions in the cerebellum as well as its established role in juxtaparanodal VGKC clustering, and the antibodies may bind preferentially to these CASPR2s.

Whether CASPR2 antibodies have a primary pathogenic role in ataxia, and their mechanisms of action, remain to be demonstrated. Although 3/9 patients had recent onset

of disease, and one of these had full recovery, six patients had a longer history at sampling; in these patients it is possible that CASPR2 antibodies represent a secondary phenomenon. However, lower levels of CASPR2 antibodies might represent the temporal tail of a higher antibody response present initially, since many of the newly-identified neurological diseases associated with antibodies to neuron surface proteins are monophasic (Graus et al., 2009; Vincent et al., 2011). There were patients studied here whose serum IgG bound to cerebellar slices or cultured cerebellar granule neurons but did not bind to CASPR2 or to VGKC-complexes, VGCCs or GAD. Although we did not attempt to perform mass spectrometry with these patients' sera, it is likely that other antigenic targets could be identified in the future. It is therefore important that future serological studies examine recent-onset patients and look for antibodies to CASPR2 and other novel antigenic targets. Immunotherapies could then be applied to those patients in whom a potentially pathogenic antibody is found, in the hope that some improvement or stabilisation will occur before permanent cerebellar damage.

Table 3. Summary of clinical and serological data in eight patients with CASPR2 and/or VGKC-complex antibodies.

Patient	**Age, sex**	**Duration at sampling**	**Onset, course**	**MRI**	**CSF**	**Extracerebellar involvement**	**Other autoimmune diseases**	**VGKC complex Ab (pM)**	**CASPR2 Ab score in CBA**	**Staining on cerebellar slices**
07-223	59, F	<3 mths	subacute, progressive	mild cerebellar atrophy, no temporal lobe changes	normal, OCB neg.	limbic encephalitis	thyroid antibodies	757	4	pos
06-26	50, F	<3 mths	subacute, progressive	mild atrophy -> severe atrophy	normal, OCB n/d	none	Sarcoidosis (mediastinal adenopathies), ANA	<100	3	pos
02-762	35, F	<3 mths	acute, full recovery	normal	n/a	n/a	n/a	<100	1	neg
A383	54, M	5 yrs	fast progressing	n/a	n/a	yes	n/a	<100	2.5	n/a
A425	55, F	14 yrs	subacute, progressive	normal	normal	none	Thyroid disease	<100	2	pos
A327	58, F	9 yrs	chronic, progressive	normal	n/d	none	no	<100	2	neg
A386	59	8 yrs	chronic, stable	n/d	n/d	none	no	<100	1	neg
A220	68, F	9 yrs	subacute, progressive	small vessel disease	n/d	mild bradykinesia	no	<100	1	pos
A201	76, F	n/a	acute/subacute	n/a	n/a	n/a	n/a	<100	1	n/a

Table 3. Summary of clinical and serological data in eight patients with CASPR2 and/or VGKC-complex antibodies.

The serum samples were obtained either at presentation or after several years. The majority had subacute onset with slowly progressive disease, except two patients, who had acute onset with full recovery. Four additional patients had low positive antibodies against voltage-gated calcium channels ((85-122 pM; nv<45 pM), two had VGKC-complex antibodies (186 pM, 168 pM) and one patient had high titres of GAD antibodies (4000 U/ml; nv<1 U/ml). Onconeural antibodies were negative in all, and tumours were not found. Sera 07-223, 06-26, 02-762 were from the Spanish ataxia cohort; all other sera were from the Welsh idiopathic ataxia cohort. n/d: not determined, n/a: not available.

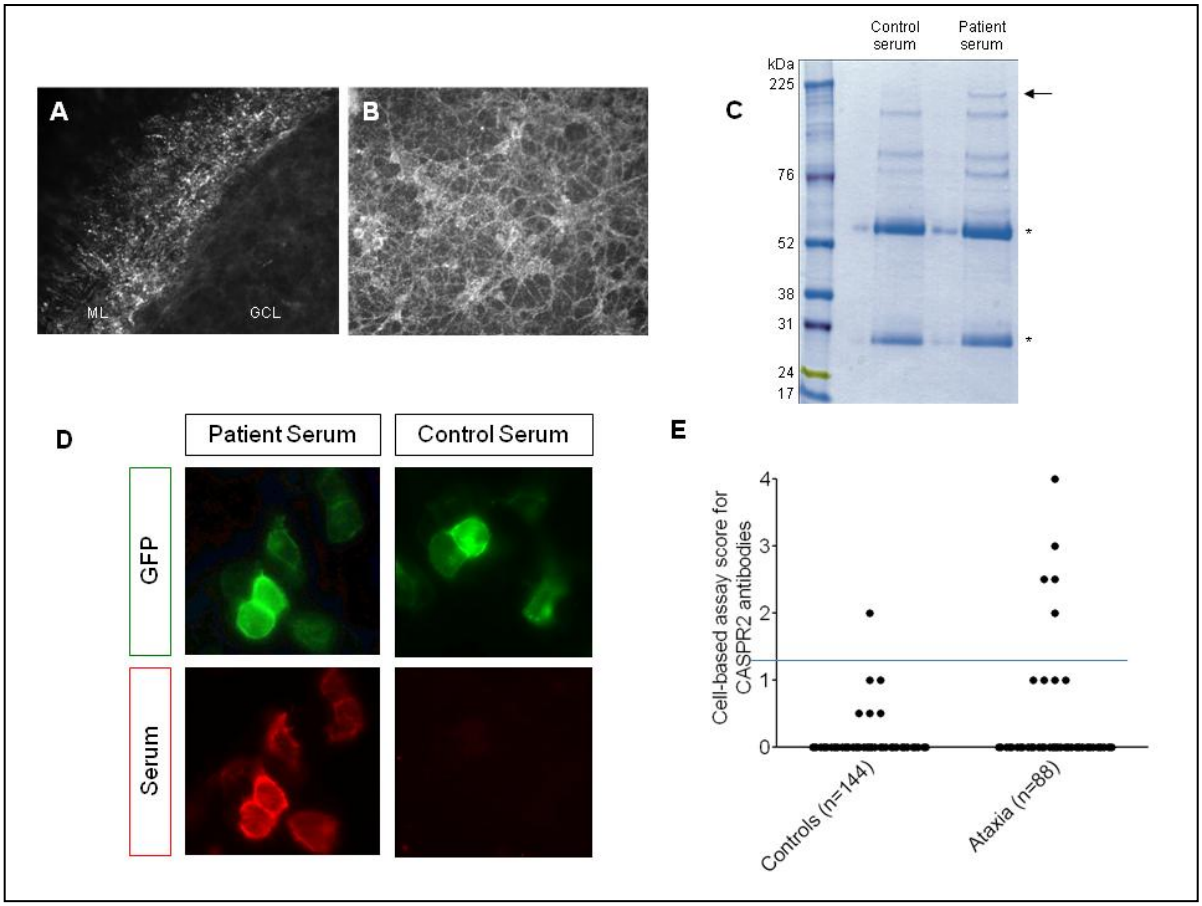

Figure 4. The serum bound strongly, in a punctuate manner, to the axons of cerebellar granule neurons in the molecular layer of unpermeabilized organotypic cerebellar slices (A) and unpermeabilized dissociated cerebellar granule neuron cultures (B). ML: molecular layer, GCL: granule cell layer. (C) Gel electrophoresis of immunoprecipitates after incubation of serum with unpermeabilized cerebellar granule neurons. The asterisks indicate heavy and light IgG chains, respectively. All precipitated proteins were excised and analysed by mass spectrometry. CASPR2, corresponding to the 180-kDa protein band selectively precipitated by patient's IgG (arrow) was the only significant membrane protein identified by mass spec (MudPIT scores>41). (D) The index patient serum IgG (red) bound strongly to the CASPR2-EGFP-transfected (green) HEK293T cells (score 4) but the control serum did not bind (score 0). (E) Binding in the cell-based assay (CBA) was determined at 1:100 serum dilution and scored visually from 0 (no binding) to 4 (very strong binding). Values of 1 and above were considered positive, as in our previous publications. The binding to CASPR2 (score 1-4) was detected in nine of 88 (10%) patient sera compared with three of 144 control samples (Fisher's exact test, p=0.011). Samples were considered positive only if 1 or greater in two independent assays. The horizontal line represents the cutoff above which the results are considered positive. To confirm some of the lower values, available sera were tested to endpoint dilution. MS sera scoring 1, and 2 titrated to 100 and 200; ataxia sera scoring 1 and 1.5 titrated to 200 and 400.

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