

DEFINING THE ROLE OF AUTOANTIBODIES IN PAEDIATRIC IMMUNE-MEDIATED ENCEPHALOPATHIES

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ABBREVIATIONS

Ab	Antibody
AChR	Acetylcholine receptor
ADAM22 and 23	A disintegrin and metalloproteinase domain-containing protein 22 and 23
ADEM	Acute disseminated encephalomyelitis
ADS	Acquired demyelinating syndrome
AED	Antiepileptic
AMPA	α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor
AQP4	Aquaporin-4
ASOT	Antistreptococcal antibody titres
BBB	Blood brain barrier
BSA	Bovine serum albumin
CASPR2	Contactin-associated protein-2
CBA	Cell based assay
CIDP	Chronic inflammatory demyelinating polyneuropathy
CNS	Central nervous system
CSF	Cerebrospinal fluid
CRION	Chronic relapsing inflammatory optic neuropathy
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
D1R	Dopamine receptor 1
D2R	Dopamine receptor 2
DNA	Deoxyribonucleic acid
DNER	Delta/notch-like epidermal growth factor-related receptor
EBV	Epstein-Barr virus
EEG	Electroencephalography
EGFP	Enhanced green fluorescent protein
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FIPA	Fluorescence-based immunoprecipitation assay
GABA	γ -Aminobutyric acid
GAD	Glutamic acid decarboxylase
GFAP	Glial fibrillary acidic protein
GlyR	Glycine receptor
GBS	Guillain-Barre syndrome
HBSS	Hanks-buffered salt solution
HEK cells	Human embryonic kidney cells
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HHV6	Human herpesvirus 6
HHV7	Human herpesvirus 7
HSV	Herpes simplex virus
HSVE	Herpes simplex virus encephalitis
IHC	Immunohistochemistry
IVIG	Intravenous immunoglobulin
IVMP	Intravenous methylprednisolone
Kv1	VGKC subunit
LE	Limbic encephalitis
LEMS	Lambert-Eaton myasthenic syndrome
LGI1	Leucine-rich, glioma inactivated 1
MAGUK	Membrane associated guanylate kinase

MEM	Minimum essential medium eagle
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
mRS	Modified Rankin Scale
MS	Multiple sclerosis
MMF	Mycophenolate mofetil
NB	Neurobasal
NGS	Normal goat serum
NMDAR	N-methyl D-aspartate receptor
NMDAR-Ab	NMDAR autoantibodies
NMO	Neuromyelitis optica
NMOSD	Neuromyelitis optica spectrum disorder
NMT	Neuromyotonia
OCB	Oligoclonal bands
OMS	Opsoclonus Myoclonus Syndrome
PANDAS	Paediatric Autoimmune Neuropsychiatric Disorders
PERM	Progressive encephalopathy with rigidity and myoclonus
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethyleneimine
RIA	Radioimmunoassay
PLEX	Plasma exchange
PLL	Poly-L-Lysine
PSA	Penicillin/streptomycin/amphotericin
RRMS	Relapsing remitting multiple sclerosis
RT	Room temperature
SC	Sydenham's chorea
SCLC	Small cell lung cancer
SEM	Standard error of mean
SD	Standard deviation
SLE	Systemic lupus erythematosus
SPS	Stiff-person syndrome
TS	Tourette syndrome
VGKC	Voltage gated potassium channel
VZV	Varicella zoster virus
WCC	White cell count

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Abstract

Autoimmune encephalopathies, mainly described in adults, are neurological syndromes characterized predominantly by impaired alertness usually with seizures, movement disorders, psychiatric features or autonomic instability. Well-characterised serum or cerebrospinal fluid (CSF) antibodies to neuronal surface proteins have been identified in these disorders. The aim was to define and extend the role of autoantibodies in paediatric encephalopathies. The clinical, paraclinical and immunological features of patients with autoimmune encephalopathies, acute encephalitis, N-methyl-D-aspartate receptor (NMDAR)-antibody and voltage gated potassium channel (VGKC)-complex antibodies, and in children with basal ganglia encephalitis were studied in detail.

In 48 children with a diagnosis of probable autoimmune encephalopathy, 21 (43%) had CNS autoantibodies, principally to NMDAR and VGKC-complexes, but children without these antibodies could also respond well to immunotherapies. Immune-mediated/autoimmune encephalitis was also the largest subgroup (34%) of encephalitis in an Australian paediatric encephalitis cohort. NMDAR-antibodies were found in children with well-defined encephalitis, but also in patients presenting without an encephalopathy, with distinct clinico-radiological white matter syndromes, or with a neurological relapse following herpes-simplex encephalitis. Children with VGKC-complex antibodies did not have autoantibodies to the complex associated proteins leucine-rich glioma inactivated 1 (LGI1) and contactin-associated protein 2 (CASPR2) as frequently seen in adults, but were more likely to have a primary neuroinflammatory disease than in patients without these antibodies. Finally, despite a recent report, dopamine receptor antibodies or an alternative autoantibody were not found in children with basal ganglia encephalitis.

Novel clinical syndromes were identified with NMDAR-antibodies, raising important questions about the role of NMDARs in white matter, and the relationship with preceding infections. Patients with basal ganglia encephalitis may have an alternative pathobiology mediating their disease. Moreover, antibody positivity is not essential for the diagnosis of autoimmune encephalitis and children with or without detectable neuronal antibodies may respond to immunotherapy.

CHAPTER 1 - Introduction

Antibodies binding to central nervous system (CNS) ion channels, receptors and other synaptic proteins are now recognised as a likely cause of neurological disorders in both adults and children. These cell-surface antigens are important for cellular function or neurotransmission and are generally expressed throughout the nervous system. Nevertheless, the resulting disease can be focal, with patients demonstrating clinical signs depending on the specific brain regions targeted (Vincent et al., 2011). The disorders can be either monophasic or relapsing, and frequently respond to immunotherapy. Some of these conditions are associated with tumours, but the frequency of malignancies differs depending on the identity of the antigen and the age of the patient (Dalmau et al., 2011).

By contrast, antibodies against intracellular antigens are generally considered unlikely initiators of disease, but are generated during tissue destruction or represent an epiphenomenon of the immune response. Unfortunately patients with these disorders do not often respond to immunotherapies. The antibodies may bind to nuclear (e.g. Hu) or cytoplasmic (e.g. Yo, Ma2) proteins, and in some cases to intracellular synaptic proteins such as glutamic acid decarboxylase 65 (GAD65) or amphiphysin (Lancaster and Dalmau, 2012).

Our understanding of CNS autoimmunity is derived primarily from autoimmune neuromuscular disorders such as myasthenia gravis, in which the role of acetylcholine receptor (AChR) antibodies has been well established by both *in vivo* and *in vitro* approaches (Vincent et al., 2006). In the peripheral nervous system (PNS) the following criteria were proposed for an antibody to be considered pathogenic (Drachman, 1990): (i) Detection of autoantibodies in patients with the disease but not in controls, (ii) Antibody interacts with the target antigen and can be detected in the target tissue, (iii) Passive transfer of antibody reproduces features of disease, (iv) Immunization with antigen produces a model disease, (v) Reduction of antibody levels in patients correlate with clinical improvement. As the CNS is protected from circulating

antibodies by the blood-brain barrier (BBB), it may be difficult to show an immediate correlation between rapid antibody reduction (e.g. following plasma exchange) and change in the clinical severity. Similarly, it can also be difficult to achieve successful transfer of antibody-mediated disease to the experimental animal. Hence, the identification of CNS disorders that are antibody-mediated currently relies on evidence demonstrating the presence of an antibody in the serum and usually also in the cerebrospinal fluid (CSF), its temporal relation to the disease state, and the success of immunological treatments.

In this Introduction, some of the general features of autoimmunity will be discussed, including the role of infections as triggers of autoimmune disorders. The targets of neuronal surface autoantibodies will be the main focus with a brief review of the associated genetic and autoimmune clinical syndromes. This is followed by some of the issues regarding antibody detection and use of immunotherapies, in patients whose management can be very challenging.

1.1 *Autoimmunity*

The immune response consists of both the initial phase, which can be either deleterious, as in autoimmune disease, or beneficial, as in vaccinations or infectious disease, and a later regulatory phase to return the immune system to homeostasis, with memory cells induced in order to ensure persistent immunity. Autoimmunity in general results from failure of the mechanisms responsible to preserve tolerance to self (Galli et al., 2012). For an antibody-mediated disease to occur an individual must express major histocompatibility complex (MHC) molecules that efficiently present peptides derived from the target self-antigen to self-reactive T cells, and this must initiate the proliferation of B-cells that can recognise the self-antigen. There are many potential mechanisms of autoimmunity that include genetic susceptibility or polyclonal activation of self-reactive T and B cells, and other factors that can make an

individual susceptible, such as environmental triggers and exposure of self-epitopes following damage. During the process of induction of autoimmunity or its results, there may be epitope spreading and bystander activation of autoimmune cells. None of these aspects will be discussed in detail as their relevance to the topic of this thesis is not yet clear, but the following provides a brief summary.

1.1.1 B-cells and the role in CNS autoimmunity

B-cells are members of the adaptive immune system and, in addition to their role in antibody production, are also important for antigen presentation, lymphocyte trafficking and cytokine production. B-cell development begins in the bone marrow and proceeds through maturation stages of pro- and pre-B cells which then exit into the circulation as naïve, antigen-inexperienced B cells (Pieper et al., 2013, LeBien and Tedder, 2008).

The majority of B cells are located in follicles. Upon stimulation by a the antigen-specific T cell, which usually occurs in germinal centres in the secondary lymphoid tissues that include lymph nodes, spleen, and mucosal sites, the activated B cell begins to differentiate. Germinal centre B cells may differentiate into memory B cells or plasma cells. The mechanism by which a B cell becomes one or the other of these is a process known as affinity maturation (Pieper et al., 2013). Most of these B cells will become plasmablasts (or "immature plasma cells"), and eventually plasma cells, and begin producing large volumes of antibodies.

Antibodies (or immunoglobulins, Ig) are proteins produced by plasma cells and consist of two heavy chains and two light chains that form a Y-shaped structure. Each of the chains has a constant and a variable region; the constant regions determine the antibody isotype and effect functions and the variable regions are important for antigen recognition. Antibodies are subdivided into five different classes depending on which heavy chain they contain - alpha (IgA), gamma (IgG), delta (IgD), epsilon (IgE) and mu (IgM). The heavy chains of IgM and IgE

are made up of four immunoglobulin (Ig) domains, and IgA, IgG and IgD contain three repeats of the Ig domain with an added hinged region that allows greater flexibility. The variable region of the heavy chain has one Ig domain. Whilst IgA molecules form dimers and IgM pentamers of the basic Y-shaped structure; the other three Ig classes are monomers. The first antibodies produced in an immune response are IgM, which are pentameric immunoglobulins with relatively low affinity, but they have a higher avidity due to the presence of ten antigen binding sites on each pentamer. After the class switch, the loss in avidity is made up by an exponential gain in affinity of the antibodies. Binding of the antigen through the B cell receptor (BCR) triggers activation, proliferation and somatic hypermutation of BCRs resulting in maturation to memory B cells. BCRs are heterodimeric proteins with the antigen-binding portion formed by the variable regions of heavy and light chains (LeBien and Tedder, 2008).

The heavy chain variable region (VH) is thought to play the primary role in antigen-recognition. The VH results from the splicing of three gene segments into a mature transcript; one copy of a variable (V), diversity (D), and joining (J) gene segment, exist as multiple copies in each genome resulting in antibody diversity due to the variation in how the gene segments are spliced together, and the somatic mutations in the complementarity determining regions (CDR) of V genes that shape the Ab response. The enzyme Activation-Induced (Cytidine) Deaminase generates random mutations in these regions of the DNA which then leads to a great number of B cells that produce antibodies that have slight variations in their CDR and thus slightly different affinities for the antigen. Following exposure to an antigen, BCRs undergo somatic diversification as their phenotype advances from naïve to memory B cells, and then to Ab-secreting plasmablasts and plasma cells (Pieper et al., 2013). The B cells with the highest affinity antibodies are then positively selected to generate plasma and memory cells.

B cells are also sophisticated and highly selective antigen-presenting cells (APCs). Once the clonally specific antigen has been recognised by the surface BCR, the antigen is then

internalised, complexed in endosomes with class II major histocompatibility complex (MHC) molecules, and then returned to the surface for antigen presentation to T cells. In the secondary lymphoid structures B cells are important in transporting cells for antigen presentations, once they have been recognised by macrophages.

Through secretion of cytokines B cells can also regulate, as bystanders, various effector immune functions mediated by both B and T cells. Some B cells support pro-inflammatory function through secretion of tumour necrosis factor alpha ($\text{TNF}\alpha$) and lymphotoxin, whereas interleukin (IL)-10-producing B cells have regulatory, anti-inflammatory, roles.

Importantly, BCRs expressed on mature naïve B cells emigrating from the bone marrow, are frequently poly-or autoreactive. Checkpoints in the bone marrow during B cell maturation ensure that B cells that bind multivalent membrane autoantigens are eliminated (Cornall et al., 1995). CD4, CD25 and FoxP3 positive regulatory T cells are responsible for the induction and maintenance of tolerance against self antigens in the periphery (Vignali et al., 2008). Defects in these mechanisms may allow antigen-presenting B cells to activate self-reactive B cell clones via self-antigen. This results in a loss in T cell tolerance, which facilitates spontaneous germinal centre (GC) reactions and promotes development of high-affinity, class-switched autoantibodies as seen in autoimmune diseases such as systemic lupus erythematosus (SLE) (Yurasov et al., 2006) and rheumatoid arthritis (RA) (Samuels et al., 2005).

Although such mechanisms are likely to influence the potential for brain directed autoimmunity, an important area for research is to determine whether cellular components of the immune system, that have escaped tolerance induction, may access the brain to recognise brain-specific antigens, and form localised specialised subarachnoid germinal centres, or whether B cells undergo antigen-stimulation and affinity-maturation in the periphery prior to

entering the CNS compartment (Ransohoff et al., 2003). Indeed, memory B cells and short-lived antigen secreting plasma blasts were detected in the CSF of patients with inflammatory CNS diseases such as multiple sclerosis and acute CNS infections (Cepok et al., 2005), and it seems that these cells can cross the blood brain barrier and enter the CNS in low numbers. Beneficial effects of peripheral B cell depletion, using drugs such as Rituximab (monoclonal anti-CD20), in CNS inflammatory disease suggest that B cells in the periphery are important for initiation and perpetuation of CNS directed inflammatory activity, but although peripheral B cell depletion will also partially deplete CSF B cells it will not affect existing antibody-producing cells in the bone marrow or in the CNS (von Budingen et al., 2011). Identification of a restricted pool of clonally related B cells participated in robust bidirectional exchange across the blood brain barrier (BBB), with some B cells undergoing different diversifications in the two compartments, was recently demonstrated using sequencing of IgG heavy chain variable region genes (IgG-VH) in paired CSF and blood samples from patients with multiple sclerosis (Palanichamy et al., 2014). Thus there is evidence for on-going stimulation and maturation to clonally restricted antigen-expressing B cells occurring primarily, but not exclusively, inside the CNS compartment.

CSF immune cell phenotyping was useful in identifying patients with CNS inflammation. In comparison to healthy individuals in whom T cells were the predominant population in the CSF (with CD4+ T cells being more prevalent than CD8+ T), elevation of B cells and plasmablasts was found in inflammatory diseases (Kowarik et al., 2014). Identification of specific immune cell profiles in patients with neuroimmune diseases may increase our understanding of the disease and provide useful biomarkers for prognosis and treatment monitoring. Nevertheless, when evaluating the diagnostic accuracy of analyses that identify inflammatory mechanisms, care needs to be taken when CNS disorders are classified only according to their primary pathology. Many of these techniques may identify secondary

inflammation; CSF oligoclonal bands, for instance, can be detected in patients with primary non-immune aetiologies (Hacohen et al., 2014e).

1.1.2 Infections and autoimmunity

The link between infections and autoimmunity is complex, comprising genetic and environmental interactions. Different infectious agents (viruses, bacteria, fungus and parasites) can trigger autoimmunity, which can be both pathogen and disease-specific. By contrast, Infections can protect individuals from some autoimmune diseases and in fact, the same infectious agent can induce one specific autoimmune disease and protect from another autoimmune disease (Kivity et al., 2009). The sequence of infections during life, may also be important, with infections during childhood relevant to the risk of autoimmunity in adulthood as shown in diseases such as multiple sclerosis (Waldman et al., 2014). Genetic susceptibility, or an underlying immune dysregulation may explain why only a subgroup of individuals will develop autoimmunity after infections (Kivity., et al 2009). This induction of post-infection autoimmunity can result from a number of mechanisms either in isolation or in combination such as molecular mimicry, bystander activation, epitope spreading and polyclonal activation of B cells (Galli et al., 2012).

(1) Molecular mimicry

One of the main mechanisms of interest is molecular mimicry, which is the generation of immune responses to epitopes (i.e. protein, carbohydrate or DNA) shared by the pathogen and the host. The criteria for this mechanism include: (i) the pathogen must be associated with the onset of the autoimmune disease; (ii) the pathogen must provoke an immune response that cross-reacts with host antigens; (iii) the shared epitope (auto-antigen) can induce the disease in an animal model by active immunisation; (iv) autoreactive T cells or auto-antibodies can

induce the disease in animal models by passive immunisation (Kivity et al., 2009). The classic example of molecular mimicry in neurological diseases is the association between Guillain-Barre syndrome (GBS) and *Campylobacter jejuni*; *C. jejuni* strains carrying the sialyltransferase gene induced the synthesis of GM1-like lipo-oligosaccharides (LOS) (Godschalk et al., 2007) and the resulting antibodies can bind ganglioside GM1.. Rabbits given *C. jejuni*-derived LOS, developed GM1 antibodies and a flaccid limb weakness. GM1Abs derived from mice immunised with LOS, or from patients with GBS, bound to human peripheral nerves and blocked muscle action potentials (Yuki, 2001). Bystander activation occurs when virally-induced tissue damage causes the release of sequestered antigen, which activates auto-reactive lymphocytes that were not directly involved in the initial reactivity to the virus (Kivity et al., 2009). Virally infected antigen presenting cells may stimulate host T-cells which can generate cytokines that lead to bystander, non-specific, killing of neighbouring (uninfected) cells and an autoimmune response (Duke, 1989). Prolonged infectivity with viruses, viral proteins or viral genome can lead to autoimmunity via continuous activation of the immune response. This polyclonal activation may result in monoclonal-specific activation and proliferation of B cells, accompanied by circulating immune complexes and eventually result in damage to self-tissues.

(2) Epitope spreading

Epitope spreading is defined as the development of an immune response to epitopes distinct from, and not cross-reactive with, the initial focused, dominant epitope, and can be extended to include other proteins within the target tissue (Vanderlugt and Miller, 2002). This phenomenon was hypothesised to occur as a consequence of attempts to clear an infections insult or may arise secondary to chronic tissue destruction. The inflammation and tissue damage then primes a hierarchical cascade of autoreactive T and B cells against self-epitopes, allowing cryptic or sequestered epitopes to be processed and presented (Vanderlugt et al., 1998).

Presentation of the primary epitope (the immunodominant self or viral epitope) occurs in peripheral lymphoid tissue resulting in activation and differentiation of autoreactive TH1 cells which migrate into the target tissue, where they encounter antigen presented by resident APCs. After antigen restimulation, the pathologic TH1 cells release a cascade of chemokines and cytokines, leading to recruitment of additional mononuclear phagocytes from the peripheral blood, which are activated along with resident APCs (Vanderlugt and Miller, 2002). Epitope spreading has a role in several viral-induced autoimmune diseases; Theiler's murine encephalomyelitis virus (TMEV) induces a life-long infection of antigen-presenting cells (APCs) in the CNS, leading to a chronic T-cell-mediated demyelinating disease in mice (Miller et al., 1997). Epitope spreading can also happen without the viral induction and was described in an experimental model of myasthenia gravis; when rabbits were immunised with peptides of part of the extracellular domain of the human AChR α -subunit, they became weak and were found to have developed antibodies specific for the intact, native rabbit AChR (Vincent et al., 1994). Although epitope spreading is primarily thought to be initiated as a result of tissue damage and to play an active role in on-going disease, there is some evidence that epitope spreading could be a component of protective immune responses, acting to enhance the efficiency of the immune response, as seen in tumour clearance, and as a mechanism to down-regulate immune responses, such as those occurring in autoimmunity.

1.2 *The pathogenic role of autoantibodies in neurological diseases*

There are clinical and experimental paradigms that have been useful when considering if a peripheral disorder is mediated by an antibody. The antibodies should be disease-specific and bind to the extracellular domain of the target antigen; they should alter the function or number of the target in a manner that explains the clinical phenotype; plasma exchange and other immune therapies should be clinically effective; and passive transfer in animal models should

lead to clinical or paraclinical evidence of the disease (Vincent et al., 2011). To date, the work in GBS and in myasthenia, form the best examples of autoimmune peripheral nervous system disorders and the evidence for the pathogenicity of the antibodies. Animal models for passive transfer and active immunisation are much more challenging in the CNS conditions. This is because the integrity of the blood brain barrier prevents adequate levels of antibodies reaching their target antigen and infusion of antibodies directly into the CSF may not access adequately the brain parenchyma. As a result most information to date comes from *in vitro* cell preparations, which cannot adequately represent the complexity of the CNS pathophysiology. The following sections concentrate on the CNS autoantigens and include the state of current knowledge regarding the pathogenicity of the antibodies.

1.3 *Nuclear and cytoplasmic neuronal antigens*

These antibodies are collectively called paraneoplastic or onconeural antibodies and are strongly associated with both specific neurological syndromes and tumours. The reported phenotypes in adults and children and the associated malignancies are summarised in Table 1-1. Paraneoplastic syndromes are neurological syndromes caused by the immune response to tumour-derived antigens. The antibodies are thought to be induced by the tumour antigens but to cross-react with neuronal antigens in the CNS and PNS, as demonstrated by binding to brain tissue sections (Graus and Dalmau, 2012). However, the antibodies are mainly target antigens associated with RNA and DNA binding proteins and are not thought to be directly pathogenic since the patients do not improve with immunotherapies. However, their presence can predate the detection of the underlying tumour.

Anti-Hu (ANNA-1) antibodies are the most common paraneoplastic antibodies and are strongly associated with small cell lung cancer (SCLC) resulting from the anti-tumour immune response that targets the HuD protein expressed by both SCLC and neurons (Dalmau et al.,

1992). Patients typically develop sensory neuronopathy, cerebellar degeneration, or limbic encephalitis, usually with additional symptoms of extensive or multifocal encephalomyelitis. Post mortem studies of patients with anti-Hu-positive paraneoplastic syndromes demonstrated antibodies within various brain regions (Dalmau et al., 1991) but antigen injection into experimental animals, or immunisation of mice (Sillevis Smitt et al., 1995), did not result in any neurological symptoms. Interestingly, less than 5% of patients with SCLC develop a paraneoplastic neurological syndrome despite 20% of patients having anti-Hu antibodies.

T-cell-mediated mechanisms are implicated in these neurological syndromes, as supported by the identification of cytotoxic Hu-directed T lymphocytes in mice immunised with the Hu protein HuD (Plonquet et al., 2003); and brain and peripheral nerve tissue from patients showing more T than B lymphocyte infiltration (Dalmau et al., 1991).

Anti-Hu antibodies are uncommon in children and in a recent study only eight out of 251 patients were under the age of 18 (3%) (Honnorat et al., 2013). In contrast to the 243 adult patients who had classical paraneoplastic syndromes, two (25%) of the children had neuroblastoma and opsoclonus-myoclonus, with the other six presenting with non-paraneoplastic limbic encephalitis (Honnorat et al., 2013). It is possible that the limbic encephalitis, in the 6 children without a tumour, was due to an undiagnosed neuroblastoma that spontaneously regressed; alternatively as Hu is strongly expressed in all CNS neuronal nuclei, Hu-Abs could have been induced in the absence of a tumour, perhaps secondary to CNS insult.

Anti-MA2 (Anti-Ta) antibodies are associated with testicular cancer and typically are found in young men with limbic and brainstem encephalitis (Graus and Dalmau, 2012). Patients present with short-term memory deficits, seizures, hypersomnia, narcolepsy–cataplexy, hypothalamic–pituitary axis dysfunction, parkinsonism, or vertical gaze paresis. The antibodies may also be found in women and in older men (in association with non-SCLC, breast cancer,

colon cancer, and lymphoma) and were reported in one 14 year-old girl with severe encephalopathy, seizures and status dystonicus in the absence of a tumour (Haberlandt et al., 2011). About a third of patients improve or stabilise following oncological treatment and some clearly respond to immunotherapies (Dalmau et al., 2004).

Anti-Yo (PCA-1) antibodies are associated with paraneoplastic cerebellar degeneration and breast/ovarian cancers. Recently these antibodies were described in a 7-year-old girl with Down's syndrome and a history of adrenocortical carcinoma at the age of 1year. She presented with subacute onset cerebellar ataxia and did not respond to immunotherapy (Philipps et al., 2014).

Anti-Ri antibodies (ANNA-2) are found most frequently with brainstem encephalitis and opsoclonus myoclonus in association with SCLC, breast carcinoma and gynaecological malignancies in older adult females. No paediatric cases have been reported to date. In a study where 59 children with opsoclonus-myoclonus-ataxia were screened for serum autoantibodies, none had anti-Hu, anti-Ri, or anti-Yo (Pranzatelli et al., 2002).

Anti-Tr antibodies are associated with a paraneoplastic cerebellar degeneration, usually identified with Hodgkin's lymphoma (Graus and Dalmau, 2012). The target is an extracellular antigen, delta/notch-like epidermal growth factor-related receptor (DNER), which is highly expressed in the Purkinje cells in a punctate somato-dendritic manner, suggesting that these antibodies may be pathogenic (de Graaff et al., 2012). This was identified in 12 patients with anti-Tr antibodies in the serum (but in only one of 246 control individuals). This specificity was further supported by immuno-adsorption of one patient's serum with DNER abolished cerebellar neuron reactivity, and knockdown of DNER in neurons prevented recognition by patient serum samples.

These antibodies have been reported in young patients, including a 12-year-old girl with Hodgkin lymphoma (HL) (de Graaff et al., 2012) and in a further case of immunotherapy

responsive neurological syndrome, which preceded the diagnosis of HL by 6 months. Recent evidence supports (1) the strong associations between anti-Tr/anti-DNER, paraneoplastic cerebellar degeneration and HL; (2) the importance of thorough and on-going evaluation for HL in these patients; (3) the potential for significant clinical improvement with immunotherapy; and (4) the transient nature of the immune response in some patients (Greene et al., 2014).

Anti-CRMP5/CV2 presents with a wide spectrum of neurological syndromes such as limbic encephalomyelitis, cerebellar degeneration, sensory neuronopathy, chorea and optic neuritis and is predominantly associated with SCLC, breast cancer and thymomas (Graus and Dalmau, 2012), and not reported in children.

	Phenotype in adults	Phenotype In children	Associated cancer
Hu	Neuropathy (often purely sensory), cerebellitis, limbic encephalitis, autonomic dysfunction and/or brainstem encephalitis ^{1,2}	8 out of 251 patients were under the age of 18 (3%) Non-paraneoplastic limbic encephalitis ³	Small-cell lung cancer ² .
Collapsin response mediator protein 5 (CRMP5/CV2)	Limbic encephalomyelitis, cerebellar degeneration, sensory neuronopathy, chorea and optic neuritis ^{1,2}	Not reported	Small-cell lung cancer, breast cancer and thymoma ^{1,2}
Yo (also known as CDR1 and CDR2)	Women with paraneoplastic cerebellar degeneration ^{1,2}	7-year-old girl with subacute onset cerebellar ataxia. No respond to immunotherapy ⁵	Breast and gynaecological cancers ^{1,2}
Ri (also known as Nova-1 and Nova-2)	Brainstem encephalitis and opsoclonus myoclonus ^{1,2}	Not reported	Breast cancer ¹
Tr (Delta/notch-like epidermal growth factor-related receptor)	Paraneoplastic cerebellar degeneration ^{4,6}	12 year old girl with Hodgkin's lymphoma ⁴	Hodgkin's lymphoma ¹
MA1	Limbic encephalitis, cerebellitis, brainstem encephalitis or polyneuropathy ¹	Not reported	Lung, skin, gastrointestinal and renal ¹
MA2 (also known as Ta)	Young men with limbic and brainstem encephalitis, parkinsonism, vertical gaze paresis ^{1,2}	14 year old girl with severe encephalopathy, seizures and status dystonicus in the absence of a tumour ⁷	Germ cell tumours (testicular teratomas) ¹
Amphipysin	Stiff-person syndrome, myelopathy and myoclonus, encephalomyelitis, sensory neuronopathy ^{1,2}	Not reported	Breast cancer and small-cell lung cancer ^{1,2}

Table 1-1 Paraneoplastic antibodies and the associated phenotypes

¹Graus and Dalmau, 2012. ²Lancaster and Dalmau, 2012. ³Honnorat et al., 2013. ⁴Greene et al., 2014. ⁵Philipps et al., 2014. ⁶de Graaff et al. 2012. ⁷Haberlandt et al., 2011.

Despite the tumour and clinical associations of the individual onconeural antibodies, multiple antibodies can be found if the assays used are very sensitive. A mathematical

analysis of 1.2 million onconeural antibody tests, performed in nearly 80,000 patients with suspected paraneoplastic neurological conditions from the Mayo Clinic, identified specific clusters of neuronal antibodies which can help to predict a patient's cancer risk and type (Horta et al., 2014). A recent report of novel treatment responsive teratoma-associated encephalopathy, in young women, with brainstem and cerebellar involvement in whom no antibody was identified, highlights the importance of considering malignancy even in the absence of an antibody biomarker (Armangue et al., 2014c)

1.4 *Intracellular synaptic antigens*

Intracellular synaptic antigens, GAD65 and amphiphysin, are expressed in the presynaptic terminals and are important for recycling of synaptic vesicles. Amphiphysin antibodies have been described in adult patients with SCLC and breast cancer presenting with stiff person syndrome (SPS), myelopathy and myoclonus, encephalomyelitis and sensory neuronopathy (Lancaster and Dalmau, 2012). No paediatric cases have been reported to date. It has been hypothesized that the antigen could be exposed to antibodies during synaptic vesicle fusion and reuptake, and can be a target of both antibodies and T-cell responses (Lancaster and Dalmau, 2012). Passive transfer of purified IgG from one adult patient with breast cancer, stiff person syndrome (SPS) and amphiphysin antibodies into rats resulted in a dose-dependent stiffness with spasms resembling human SPS (Sommer et al., 2005), and thought to be due to inhibition of inhibitory synaptic activity. However, it is not clear whether amphiphysin is exposed during synaptic vesicle exocytosis, and whether it could take up the antibodies by this mechanism, or how they would interfere with release of transmitter only in the inhibitory neurons as amphiphysin is expressed also in excitatory neurons.

The neurological presentation most frequently seen with antibodies to GAD65 is SPS. GAD65, in addition to being the rate-limiting enzyme for the synthesis of GABA, is also

expressed in pancreatic islet cells and these antibodies are seen, generally at relatively low levels, in the majority of patients with type 1 diabetes mellitus and also in patients with other autoimmune diseases but without any neurological features (Hallengren et al., 1996, Saiz et al., 1997). However, CNS mediated diseases occur with GAD65-Ab levels over 100 times higher (CSF levels > 2000 U/ml, Saiz et al., 2008), which suggests that they may have some relevance to CNS disease. Although not thought to be directly pathogenic these antibodies are a useful marker in adult patients, not only for SPS, but also in patients with immune mediated epilepsy, cerebellar ataxia and limbic encephalitis, with variable degree of immunotherapy responsiveness (Saiz et al., 2008). These antibodies are rare in children but have been reported with limbic encephalitis and refractory seizures (Korff et al., 2011, Haberlandt et al., 2011).

Immunisation of mice with GAD65 resulted in a highly characteristic pattern on CNS tissue with intracellular binding to GAD, some loss of GABAergic neurons in the brainstem and, surprisingly, also resulted in antibodies that bound not only to GAD65 but also to the surface of GAD65/GABA-expressing cultured cerebellar neurons. Although these experiments did not produce any behavioural abnormality in the mice, it appeared that generating an immune response to GAD65 could induce antibodies to other antigens expressed on the surface of the GAD65 positive neurons (Chang et al., 2013).

1.5 *Neuronal cell-surface antigens*

Autoimmune encephalopathies are neurological syndromes characterised predominantly by impaired alertness, often with seizures, movement disorders, psychiatric features or autonomic instability, but without focal neurological deficits. Some patients with these encephalopathy syndromes have detectable serum or CSF antibodies directed against ion channels, receptors and synaptic neuronal proteins. MRI can be normal, although abnormal MRI features can

occur, as illustrated in this Thesis. In all such patients, exclusion of active CNS infection is essential (Gable et al., 2012, Hachon et al., 2013, Armangue et al., 2014a, Kneen et al., 2012). The clinical phenotypes in both adults and children, and the proposed pathogenic mechanism are summarised in Table 1-2 and Figure 1.1. In the following sections, the target antigens and the clinical syndromes that are associated will be described.

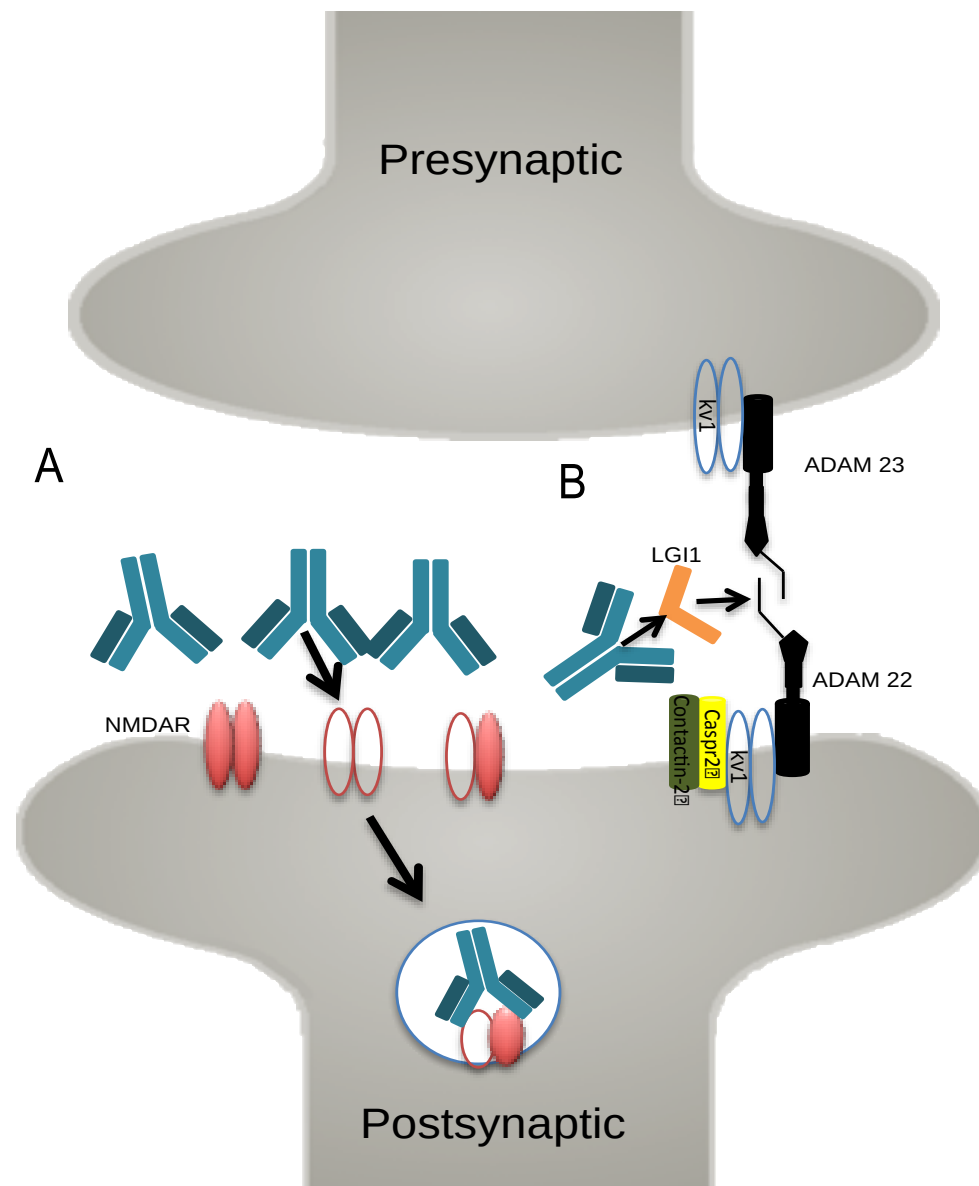


Figure 1.1 Pathogenic mechanism of CNS autoantibodies.

Multiple pathogenic mechanisms have been identified (A) In NMDAR-Ab encephalitis, antibodies bind the receptor and cause receptor internalisation. This result in an overall reduction of surface NMDAR, and the clinical symptoms of primarily NMDAR hypofunction (Moscato et al. 2014). (B) In LGI-Ab mediated limbic encephalitis, the antibodies bind to the secretory protein LGI, prevents interaction between LGI1 and ADAM22/23 and reversibly reduced synaptic AMPAR clusters (Ohkawa et al. 2013) The lack of complement activation or activation of further downstream inflammatory cascades amplifying neurodegeneration, in both diseases, may explain the good recovery seen in some patients following removal of the antibodies with immunotherapies.

	Main phenotypes in adults	Phenotypes in children	Pathogenic mechanism
NMDAR	Encephalopathy with psychiatric features, movement disorder, seizures and dysautonomia ¹ .	Similar phenotypes to adults. 40% of all patients are children ¹ .	Cross-linking and internalization of the receptors and reduction in NMDAR surface expression ² .
AQP4	NMO NMOSD ³	NMO NMOSD ⁴	Astrocyte damage by complement-dependent cytotoxicity, causing leukocyte infiltration, cytokine release and BBB disruption, which leads to oligodendrocyte death, myelin loss and neuron death ⁵
MOG	NMOSD ⁶	ADS NMOSD Monophasic/recurrent ON ADEM-ON ⁶	Induce/contribute to demyelination <i>in vitro</i> cultures and animal models ⁶
VGKC-complex	LE, neuromyotonia, Morvan syndrome ⁷	Autoimmune encephalitis, seizure disorders, demyelination (less frequently than adults) ⁸	Unknown
LGI1	LE ⁹	Not reported	Inhibit the ligand-receptor interaction between LGI1 and ADAM22/23 and reversibly reduced synaptic AMPA receptor clusters ¹⁰
CASPR2	Peripheral nerve hyperexcitability ¹¹	Not reported	Unknown
Contactin-2	In association with VGKC, LGI1 and CASPR2 ⁹ .	Not reported	Unknown
D2R	Not reported	Basal ganglia encephalitis, SC, TS ¹²	Unknown
GlyR	PERM, Stiff-person syndrome ¹³	PERM ¹⁴ , SPS ¹⁵ , autoimmune encephalitis ⁸	Cross linking and internalization of the receptor ¹³
GABA _B R	Encephalitis with prominent seizures ¹⁶ .	2 patients reported; 16yr monophasic LE and 3yr with refractory seizures (died) ¹⁶	Unknown
GABA _A R	Encephalopathy with refractory seizures and status epilepticus ¹⁷ .	3/6 patients reported are <18yr ¹⁷	Selective reduction of synaptic GABA _A receptors clusters at synapses, but not along dendrites, without altering NMDAR and the anchoring protein gephyrin ¹⁷
DPPX	Cognitive dysfunction, psychiatric features, resting tremor and myoclonus(Boronat et al., 2013) PERM ¹⁹	Paediatric onset (age 15year) in one reported case ¹⁸	Possible molecular mimicry between a gut pathogen and DPPX ¹⁸
mGluR1	Cerebellar ataxia +/- HL ²⁰	Not reported	Loss of Purkinje neurons (unknown mechanism) ²⁰
mGluR5	Limbic encephalitis and HL (Ophelia syndrome) ²¹	15yr male with Ophelia syndrome ²¹	Paraneoplastic syndrome ²¹
AMPA	LE ²²	Not reported	Cross linking and internalization of the receptor ²²
GAD	SPS ²³	Very rare in children but have been reported in patients with LE and refractory seizures ²⁴	Unknown.

Table 1-2 Cell-surface autoantibodies and the associated phenotypes

Abbreviations: **ADEM-ON** Acute Disseminated Encephalomyelitis followed by optic neuritis **ADS** acquired demyelinating syndromes **HL** Hodgkin lymphoma **LE** limbic encephalitis **NMO** neuromyelitis optica **NMOSD** NMO spectrum disorder **PERM** progressive encephalomyelitis with rigidity and myoclonus **SC** Sydenham's chorea **TS** Tourette's syndrome **SPS** stiff person's syndrome.

¹Titulaer et al. 2013. ²Moscato et al. 2014. ³Wingerchuk et al. 2006. ⁴Tillema and McKeon. 2012. ⁵Papadopoulos and Verkman, 2013. ⁶Reindl et al. 2013. ⁷Irani et al. 2010. ⁸Hacohen et al. 2013. ⁹Lai et al.2010. ¹⁰Ohkawa T et al. 2013. ¹¹Lancaster et al. 2011. ¹²Dale et al. 2012.

¹³Carvajal-Gonzalez et al. 2014. ¹⁴Damasio et al. 2013. ¹⁵Clardy et al. 2013. ¹⁶ Lancaster et al.2010. ¹⁷Petit-Pedrol et al. 2014. ¹⁸Boronat et al. 2013. ¹⁹Balint et al. 2014. ²⁰Marignier et al. 2010. ²¹Lancaster et al. 2011. ²²Lai et al. 2009. ²³Vincent et al.1997 ²⁴Haberlandt et al. 2011.

1.5.1 N-methyl-D-aspartate receptor (NMDAR)

NMDARs are glutamate-gated ion channels and are crucial for neuronal signalling. They form tetrameric complexes that consist of several homologous subunits (reviewed in Paoletti et al., 2013). Seven NMDAR subunits have been identified: GluN1, GluN2A-D and GluN3A and B, with 2 GluN1 subunits in each receptor. NMDARs are diverse in their subunit composition, their biophysical and pharmacological properties, their interacting partners and their subcellular localization. Subunit composition varies across CNS regions during normal development and in many diseases of the CNS. The different regulatory binding sites (glycine, polyamine, Mg^{2+} and Zn^{2+}) determine either a negative or positive control over receptor function and each individual receptor subtype is responsible for specific functions in the CNS in both normal and pathological conditions (Paoletti et al., 2013). During early postnatal development the subunit composition switches from GluN2B to GluN2A, and GluN2D and GluN3A subunits also drop markedly, suggesting that these subunits are particularly important during synaptogenesis and synaptic maturation (Pachernegg et al., 2012, Sheng et al., 1994).

Pharmacological blockade or genetic reduction of NMDARs alters learning and memory. Defects in glutamate signalling have been linked to neuropsychiatric disorders and NMDAR hypofunction has been proposed to be part of the pathophysiological mechanisms underlying schizophrenia (Paoletti et al., 2013). Interestingly, mutations in the NMDA receptor subunits GluN2A, equivalent to NMDAR NR2a, were recently reported in children with epilepsy–aphasia spectrum disorders ranging from benign epilepsy with centrotemporal spikes (the most common childhood epilepsy, also known as Rolandic epilepsy), through acquired epileptic aphasia (Landau–Kleffner syndrome [LKS]), to continuous spike and wave during slow-wave sleep syndrome (CSWSS) (Lemke et al., 2013, Lesca et al., 2013, Carvill et al., 2013). Although genetically predetermined, these conditions do not manifest at birth and

present later, possibly related to the age dependent physiological switch from GluN2B to GluN2A (Paoletti et al., 2013).

NMDAR antibodies were first described in 2007 in young females presenting with severe encephalopathy, psychiatric features, movement disorder and autonomic dysfunction associated with an ovarian teratoma (Dalmau et al., 2007). NMDAR-Abs are the commonest CNS antibody mediated disease with incidence exceeding that of any single viral aetiology in young patients (Gable et al., 2012). The frequency of tumours is age and sex dependent and 96% are ovarian teratomas (Titulaer et al., 2013). The characteristic clinical syndrome of a polysymptomatic encephalopathy affects both adults and children with around 40% of the patients presenting under the age of 18 (Titulaer et al., 2013, Florance et al., 2009). Infants and children are reported to present with more neurological (e.g. seizures, movement disorder) than psychiatric symptoms, possibly because psychiatric symptoms may go unrecognised in the young (Armangue et al., 2013). Despite the severity of the encephalopathy, with 69% of the patients being admitted to intensive care (Titulaer et al., 2013), the neuroimaging is normal in the majority. Similarly, the cerebrospinal fluid (CSF) analysis reveals only moderate lymphocytic pleocytosis, normal protein concentration. Intrathecal oligoclonal bands (OCB) are present in up to 60% of patients (Dalmau et al., 2011), but not necessarily at onset of the disease (Irani et al., 2010b).

The EEG is encephalopathic in the majority, with generalised rhythmic delta activity with or without epileptic discharges (Dalmau et al., 2011). Extreme “delta brush,” a continuous EEG pattern that may be unique to patients with NMDAR-Ab encephalitis has been reported in some of the patients (Schmitt et al., 2012) and, when found, could be a clue to the diagnosis. Early electro-clinical features may also correlate with severity; persistence of normal physiological background activity in at least one hemisphere is suggestive of moderate severity

and better outcomes, whereas diffuse abnormalities, with lack of physiological background activity, indicates severe neurological impairment and poor prognosis (Gitiaux et al., 2013).

Acute first line immunotherapy consists of steroids, intravenous immunoglobulins (IVIG) and plasma exchange (PLEX), given alone or in combinations, with second line treatment with rituximab and/or cyclophosphamide if improvement is not clear or sustained. Azathioprine or mycophenolate mofetil (MMF) are generally given if patients remain symptomatic or require maintenance immunotherapy over a prolonged course.

There have been no controlled trials to provide high quality evidence on the optimal therapeutic strategy for NMDAR-Ab encephalitis. A large cohort analysis suggested the additional benefits of second line therapy (rituximab, cyclophosphamide), when first line therapy fails. However, spontaneous improvement following only 1st line therapy was also observed (65% with normal outcome) (Titulaer et al., 2013). Importantly, when evaluating efficacy of different treatment regimes, a major confounder in the interpretation of the results is the phenomenon whereby the severity of the conditions influences the physician choice of therapy. This can result in a paradox in which the more severely affected patients receive more treatments but may, nevertheless, have the worst outcomes.

The recovery process can be slow, up to 18 months or more, and appears to be in reverse order of symptom presentation with cognitive (verbal fluency and executive function) and behavioural problems last to improve (Dalmau et al., 2011, Irani et al., 2010b). Relapses occur in 12% of patients typically with a less severe presentation to the initial one (Titulaer et al., 2013). Complete recovery is seen in 75% of the patients (Titulaer et al., 2013).

The similarities between the clinical presentation of NMDAR-Ab encephalitis and NMDAR agonists such as phencyclidine and ketamine (which are psychotomimetic and cause stereotypic movements and autonomic instability, and seizures) suggest that the antibodies cause loss of function (Figure 1.1) with a decrease of NMDAR in inhibitory GABAergic neurons

and glutamatergic synapses. This is supported by *in vitro* studies showing that patient antibodies cause a selective, reversible decrease of NMDAR surface density, synaptic localisation, and currents (Moscato et al., 2014). In an animal model of anti-NMDAR encephalitis, continued intraventricular infusion of patient's CSF for 14 days, resulted in progressive decrease in memory and depressed behaviour and anhedonia, reaching a maximal effect on day 18 and normalising on day 25. These effects correlated with reversible decrease of hippocampal NMDAR (Planaguma et al., 2015).

1.5.2 VGKC-complex and the associated proteins

The VGKC shaker family are delayed rectifiers, which open to allow K⁺ efflux in response to membrane depolarisation. During action potentials they play a crucial role in returning the depolarized cell to a resting state. Kv1 channels are composed of α and β -subunits. There are 12 known Kv1 α subunits (Kv1.1-1.12) and three β subunits (Kv β 1-3). Each α -subunit contains six transmembrane domains (S1-6) and has intracellular N- and C-termini with the S4 region acting as a charge sensor and the four β -subunits (S5-6) forming the pore-containing domain. The channel has four small extracellular loops. The channel subunits Kv1.1, 1.2 and 1.6 bind the epileptogenic snake-venom derived toxin α -dendrotoxin (DTX), which when radioactively labelled forms the basis for the radioimmunoprecipitation assay used to detect the antibodies. Kv1.1, 1.2, 1.4 and 1.6 channels show >90% identity between humans and other mammals. These subunits are believed to represent the neuronal predominant composition and are critical for regulating neuronal excitability (Coleman et al., 1999).

VGKCs are highly expressed in the cerebellum and hippocampus and are concentrated in the juxtaparanodal region in myelinated nerves. Kv1 proteins can be detected in both the dendrites and the axons but, when exogenously expressed in neurons, detection of surface-expressed Kv1 channels by electrophysiology has only been identified in the axonal

membrane (Jensen et al., 2011). In axons, Kv1 channels are clustered at a variety of functionally important sites including axon initial segments, juxtaparanodes of myelinated axons, and cerebellar basket cell terminals (Rasband, 2010). Coupling of the Kv1 channels with cell adhesion molecules and scaffolding proteins has a direct effect on the channel clustering and surface expression, and plays an important role in recruiting K⁺ channels to distinct axonal domains (Rasband, 2010).

Mutations in the KCNA1 gene encoding Kv1.1 have been identified in episodic ataxia type 1 (EA1) (Browne et al., 1994) characterized clinically by brief episodes of ataxia and continuous and spontaneous motor unit activity (myokymia and neuromyotonia). Of note, individuals with EA1 have increased incidence of epilepsy (Zuberi et al., 1999). Variation of the phenotypes does occur and patients may have the peripheral syndrome only, presenting with neuromyotonia (Rajakulendran et al., 2007).

Antibodies that immunoprecipitate 125I- α -dendrotoxin-labelled voltage-gated potassium channels (VGKC) extracted from mammalian brain tissue have been detected in patients with neuromyotonia, Morvan's syndrome, limbic encephalitis and a few cases of adult-onset epilepsy (Vincent et al., 2011). These conditions often improve following immunomodulatory therapies. Earlier studies suggested that the patients' antibodies bind to the VGKC subunits KV1.1, KV1.2 and KV1.6 (Hart et al., 1997) but it is now recognised that antibodies identified in the radioimmunoassay mainly target neuronal proteins that co-immunoprecipitate with the detergent solubilised VGKCs, particularly the secreted synaptic protein leucine-rich glioma inactivated 1 (LGI1) and the transmembrane axonal protein contactin-associated protein 2 (CASPR2). Two studies looking at adult patients with VGKC-complex antibodies identified LGI1 and/or CASPR2 in only 26% and 16% suggesting that the majority of the sera bind to novel autoreactive target(s) within the VGKC-complex (Klein et al., 2013) which could include intracellular epitopes on the VGKC subunits or other components of

the complex. Antibodies to LGI1 and CASPR2 appear even less common in children (Wong-Kisiel et al., 2012).

1.5.3 Leucine-rich glioma inactivated 1 (LGI1)

The secreted protein LGI1 binds both ADAM22 and ADAM23 (a disintegrin and metalloproteinase domain-containing protein, 22 and 23). Mutations in LGI1 result in autosomal-dominant partial epilepsy with auditory features (Kalachikov et al., 2002), also known as autosomal dominant lateral temporal lobe epilepsy (Gu et al., 2002). Patients with this epileptic syndrome suffer from partial seizures and auditory or visual hallucinations. LGI1 is also clearly part of the VGKC-complex as antibodies to LGI1 precipitate the VGKC-complex (Irani et al 2010a). LGI1 is thought to interact both pre-synaptically with VGKC and post-synaptically with AMPARs (Ohkawa et al., 2013; Figure 1.1).

LGI1 antibodies are associated with immunotherapy responsive, non-paraneoplastic limbic encephalitis (Irani et al., 2010a, Lai et al., 2010) and faciobrachial dystonic seizures (FBDS) (Irani et al., 2011). Recognising that the VGKC-complex antibodies bind LGI1 in these patients has increased our understanding of these disorders. Typically, patients present with acute to sub-acute onset memory loss, confusion, medio-temporal lobe seizures and psychiatric features evolving over days or weeks (Vincent et al., 2011). Median age at presentation is around 60 year and men predominate (Irani et al., 2010a, Lai et al., 2010). A low plasma sodium should alert the clinician to the possibility of LGI1-Ab associated limbic encephalitis but the hyponatraemia is uncommon in patients during FBDS if they are tested before they progress to limbic encephalitis (Irani et al., 2011).

Purified IgG from a patient with limbic encephalitis and LGI1 antibodies caused an epileptogenic effect on neuronal excitability in hippocampal slices in a similar way to α -dendrotoxin suggesting the antibodies act as Kv1.1, 1.2 and/or 1.6 antagonists (Lalic et al.,

2011). A more recent study looking at postsynaptic AMPAR distribution and function in neuronal cultures showed that the antibodies inhibited the interaction between LGI1 and ADAM22 and reversibly reduced synaptic AMPA receptor clusters (Ohkawa et al., 2013) (Figure 1.1). The effects on VGKC distribution or function were not examined.

1.5.4 Contactin-associated protein 2 (CASPR2)

The CASPR2 protein belongs to the neurexin-family that is known to be involved in the juxtaparanodal clustering of potassium channels in myelinated neurons (Poliak et al., 2003). It has a large extracellular domain, and an intracellular domain that interacts with axonal cytoskeleton proteins at the juxtaparanodes in myelinated axons (Salzer et al., 2008). Homozygous mutations in CNTNAP2, the gene coding CASPR2, were reported in Old Order Amish children with cortical dysplasia, focal epilepsy, relative macrocephaly, and absent tendon reflexes (Strauss et al., 2006). More recently gene polymorphisms were linked to heritability patterns of autistic spectrum disorders (Alarcon et al., 2008, Bakkaloglu et al., 2008).

Antibodies to CASPR2 were first identified in patients with positive VGKC-complex antibodies but also in isolation. VGKC-complex/CASPR2-Ab patients have been reported with autoimmune encephalitis, peripheral nerve hyperexcitability and Morvan syndrome, occasionally co-occurring with LGI1 antibodies (Irani et al., 2012). CASPR2-Ab were also found, mainly with negative VGKC-complex antibodies, in 10% of patients with idiopathic cerebellar ataxia (Becker et al., 2012), Guillain-Barre syndrome (GBS) (Tuzun et al., 2013) and childhood epilepsies (Suleiman et al., 2013). Most patients respond to immunotherapy but patients with peripheral nerve hyperexcitability are frequently adequately treated with antiepileptic therapies. An antibody-mediated immune response against CASPR2 could impair

its ability to cluster juxtaparanodal membrane components but it remains unclear what determines the distribution of disease between the CNS and PNS phenotypes.

1.5.5 Contactin-2

Contactin-2, a cell-adhesion molecule of the immunoglobulin superfamily, exists both as a glycosphosphatidylinositol-linked cell surface isoform and as a released form (Poliak et al., 2003), and is expressed on the glial membrane at the juxtaparanodes of myelinated axons where it forms heterodimers with CASPR2 (Derfuss et al., 2010). Antibodies to contactin-2 failed to induce demyelination in experimental animals with EAE induced by transfer of contactin-2-specific T-cells (Derfuss et al., 2010) and it is likely that the intact myelin sheath normally prevents antibodies from gaining access to contactin-2 in the juxtaparanodal region. Contactin-2 mutations (CNTN2 gene) were recently reported in a consanguineous Egyptian family with familial adult myoclonic epilepsy (Stogmann et al., 2013). Sequencing of the CNT2 gene in 189 patients with various epilepsy syndromes did not identify any pathogenic mutations.

Antibodies to contactin-2 were first identified in five patients with VGKC-complex antibodies; two were also CASPR2-antibody positive. Of the other three, one had LGI1-Abs and one had Kv1.2-Abs (Irani et al., 2010a). These antibodies were also identified in 8% of patients with relapsing-remitting multiple sclerosis but were not associated with a specific clinical-radiological phenotype (Boronat et al., 2012).

1.5.6 Dopamine receptor 2 (D2R)

Dopamine (or 3,4-dihydroxyphenethylamine) is an important neurotransmitter in the brain with several potential functions and distinct distributions. It is recognised to be involved in a number of human diseases such as Parkinson's disease, Tourette's syndrome and schizophrenia

(Beaulieu and Gainetdinov 2011). Dopamine signalling is mediated through dopamine receptors, which are G-protein-coupled seven-transmembrane domain receptors. Most dopaminergic receptors are located both presynaptically and postsynaptically. The presynaptic dopamine receptors play an important role in modulating and monitoring the release and synthesis of dopamine. Five receptors have been identified in humans and are divided into two groups: D1- (DRD1 and DRD5) and D2-class receptors (DRD2, DRD3 and DRD4) (Kebabian and Calne, 1979). D1- and D2-class receptors are widely distributed in the brain and have high expression in basal ganglia, cortex and hippocampus (Beaulieu and Gainetdinov, 2011). Two isoforms have been described for the D2R receptor; D2RS and D2RL (termed “short” and “long”). D2RL is 444 amino acids long and has an additional 29 amino acids in the third intracellular loop (vs. 415 amino acid in D2RS). D2RL isoforms are relatively more abundant in the extrapyramidal basal ganglia whereas more D2RS are found in the cortex, hippocampus and brain stem.

Modulation of D2R expression in the basal ganglia has been reported in patients with schizophrenia, depression and movement disorders (Nikolaus et al., 2009b, Nikolaus et al., 2009a, Beaulieu and Gainetdinov, 2011). Mutations in the D2R gene have been reported in myoclonus dystonia (Klein et al., 1999) and D2R antagonists are known to cause dystonia-parkinsonism. More recently an animal model of exposure to streptococcal antigens has shown behavioural changes, elevation of dopamine in the basal ganglia and elevated D1R and D2R antibodies in the serum detected using an enzyme linked immuno-adsorbent assay (ELISA) (Brimberg et al., 2012).

Antibodies to the D2R were recently described in children with basal ganglia encephalitis (Dale et al., 2012) and also in children with Sydenham chorea (SC), Tourette's syndrome (TS)(Dale et al., 2012) and paediatric autoimmune neuropsychiatric disorders associated with streptococcal infection (PANDAS) (Brimberg et al., 2012). These groups of

post-infectious conditions (frequently associated with streptococcal infections) that target the basal ganglia range from SC where evidence of autoimmunity is derived from both clinical studies and identification of specific autoantibodies (Brilot et al., 2011), to diseases where the autoimmune link is less clear such as PANDAS and TS (Dale and Brilot, 2012). In a cohort of children with basal ganglia encephalitis (who were negative for NMDAR-Ab) 12 out of 17 had elevated D2R-Abs compared to none of the 67 controls (Dale et al., 2012), these patients respond to immunotherapy with temporal relation between the clinical state and antibody titres (Ramanathan et al., 2013). Although the antibodies bind to the extracellular domain of D2R, antibody levels were low, and their pathogenicity has not yet been demonstrated.

1.5.7 Glycine receptors (GlyR)

The GlyR family represents pentameric ligand-gated ion channels responsible for opening a chloride-selective central pore lined with five transmembrane segments. This results in hyperpolarisation of the neuronal membrane (Avila et al., 2013). Activation of glycine receptors causes inhibition in the spinal cord and the brainstem. Four different isoforms of the α -subunit have been described. These isoforms are expressed in different area of the CNS and in different stages during development. Mutations in the $\alpha 1$ subunit of the GlyR gene *GLRA1* have been identified in hereditary hyperekplexia (Shiang et al., 1993). The response to immunotherapy, together with the phenotypic similarities to the genetic syndrome, suggests a pathogenic effect of the antibodies.

Antibodies to GlyR are most commonly associated with progressive encephalomyelitis with rigidity and myoclonus (PERM), which comprises part of the stiff-person syndrome (SPS) spectrum. Patients with this condition present with rigidity, excessive startle response, brainstem involvement with oculomotor dysfunction and potentially life threatening respiratory arrests. The clinical spectrum of 45 patients associated with these antibodies is expanding and

now include both seizure disorders and demyelination (Carvajal-Gonzalez et al., 2014) although the majority of patients have PERM. The antibodies bind to the extracellular domain of the $\alpha 1$ subunit and cause receptor internalisation (Carvajal-Gonzalez et al., 2014). Previous studies of patients with SPS, found some with GlyR-Abs as well as GAD65 (McKeon et al., 2013), but these were uncommon in the larger cohort defined by the antibodies.

GlyR-Ab are rare in children but have been reported in a 14 month old girl who developed startle-induced episodes of generalised rigidity and myoclonus and axial hyperextension, without impairment of consciousness. She made an excellent response to immunotherapy (Damasio et al., 2013). In a cohort of eight children with SPS, three had GlyR-Ab (of which 2 were only detected in the CSF) and improved with immunotherapy (Clardy et al., 2013), but a number of children have now been identified with GlyR-Abs and either PERM or encephalopathies (Carvajal-Gonzalez et al. 2014).

1.5.8 AMPA receptors

AMPA receptors are ionotropic glutamate receptors, which are widely distributed in the brain particularly in the hippocampus, cerebral cortex, basal ganglia, and cerebellum. The relative abundance of the different receptor subunits (GluR 1-4) and the physiological properties of the receptors vary and elicit different responses depending on the subunit composition expressed in a particular type of neurons (Wang et al., 2012).

Antibodies to AMPARs have been reported in adult patients with otherwise typical limbic encephalitis often associated with relapses (Lai et al., 2009). This is seen more commonly in female patients with different tumours who respond well to tumour treatment and immunotherapies. To date, no paediatric cases of AMPAR antibodies associated with limbic encephalitis have been reported. The antibodies target the extracellular domain of GluR1 and GluR2 subunits causing a reversible decrease in synaptic AMPARs and associated synaptic

currents (Lai et al., 2009). Earlier reports of antibodies to the AMPAR (GluR3) subunits in paediatric patients with Rasmussen's encephalitis (Rogers et al., 1994) have not been replicated in further studies.

1.5.9 GABA_b receptors

The GABA_b are G-protein coupled receptors, rather than ion channels, are expressed both presynaptically and postsynaptically, and are particularly prominent in the hippocampus, cerebral cortex, the thalamus, the superior colliculus, the cerebellum and the dorsal horn of the spinal cord. The receptor is a heterodimer consisting of two subunits; a $\beta 1$ subunit (agonist binding site) and a $\beta 2$ subunit (intracellular signalling). Genetic or pharmacological perturbation of the receptor function results in seizures and cognitive impairment (Bettler et al., 2004).

Patients with antibodies to the GABA_b receptor most frequently present with limbic encephalitis (Lancaster et al., 2010) Although initially thought to be a paraneoplastic syndrome with strong association with small cell lung cancer, it appear that around half of the patients have a non-paraneoplastic syndrome and show complete or partial improvement following immunotherapy (Hoftberger et al., 2013). The antibodies bound to the extracellular domain of the $\beta 1$ subunit, and did not appear to cause receptor internalisation (Lancaster and Dalmau, 2012) and may act by disruption of receptor signalling and hence receptor loss of function.

These antibodies are typically seen in adult patients with a median age of 60 but they were also reported in a 16 year-old boy presenting with limbic encephalitis, who made a complete recovery following treatment with steroids, IVIG and PLEX (Hoftberger et al., 2013), and more recently in a 3 year-old boy with a mixed movement disorder (opsoclonus, ataxia, and chorea) and refractory seizures and was treated with intravenous corticosteroids and immunoglobulins but died of overwhelming sepsis (Kruer et al., 2014).

1.5.10 Dipeptidyl-peptidase-like protein-6 (DPPX)

DPPX is a cell-surface auxiliary subunit of the Kv4.2 potassium channel. Kv4.2 is the major α -subunit underlying the somatodendritic A-type (I SA) current in neurons (Colinas et al., 2008), which plays a role in the regulation of both neuronal and dendritic membrane excitability. A Kv4.2 truncation mutation has been reported in one patient with temporal lobe epilepsy (Singh et al., 2006). The channel function is dependent on 2 subunits; the intracellular Kv channel interacting protein and the extracellular DPPX, which is predominantly expressed in the hippocampus and the cerebellum (Kim et al., 2008)

Antibodies to DPPX have been reported in four adult patients with encephalopathy, cognitive dysfunction, psychiatric features, resting tremor and myoclonus who responded to intensive immunotherapy. Patients were identified from a cohort of 1492 seronegative autoimmune encephalopathies suffering predominantly from seizures. Of the 11 who additionally had prodromal or concurrent diarrhoeal illness, three were positive (in both serum and CSF) for DPPX using DPPX-expressing HEK293 cells (Boronat et al., 2013). All four patients' sera/CSFs had similar immunostaining patterns on rodent hippocampus and cerebellum, and bound the cell surface of hippocampal neurons. DPPX was also expressed in the myenteric plexus, raising the possibility that in some patients the immune response may derive from molecular mimicry between a gut pathogen and DPPX. These antibodies have also been reported in three patients with PERM presenting with a distinct syndrome of subacute onset hyperekplexia, abnormal eye movements and variable trunk stiffness. Additional symptoms comprised allodynia, neurogenic pruritus, and gastrointestinal symptoms. All patients had pleocytosis in the CSF and positive OCBs. The patient responded to immunotherapy but an aggressive treatment was required for symptom control (Balint et al., 2014). Of note, one of the three patients presented aged 15 years.

1.5.11 GABA_A receptors

The GABA_A receptor is a member of the cys loop ligand-gated ion-channel superfamily, located on the majority of neurons in the central and peripheral nervous system, where they mediate important actions of the inhibitory neurotransmitter GABA. The GABA_ARs are pentamers, with five subunits of different isoforms (α 1–6, β 1–3, γ 1–3, δ , ϵ , π 1–3). These 19 subunits combine in different ways to form functional receptors which determine the affinity for GABA_AR conductance, kinetics, and modulation (Tretter and Moss, 2008). The receptors are regulated by both positive (barbiturates, benzodiazepines) and negative (picrotoxin, bicuculline) allosteric modulators, providing several models of GABA_AR-antagonist induced-seizures (Vazquez-Lopez et al., 2006, Kapur and Macdonald, 1997, Gonzalez, 2013) and mutations in the α 1 and α 3 subunits are associated with generalised epilepsies (Zhou et al., 2013, Tanaka et al., 2008).

These antibodies were recently identified in patients with encephalitis with predominant seizures who showed specific immunostaining patterns on rat brain sections, similar to those seen in patients with antibodies to GABA_B receptor (Petit-Pedrol et al., 2014).

Immunoprecipitation of two of the index cases identified GABA_A receptor sequences. Screening a cohort of 140 patients with unknown neuronal antibodies, presenting with encephalopathy and seizures identified a total of six patients (3 children, 50%). Patients presented with refractory seizures and status epilepticus, had extensive cortical and subcortical MRI abnormalities and interestingly some patients had other markers of autoimmunity with positive thyroid peroxidase antibody (TPO) and GAD65 antibodies. Although immunotherapy was beneficial, the long-term outcome was poor and one out of six reported cases died (Petit-Pedrol et al., 2014). Low titre antibodies, detected in the serum only, were also reported, frequently in patients with other autoimmune diseases presenting with a broader spectrum of symptoms and the clinical relevance of this is not yet clear (Petit-Pedrol et al., 2014). The high titre antibodies caused a selective reduction of synaptic GABA_A receptor clusters at synapses,

but not along the dendrites, without altering NMDA receptors or the anchoring protein gephyrin (Petit-Pedrol et al., 2014). Similar GABA_A antibodies have been identified in Oxford but the clinical syndromes are not well defined and immunotherapy responses undetermined in the majority (Pettingill et al., 2015)

1.5.12 Metabotropic glutamate receptors (mGluR)

Antibodies to the metabotropic glutamate receptor were reported in patients with Hodgkin's lymphoma (HL). mGluR5 antibody is strongly associated with limbic encephalitis (which when seen in association with HL is termed Ophelia syndrome) (Lancaster et al., 2011b, Paoletti et al., 2013) whereas mGluR1 antibody is seen in patients with cerebellar ataxia (Marignier et al., 2010). These patients are often young and paediatric presentations have been reported (Lancaster et al. 2011b). Patients respond to tumour resection and immunotherapy. While sera of patients with mGluR1 antibodies show robust staining of the cerebellum, and this autoimmunity associates with cerebellar ataxia, the sera of patients with mGluR5 antibodies show intense immunolabeling of the hippocampus and other brain regions (with the lowest reactivity in brainstem and cerebellum), consistent with the predominant limbic dysfunction that characterizes the Ophelia syndrome (Lancaster et al., 2011b).

1.6 *Antibodies in acquired demyelination syndromes (ADS)*

There is a growing interest in the role of B-cells and central nervous system (CNS) antibodies in demyelination (Krumbholz et al., 2012). Histopathological studies of both animals and humans have identified immunoglobulin and complement deposition in actively demyelinating lesions and immunoglobulins derived from a subset of multiple sclerosis (MS) patients appear to induce demyelination and axonal injury (Elliott et al., 2012). Additionally patients with MS

show clinical responses to B-cell depleting treatment such as rituximab (Krumbholz et al., 2012).

1.6.1 Aquaporin 4

The aquaporins (AQPs) are plasma membrane water-transporting proteins. AQP4 is the principal member of this protein family in the CNS, where it is expressed in astrocytes and involved in water movement, cell migration and neuroexcitation (Papadopoulos and Verkman, 2013). Antibodies against the astrocyte water channel protein, aquaporin-4 (AQP4) were first described in 2004 in patients with the rare inflammatory demyelinating disease, neuromyelitis optica (NMO)(Lennon et al., 2004). Initially thought to be a variant of MS that preferentially affects the optic nerves and spinal cord, the discovery of these antibodies, which are generally not found in patients with MS (Lennon et al., 2005), has directly influenced the diagnosis and management of these patients. The clinical features, MRI abnormalities and AQP4-Ab seropositivity reported in children with NMO are similar to the adult phenotype (Banwell et al., 2008, Huppke et al., 2010, McKeon et al., 2008). Children with NMO may have a less clinically severe disease, and may take longer to reach disability than adults with NMO (Collongues et al., 2010), but many children are left with visual or motor deficits after a single NMO attack. Studies *in vitro* and *in vivo* have demonstrated the pathogenic potential of these autoantibodies. Antibodies are predominantly of the immunoglobulin G1 (IgG1) subtype and bind three-dimensional conformational epitopes on the extracellular loops of AQP4, producing astrocyte damage by complement-dependent cytotoxicity this leads to blood–brain barrier disruption causing leukocyte infiltration, and cytokine release resulting in damage to oligodendrocyte, myelin and neurons (Papadopoulos and Verkman, 2013).

1.6.2 Myelin oligodendrocyte glycoprotein (MOG)

MOG is exclusively expressed in the central nervous system (CNS). Although only a minor component (0.05%) of the myelin sheath, its location on the outermost lamellae (Hemmer et al., 2002) and on the cell surface of oligodendrocytes, makes it available for antibody binding (Reindl et al., 2013). Antibodies to MOG have been known to induce or contribute to demyelination in animal models (Iglesias et al., 2001)

Earlier studies, which looked at antibodies to the linear epitopes of the denatured MOG protein using ELISA and western blotting, resulted in inconsistent results and positivity in healthy controls (O'Connor et al., 2007). Additionally, even when using the more appropriate cell-based assays, differences in positivity across cohorts were seen when the full-length protein construct is used, compared to the truncated form that lacks the intracellular domain. The reason for this difference is unknown, but recent studies suggest that the full-length protein can provide a specific and more sensitive assay for antibodies to MOG in patients with demyelinating disorders (Waters et al submitted).

Using the full-length MOG protein assay, antibodies are found in 30-50% of children at first presentation of ADS (Reindl et al., 2013) and in AQP4-negative NMO adult and children (Kitley et al., 2012). These antibodies have been reported in both monophasic and relapsing diseases (Reindl et al., 2013), with persistence of antibodies in relapsing demyelination including MS, and normalization in monophasic diseases (Probstel et al., 2011). Currently, it remains unclear if the presence of these antibodies requires a different therapeutic approach to that used in patients with AQP4 antibodies. Recently, from a cohort (n=65) of children with ADS from the UK and France MOG-Ab were found in 35% of patients across a range of demyelinating disorders. Interestingly despite the clinical features and patients' demographics being indistinguishable between antibody positive and negative patients at first presentation, the identification of MOG antibodies predicted a non-MS course at 1 year follow-up with 2/23

MOG-Ab patients were diagnosed with MS compared to 16/42 MOG-Ab negative patients ($p=0.02$, Fisher's exact). Importantly, as MOG antibodies were only tested retrospectively, identification of the antibodies had not influenced the final diagnosis (Hacohen et al. In press)

1.7 *General features of neuronal antibodies and their detection*

1.7.1 Antibody detection methods

The different antibody detection methods are now an essential laboratory investigation in evaluating patients with inflammatory CNS disorders, with diagnostic, prognostic, and therapeutic implications. Interpreting these assays requires the knowledge of the methodological strengths and weaknesses of each respective technique and is different for the different antigens identified (intracellular vs. extracellular). Methods, such as immunohistochemistry on brain tissue and immunoblotting of brain homogenates or of recombinant proteins, frequently pick up intracellular epitopes in addition to extracellular components of the molecule of interest. Although immunohistochemistry has the advantage of detecting novel antigens, tissue fixation can potentially alter antigen epitopes or reveal cryptic epitopes, and the results may be difficult to interpret when several antigens co-exist (Lancaster and Dalmau, 2012). Similarly, with immunoblotting, the detergents used may alter protein conformation and break disulphide bonds and will react preferentially with linear epitopes.

Immunoprecipitation assays (such as radiomunoassay and fluoroimmunoprecipitation assays) are performed by labelling the antigen with either radioactivity or fluorescent molecules, such as enhanced green fluorescent protein (EGFP), and incubating with the patient sample in solution. Following precipitation of antibody-antigen complexes with a secondary anti-human IgG, the radioactivity or fluoresce intensity in the pellet provide a measure of the antibody levels. As the antigens are in soluble form, these assays are not specific for detection of extracellular binding and could also detect cytosolic binding

(Waters and Vincent, 2008). In order to optimise specificity for antibodies to extracellular epitopes on neuronal surface antigens in neurological autoimmune diseases, cell-based assays were established (Dalmau et al 2008; Leite et al 2008; Waters et al 2008; Hutchinson et al 2008).

For detection of the NMDAR-Abs, sera and CSF were tested in three different assays; immunohistochemistry on brain tissue demonstrated the characteristic staining pattern to the molecular layer of the hippocampus (Dalmau et al., 2008), binding of the patients' antibodies to the surface of live primary neuronal cultures and finally a cell-based assays (CBAs) expressing the NMDAR NR1 and NR2b subunits. However, the latter test was performed with the cells fixed and permeabilised, which could potentially identify binding to intracellular epitopes, which will also be exposed on the fixed permeabilised brain tissue sections. By contrast, for AQP4 antibodies, live cell-based assays proved the most sensitive method (Waters et al., 2008)

However, use of three tests would be very time consuming for routine clinical practice when hundreds of samples are sent for different tests each week. Many laboratories use a commercial fixed cell assay (Euroimmun AG, Germany) which allow testing for a range of antigens simultaneously ("antibody panels"), but the Oxford laboratory uses live CBAs for all neuronal antigens because they are more likely to express the antigen of interest in their true conformational state (Vincent et al., 2011). For these CBAs, the binding of serum IgG to the surface of human embryonic kidney cells, transfected with cDNAs encoding the auto-antigens, is followed by fluorescence-labelled secondary antibody, and the fluorescence is scored visually. Fluorescence activated cell sorting (FACS) can also be used and is more quantitative and slightly more sensitive (Waters et al., 2012) but has not been shown to be helpful for all antigens (P Waters unpublished observation).

1.7.2 Are the diseases driven by serum or intrathecal antibodies?

Autoimmune induction is thought to occur outside of the CNS in the peripheral immune system and not surprisingly autoantibodies are detected in the serum, which is considered to be adequate for detecting antibodies to onconeural antigens, AQP4, VGKC-complexes, LGI1 and CASPR2 (Vincent et al., 2011). However, during the course of those CNS diseases that are antibody-mediated, the antibody undoubtedly has to be present in the brain. It has been suggested that the activated lymphocytes, including B-cells, plasmablasts and plasma cells, need to cross the blood brain barrier where they interact with their autoantigen, produce the antibodies and mediate this pathogenic effects.

Information regarding the amount of antibodies produced locally can be gleaned from measuring the intrathecal synthesis of antigen-specific antibody. This is obtained by calculating the amount of the specific antibody in the CSF relative to the total IgG, and comparing with a similar calculation in the serum. This ratio would be 1 in a healthy individual, irrespective of the antibody, and can be much higher in the CNS antibody-mediated diseases indicating intrathecal synthesis (Vincent et al., 2011). However, whether this is necessary at disease onset or whether the antibodies first gain access via diffusion across a healthy or temporally breached blood brain barrier is more contentious. As is whether CSF antibodies are more clinically relevant than serum antibodies.

1.8 Immunotherapies

Treatment of autoimmune neurological disorders requires removal of the systemic antibodies and immunosuppression. Currently there are no evidence-based guidelines for the treatment of children or adults with antibody-mediated encephalopathies. A rational approach to treatment has been developed, particularly for diseases in which there is a pathogenic disease biomarker such as in NMDAR-Ab encephalitis. Assessing treatment response with pre and post neurological assessments (in addition to neuropsychology or EEG) has been used in some

centres to support therapeutic efficacy (Wong-Kisiel et al., 2012). Treatment can be divided into acute and maintenance immunotherapies and symptomatic treatment.

First line immunotherapy normally consists of intravenous steroids, IVIG and PLEX (in isolation or combination). PLEX with five exchanges on alternative days is the best way of reducing antibody levels if urgently required, but not all centres will have the facilities. Steroids (intravenous methylprednisolone and/or high dose oral prednisolone) are thought to be very useful in reduction of inflammation, sealing of the blood brain barrier and overall reduction of peripheral and intrathecal antibody production. Although originally avoided in patients with suspected viral encephalitis, a randomised controlled trial using Dexamethasone in Herpes Simplex Virus Encephalitis (Tom Solomon, personal communication) hypothesise a beneficial effect. The use of steroids to treat seizures as a diagnostic tool for autoimmunity (in addition to EEG and neurocognitive testing) is controversial as the physiologic effects of steroids may extend beyond immunosuppression, such as the antiepileptic properties via GABA receptors (Murashima and Yoshii, 2010) The mechanisms of action by which IVIG acts are complex and thought to have both immunoregulatory and neuroinflammatory actions beyond their effect on antibody levels which are not well documented. IVIG has been successfully used in a range of immune-mediated conditions (Dalakas, 2014).

Immunosuppression with MMF, azathioprine, rituximab and cyclophosphamide can be added acutely to more severe patients who do not respond to first line therapy (steroids, IVIG) or as a “steroid-sparing” agent, to expedite remission and reduce the risk of relapse. The beneficial effect of long-term treatment, particularly without clear evidence, need to be weighed against the side effects and risk of long-term immunosuppression particularly in children. Clinical trials are required to answer some of these questions.

In conjunction to disease-specific immunotherapies, symptom management is essential in reducing secondary medical morbidities, and improving functional outcomes. A

significant proportion of patients require admission to the intensive care unit for management of encephalopathy and reduced consciousness, seizures, dysautonomia, and respiratory failure.

1.9 *Causes of encephalitis*

Encephalopathy in children and adolescence is associated with a high rate of morbidity and mortality and poses difficult diagnostic and therapeutic challenges (Davies et al., 2012, Glaser et al., 2003). The differential diagnoses are diverse and include infectious, para-infectious, metabolic, genetic, traumatic, malignant and toxic disorders (Davies et al., 2012, Granerod et al., 2010b). The clinical features of these disorders overlap and, in many cases, the cause will not be apparent from the history and examination at presentation. Rigorous clinical observations, alongside extensive investigations and empirical treatment of multiple, potentially life-threatening causes, are often effected simultaneously in patients presenting with encephalopathy (Davies et al., 2012).

Worldwide data report an annual incidence of acute encephalitis ranging from 3.5 to 7.4/100 000, rising to 16/100 000 in children (Thompson et al., 2012). In the UK, the Health Protection Agency identified an annual rate of 1.5 cases/100 000 in the general population and 2.8/100 000 in children (Granerod et al., 2010b). In a large UK study of 203 patients with encephalitis recruited over 2 years, of which 76 (37%) were under the age of 20 years, 86 patients (42%) had infectious causes, including 38 (19%) herpes simplex virus 1 (HSV-1), ten (5%) varicella zoster virus (VZV), and ten (5%) *Mycobacterium tuberculosis*. Similarly in the California encephalitis study, which recruited 334 patients over 2.5 years, a confirmed or probable viral agent of encephalitis was only identified in 31 cases (9%; 11 HSV-1, 5 enterovirus, 4 Epstein–Barr virus and 3 VZV), a bacterial agent was found in 9 cases (3%), and a parasitic agent was found in 2 cases (1%). A possible aetiology was identified in 41 cases (12%) and a non-infectious aetiology was identified in 32 cases (10%); despite extensive testing and evaluation 62% remained unexplained (Glaser et al., 2003). Recent advances in

diagnostic methods have led to the identification of novel pathogens responsible for infections of the central nervous system. Since the publication of these two large studies the list of emerging and reemerging pathogens identified as the cause of neurological syndromes has expanded. It is also possible that several recognised pathogens have expanded their geographic distribution into previously non-endemic regions, but advances in diagnostics is likely to be an important factor (Chow and Glaser, 2014). The strength of association between infectious agents and encephalitis is a topic of on-going debate; the majority of the published literature defines this association as confirmed, probable, or possible, on the basis of the type of specimen in which the potential etiologic agent is detected, the strength of the previously established associations between the agent and encephalitis, and the clinical and epidemiologic characteristics of the disease. Importantly, evidence of an acute infection without sufficient evidence to establish a causal relationship to the illness, as often occurred with respiratory pathogens, in the absence of a known neurotropic agent or confirmation by tissue analysis, makes the diagnosis only presumptive and cannot rule out an alternative diagnosis such as post or para-infectious autoimmune aetiology.

1.10 *Challenges of the autoimmune encephalopathies*

The discovery that several forms of encephalitis result from neuronal antibodies, which are immunotherapy responsive, has led to a paradigm shift in the diagnostic approach. However, despite the rapidly expanding subset of autoimmune encephalitis and the discovery of a number of novel infectious pathogens, up to 50 % of encephalitis cases typically remain without an identified aetiology (Venkatesan, 2013). The lack of consensus definitions and standardised diagnostic approaches is thought to be a limiting factor, particularly the clinical overlap between encephalitis and encephalopathy, terms often used interchangeably in the literature (Venkatesan et al., 2013).

Encephalopathy refers to a clinical state of altered mental status, manifesting as confusion, disorientation, behavioural changes, or other cognitive impairments, with or without inflammation of brain tissue. By contrast the definition of encephalitis does require inflammation of the brain parenchyma in addition to the neurologic dysfunction. As neuropathological examination of brain tissue is rarely performed an inflammatory response in the CSF or the presence of parenchymal abnormalities on neuroimaging is often used as surrogate markers. Nevertheless, normal neuroimaging and unreactive CSF are not uncommon in antibody-mediated encephalopathies (Vincent et al., 2011) and may also be a feature in infective encephalitis albeit at a lower frequency (Granerod et al., 2010a).

Recognising the expanding clinical phenotypes associated with the antibodies detailed above will aid early recognition and early institution of treatment. However, there are increasing numbers of patients who present with similar clinical phenotypes but in whom no known CNS autoantibody can be detected and, conversely, patients with a known CNS antibody in whom the clinical relevance of the antibody is questionable. These challenge the diagnosis and management of these children.

The aims of this thesis were to begin to define better the different clinical cohorts and explore the range of clinical features associated with specific antibodies.

Two different approaches were used:

- a) Studying a group of patients with a (i) strict clinical phenotypes with or without known CNS autoantibodies (autoimmune encephalopathy and basal ganglia encephalitis described in Chapter 3 and 6 respectively) and
- b) Studying the clinical features of patients with specific CNS autoantibodies (NMDAR and VGKC-complex antibodies described in Chapter 4 and 5).

CHAPTER 2 - Methods

2.1 Patients selection

The Clinical Neuroimmunology Service at the Oxford Radcliffe Hospital Trust receives all national requests for serum and/or cerebrospinal fluid (CSF) central nervous system (CNS) autoantibody testing and/or clinical advice. This allowed the identification of children who were referred to the clinical neuroimmunology service for antibody testing from London, Birmingham and Oxford. The first cohort to be identified was of children with a presumed diagnosis of autoimmune encephalopathy that presented between 2007-2010 (Chapter 3). Following the identification of this cohort, patients were recruited to three separate cohorts; two of which were based on antibody positivity only (NMDAR-Ab and VGKC-complex-Ab, Chapter 4 and 5 respectively) and one based on a clinical diagnosis of basal ganglia encephalitis (Chapter 6). All patients presented between 2007-2013. These children underwent extensive diagnostic studies as indicated by the clinical history to exclude infective, genetic and metabolic disorders. An additional Australian cohort of children with acute encephalitis was studied in collaboration with Prof Russell Dale, Children's Hospital of Westmed, Sydney University. Ethical approval for these studies was from the Oxfordshire Regional Ethical Committee A (07/Q1604/28).

2.1.1 Autoimmune encephalopathy cohort

The children, all under 18 years, were seen at the Evelina Children's Hospital, London (n=36), Great Ormond Street Children's Hospital, London (n=30), Birmingham Children's Hospital, Birmingham (n=28), St George's Hospital, London (n=10) and King's College Hospital, London (7) between 2007 and 2010. Serum samples from these 111 patients had been sent to the Oxford laboratory for testing for CNS autoantibodies. Clinical data, comprising demographic information, clinical features at presentation, discharge and follow up, and results of laboratory,

electrophysiological and neuroimaging testing, were compiled and presented anonymously, without revealing available antibody positivity, to a multi-centre review panel of four paediatric neurologists. Patients were considered to have a probable immune-mediated encephalopathy if they presented with encephalopathy (depressed or altered level of consciousness, lasting more than 24 hours, lethargy, or change in personality or behaviour) and at least one of the following features i) neuropsychiatric symptoms, ii) seizures, iii) movement disorder, or iv) cognitive dysfunction. Patients were excluded if an alternative diagnosis was made, but included even if the diagnosis of probable autoimmune encephalopathy was made retrospectively. Four patients, who were excluded from the study because they were not encephalopathic, were positive for autoantibodies in the serum; two had VGKC-complex antibodies (one Guillain–Barré syndrome and one myositis) and two had NMDAR-Ab (one with predominantly movement disorder and with one optic neuritis). In addition, we chose to exclude patients with demyelinating disease who were being studied separately (Hacohen et al., 2014b) and two with Rasmussen’s encephalitis, as well as those whose encephalopathy were given an alternative diagnosis (See Figure 3).

2.1.2 NMDAR-Ab positive cohort

The neuroimmunology service in Oxford is the national referral centre for NMDAR-Ab testing and between 2009-2013; sera and/or CSF were referred for NMDAR-Ab testing from 662 children from six paediatric neurology centres. Fifty children were positive for NMDAR-Ab; Evelina Children’s Hospital, London (n=17), Great Ormond Street Children’s Hospital, London (n=10), Birmingham Children’s Hospital, Birmingham (n=8), Oxford University Hospital (n=7), St George’s Hospital, London (n=5), and King’s College Hospital, London (n=2). Clinical data, comprising demographic information, clinical features at presentation, discharge and follow up, and results of laboratory, electrophysiological and neuroimaging testing, were reviewed.

2.1.3 VGKC-complex antibodies positive children

From 2008-2013, 363 serum samples were referred from the Evelina Children's Hospital (n=241) and Great Ormond Street Children's Hospital (n=122) to the Clinical Neuroimmunology service at the Oxford Radcliffe Hospital Trust for VGKC-complex antibodies. Patient case notes were retrospectively reviewed and patients were classified using the International Classification of Diseases (ICD-10) into inflammatory diseases of the central and peripheral nervous system (n= 158) and non-inflammatory aetiologies (n=205). Demyelinating phenotypes were classified based on IPMSSG criteria (Krupp et al., 2013) to monophasic ADS (ADEM, optic neuritis, transverse myelitis or clinically isolated syndrome – other) and relapsing phenotypes. Patients with autoimmune encephalopathies have fulfilled diagnostic criteria described in section 2.1.2. Two patients were diagnosed with presumed viral encephalitis although the relevant serum and CSF investigations were not available to confirm the specific diagnosis.

2.1.4 Basal ganglia encephalitis cohort

Basal ganglia encephalitis was defined as encephalopathy associated with predominant basal ganglia symptoms (movement disorders, psychiatric or sleep disorders), and/or neuroimaging findings of basal ganglia abnormalities (Dale and Brilot, 2012). In view of the phenotypic similarity between basal ganglia encephalitis and NMDAR-Ab encephalitis (Dale et al., 2009), identifying a group of patients in whom clinicians had suspected the diagnosis of NMDAR-Ab encephalitis, would appear to be a good surrogate strategy to identify patients with potential basal ganglia encephalitis. Case notes of the 662 patients referred to above (2.1.2) were retrospectively reviewed, the NMDAR-Ab positive patients were excluded, and 25 patients fulfilled the inclusion criteria proposed by Dale and Brilot (2012).

2.1.5 Australian acute encephalitis cohort

These patients were identified by Dr Sekhar Pillai and Prof Russell Dale who collected the clinical data, comprising demographic information, clinical features at presentation, discharge and follow up, and results of laboratory, electrophysiological and neuroimaging testing. All antibody testing was done in Oxford (YH). Patients with encephalitis seen between January 1998 - October 2010 at the Children's Hospital at Westmead, the largest children's hospital in New South Wales Australia, were included. Patients were identified retrospectively using International Classification of Diseases version 10 (ICD-10) coding of encephalitis or encephalomyelitis at discharge, plus screening of consultant correspondence from the Department of Neurology database, and review of cerebrospinal fluid (CSF) polymerase chain reaction (PCR) testing from the Department of Infectious Diseases and Microbiology. The following patients were excluded: neonates (<1 month), patients >16 years, known or suspected cases of immunodeficiency, and meningitis (bacterial and viral). Patients with infection-associated encephalopathy (influenza and rotavirus) were included. 164 patients fulfilled the diagnostic criteria for acute encephalitis (Granerod et al., 2010)

2.2 Defining immunotherapy responses

An immunotherapy response was defined as "definite" if the response could not be attributed to another factor or when patients were treatment-dependent and relapsed on discontinuation. A "probable" response was when a confounder such as anti-epileptic drugs (AED) or possible spontaneous improvement could not be excluded. The outcomes, as measured by the range of difficulties the patients were experiencing (cognitive, behaviour, seizure, and motor), were retrieved from the patient's medical records or, if unavailable, were obtained directly from the patient's primary paediatric neurologist. Full recovery was defined by the absence of medical,

educational and social concerns reported from parents, school and the patient's primary clinician at follow up (minimum follow up time of 1 year).

2.3 *Modified Rankin Scale (mRS) for children*

We applied the modified Rankin Scale (mRS) for children, as previously used (Bigi et al., 2011). Disability was scored retrospectively using the mRS with age-specific modification based on information from the patients' clinical records and neuropsychology when available. Details on modifications are shown below.

Score	Modified Rankin Scale (mRS) for children
0	No symptoms at all
1	No significant disabilities despite symptoms; behaviour appropriate to age and normal further development
2	Slight disability; unable to carry out all previous activities, but same independence as other age- and sex-matched children (no reduction of level at the gross motor function scale)
3	Moderate disability; requiring some help, but able to walk without assistance; in younger patients adequate motor development despite mild functional impairment (reduction of 1 level on the gross motor function scale)
4	Moderate severe disability; unable to walk without assistance; in younger patients reduction of at least 2 levels on the gross motor function scale
5	Severe disability; bedridden, requiring constant nursing care and attention
6	Dead

2.4 *Autoantibody detection method*

2.4.1 *Cell based assays (CBAs) by visual inspection*

This method was used for detection of cell surface antibodies to NMDAR, leucine-rich glioma inactivated 1 (LGI1), contactin-associated protein 2 (CASPR2), contactin-2, glycine receptor (GlyR), and the dopamine receptors 1 and 2 (D1R and D2R).

Human embryonic kidney cells (HEK293) were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin-amphotericin (PSA). The HEK 293 cells were plated at a concentration of

approximately 400,000 cells per well in 6 well plates containing poly-L- lysine (PLL) coated glass cover slips for transfection 24 hours later. Each well was transfected with a total of 3µg of cDNA of the antigen of interest using polyethylenimine and glucose. A plasmid encoding for green fluorescent protein (EGFP) was co-transfected to allow visualisation of cell uptake of the cDNA in the NMDAR-Ab CBA. After 16 hours the medium was replaced by fresh DMEM. In the NMDAR transfected cell plates, 500µM of ketamine was added to prevent cell death due to activation of receptors by glutamate in the medium.

Forty-eight hours post transfection cover slips containing live antigenic protein expressing HEK cells were incubated in 24 well plates with patient sera at 1:20 or 1:100 (for CASPR2) dilution with wash buffer (DMEM supplemented with 1% bovine serum albumin [BSA, sigma) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)] for 60 minutes. Cells were then washed and fixed in 3% formaldehyde for 10 minutes. Alexa Fluor 568-conjugated goat anti-human IgG secondary antibody (A-21090) was applied for 45 minutes at room temperature at 1:750. Cover slips were mounted using aqueous mounting medium (DakoCytomation, Cambridge, UK) containing a 1:1000 dilution of 4',6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain. Cells were visualised using a Leica DM2000 fluorescence microscope, by two independent observers. Binding was scored on a scale from 0-4 where 0 is “no-binding” and 4 is “very strong binding” (Irani et al., 2010b, Waters and Vincent, 2008, Leite et al., 2008a). Positive serum samples were further diluted to determine their end-point titers.

For detecting binding of patient's IgM, IgA or IgE antibodies to the NMDAR, subtype-specific goat anti-human secondary antibodies [Alexafluor 568 goat anti-human IgG (Molecular Probes, USA) or goat anti-human IgA, IgM or IgE (Vector, USA); 1:750] were used followed by a third layer of fluorescein-conjugated rabbit anti-goat IgG (Molecular Probes, USA; 1:750) for IgA, IgM and IgE visualization.

2.4.2 Cell-based assays by Fluorescence-activated cell sorting (FACS)

This was performed only for D2R antibodies. Thirty-six hours after HEK293 cells were transfected with a plasmid encoding both human D2R and the fluorescent protein dsRed, the cells were trypsinized, resuspended in Dulbecco's modified Eagle's medium, 1% fetal calf serum, and 1mM EDTA (FACS buffer) at 1.0×10^6 cells/mL, and rotated at 4°C for 2 hours. Patient serum (diluted 1:10 in FACS buffer) was mixed with 1.0×10^5 cells (100 μ L). After holding at 4°C for 15 minutes, the cells were washed, and bound IgG was detected with Alexa 488-labelled antihuman IgG (diluted 1:500 in FACS buffer). The cells were resuspended 30 minutes later in 400 μ L phosphate-buffered saline/5mM EDTA and analysed by FACSCalibur. The level of transfection was determined by measuring dsRed intensity (PE-Texas Red channel) in live cells. Two gates were created: the higher gate captured cells expressing high levels of dsRed; the lower gate captured untransfected cells and served as a negative control for each sample. Bound IgG was measured in the green channel (a shift to the right on the x-axis). A score for each serum was determined by subtracting the median green fluorescence in the lower gate from the median green fluorescence in the higher gate.

2.4.3 Radioimmunoprecipitation assay

This was performed as previously described (Vincent et al 2004) and is the current diagnostic assay for VGKC-complex antibodies. VGKC complexes were extracted from rabbit whole brain membranes solubilised using 2% digitonin (Calbiochem, USA) in DTXbuffer (100mM NaCl, 20mM Tris, 5mM KCl adjusted to pH 7.12) at 37°C for 20 minutes and then centrifuged at 15,000g for 15 minutes at room temperature (RT). The supernatant was harvested and diluted 1:2 with PTX (0.02M phosphate buffer and 0.1% Triton X100, pH 7.2) and incubated with ^{125} I- α DTX (specific activity 2000 Ci/mmol, Perkin Elmer, USA). This extract was further diluted

with DTX-buffer to give 1 million counts per minute (cpm) per ml (an approximate concentration of 250 fmoles of ^{125}I - α DTX /ml).

50 μl of ^{125}I -D α TX-labelled extract was incubated with 5 μl serum or 1 μl commercial antibodies (made up to a constant volume of 50 μl with PTX) overnight at 4°C. Secondary antibodies (anti-human IgG, RSR Ltd, UK or anti-goat IgG, Jackson ImmunoResearch Laboratories Inc., USA) were added. The addition of normal human or goat carrier serum (1-2 μl) was added for precipitation with commercial antibodies. After 90 minutes at RT, 0.5ml of PTX was added and the precipitates were spun at 15,000g for 5 minutes and resulting pellets washed twice in PTX and read on a gamma counter (Cobra 2, Perkin Elmer). When expressed as specific cpm, results have the non-specific binding, determined by precipitation in the presence of healthy control serum, subtracted.

2.5 Protein extraction and labelling

To look for antibodies binding to the VGKCs themselves the KV1s subunit were extracted and labelled. These extracts were then used in radioimmunoprecipitation assays as performed for the digitonin brain-extracted VGKC complexes.

HEK cells (seeded at around 10 million /flask) were grown in 175cm² flasks until 40% confluent, and transfected with a total of 60 μg of cDNA, 30 μl of PEI and 25 μl of 20% glucose. At 15 hours post-transfection, the medium was changed. At 48 hours post transfection, the medium was removed; flasks were washed with PBS and lysed in 3mls of 2% digitonin in DTX-buffer, with 1:100 protease inhibitor cocktail. Lysates were gently rotated for one hour at 4°C, spun (13,000rpm for 5 minutes) at 4°C and labelled with ^{125}I - α DTX, followed by immunoprecipitation with human sera (1-5 μl) or commercial antibody (1 μl) as described above.

2.6 *Immunofluorescence on brain sections*

Juvenile rats were sacrificed by terminal anaesthesia and the brains were snap-frozen. Regions of interest/axial sectional/coronal sections were mounted on Tissue-Tek OCT compound (Sakura, EU) and 12µm thickness sectioned. To identify binding of human sera on sections, each slide was prepared and blocked in 10% normal goat serum (NGS) for 45 minutes followed by incubation with patient sera (1:200-1:1600) or CSF (1:10), diluted in 5% NGS for 1hr. Sections were then washed and incubated with a goat-anti human Alexa Fluor488 (1:1000) for 1hr at room temperature. Binding was visualised on a fluorescence microscope. Sections were counterstained with DAPI.

2.7 *Primary neuronal and astrocytic cultures*

Hippocampal neurons were prepared from P0/P1 decapitated rat pups, and cerebellar neurons from P6 pups. Hippocampi or whole brain without the cerebellum (whole brain neurones/astrocytes) were dissected out and incubated in trypsin-HBSS for 20 minutes at 37°C prior to trituration in Minimal Essential Medium (MEM) with added FCS (10%) and PSA (1%). After gentle centrifugation at 1000rpm for five minutes and re-suspension in MEM, the cells were seeded out into six well plates with PLL coated cover slips at approximately 100,000 viable cells per cover slip. They were placed in a humidified 37°C, 5% CO₂ incubator. Between 2-4 hours later, the MEM was replaced with Neurobasal (NB) media supplemented with 1% B27, 200 mM L-glutamine and 1% PSA. Twice a week half of the media was replaced by fresh NB. Neuronal cells were assayed between days *in vitro* (DIV) 14-17.

For cerebellar neurons, the cerebellums were extracted and incubated with trypsin-DNase solution for 10 minutes at 37°C. DNase was added prior to titration. Centrifugation and seeding cells was as for hippocampal neurons except Basal Medium Eagle (BME) replaced

NB. 24 hours later, cytosine arabinoside (AraC) was added. On DIV 3, 1M glucose was added to cells with AraC. Media was refreshed with BME twice a week. Cerebellar neurons were assayed at DIV 6-7.

Cells were incubated for 1 hour with patient serum at a dilution of 1 in 100 in NB/HEPES/BSA 1%. Cells were then washed and fixed in formaldehyde 4%. After washing, cells were incubated with anti-human IgG-Alexa Fluor 488-conjugated secondary antibody (A-11013) at 1:1000 for 45 minutes. Cells were washed, then permeabilised with 0.25% PBST (0.25% Triton X-100 in PBS) for 15 minutes at room temperature. Mouse monoclonal anti-MAP2 antibody was added for 1 hour at room temperature to identify the neuronal cells. After washing, cells were incubated with anti-mouse IgG-Alexa Fluor 568-conjugated secondary antibody at dilution 1:1000. Cells were then washed and mounted as described for CBA.

2.8 *Molecular genetics for the dopamine receptor 2 (D2R)*

Using the Nucleotide resource of the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/nucleotide/>), the sequence for human Dopamine receptor 2 isoform 1 (long isoform) was located. D2R contain 434 amino acids (1302bp). pIRES2 DsRed-Express2 is a bicistronic expression vector that allows the simultaneous expression of the protein of interest and DsRed-Express2 from the same mRNA transcript. This vector is designed to allow for efficient flow cytometric detection of transiently transfected mammalian cells expressing either DsRed-Express2 and a protein of interest, or DsRed-Express2 alone (at lower signal intensity). This part of the work was supervised closely by Dr P Waters.

The forward and reverse primers are designed to anneal on opposite strands of the target sequence so they will be extended towards each other by addition of nucleotides to their 3'-ends. The target region should be 18-30nt. The restriction site XhoI and XmaI were used as they are not found on the sequence chosen for amplification, the pIRES2-DsRed2 Vector and

the PgmeTeasy vector. The forward primer was designed at the start of the D2R sequence. It included a kozak sequence and an XhoI (CTCGAG) restriction site. The melting temperature of the primer was calculated as 63.55. 4 nucleotides 'gatc' were included at the 5' end of the primer to protect the restriction site from loss of individual nucleotides in the primer. The reverse primer contains the 3' end of the D2R gene with a stop codon and an XmaI(CCCGGG) restriction site. The melting temperature of this primer is 62.55. The PCR DNA sequencing was then performed by the in-house DNA sequencing service.

Forward primer: GATC CTCGAG CCACC ATG GAT CCA CTG AAT CTG TCC AT:12 GC:12
Tm:63.55 XhoI

Reversed primer: GATC CCCGGG TCAG CAG TGG AGG ATC TTC AGG AT:10 GC:12
Tm:62.55 XmaI

Polymerase Chain Reaction (PCR) was used to amplify the DNA insert. All PCR reactions were performed using an Applied Biosciences® Veriti™ Thermal Cycler. PCR amplification of D2R was achieved with the Forward and Reverse primers and PfuUltra™ II polymerase (Agilent®) using the following reaction protocol:

- 1 Cycle: 95°C for 3 minutes (denaturing)
- 32 Cycles: 95°C 1 minute (denaturing),
65°C 1 minute (primer annealing),
72°C 30 seconds (extension with DNA polymerase)
- 1 Cycle: 72°C 5 minutes (final extension)

To find the optimal hybridisation temperature for the primers, the mix was divided up into 4 individual 500µl eppendorf tubes and the gradient option of the thermal cycler was used to test the hybridisation of oligonucleotide primers at a range of annealing temperatures (50-70°C).

A-tailing is a subcloning technique that avoids the use of restriction enzymes. The technique relies on the ability of adenine (A) and thymine (T) (complementary basepairs) on different DNA fragments to hybridize and, in the presence of ligase, become ligated together. To ligate the DNA into the pGEM-Teasy plasmid which contains poly(dT) as 3' ends, it is necessary to poly-adenylate the ends of the purified DNA. **For** poly-adenylation the mix was incubated at 72°C for 30 min: DNA template 6µl, Taq polymerase 1µl, 10x Taq polymerase buffer 1µl, dATP 1µl, MgCl₂ 1µl.

Ligation of the DNA into the pGEM-Teasy plasmid was performed according to pGEM®-TEasy manufacturer's instructions. Ligation mix (DNA 3µl, ligation buffer 5µl, pGEM-Teasy vector 1µl, DNA ligase 1µl, nuclease free water 1µl) incubated for 1hr at room temperature.

For transformation, JM109 bacterial cells were thawed on ice and 1µl of the DNA was added to 40µl of the cells. The mixture was put on ice for 15min before placing it into a preheated water bath (42°C) for exactly 1min. The heat shock forces the DNA into the bacterial cells. The cells were put on ice immediately for 2min. Then 250µl of SOC solution were added to the mixture and the cells were shaken for 1hr at 37°C. The bacteria were then plated out on antibiotics containing LB plates amplified with IPTG and Xgal and incubated overnight at 37°C.

The pGEM-Teasy vector is designed for blue-white screening which is a method used in identifying which colonies contain the vector. Blue colonies were inoculated into 5 ml LB containing antibiotic. The cultures were shaken overnight at 37°C. Subsequent purification of DNA was performed using Miniprep kit (according to QIAprep™ Miniprep kit QIAGEN® manufacturer's instructions).

The restriction enzymes XhoI and XmaI were used to excise the D2R from the pGEMT-easy vector. A mixture of 3µl DNA, 0.5µl XhoI, 0.5µl XmaI, 1µl Buffer 4, 1µl BSA, 4µl H₂O incubated for 1-2 hr., 37°C. pIRES2-DsRed2 was also cut in a similar manner.

For gel extractions, Gel was prepared by dissolving agarose (1%) in TBE (0.9 M Tris Base, 0.9 M Boric acid, 10 µM EDTA in dH₂O). The melted agarose/TBE was poured in Thermo Scientific® Easycast chambers™, 1.5 µl of ethidium bromide was added once the gel had cooled, and allowed to set in a fume hood. Samples were prepared by the addition of 10X loading buffer. Samples were then loaded alongside 5µl of HyperLadder™ 1 (Bioline®) (marker of DNA size). Gels run at 90-120V for 45-60 min. DNA bands were visualised and photographed under low energy UV light. The digested D2R insert and pIRES2-DsRed2 vector backbone were visualised on a UV transilluminator and excised from the gel using a scalpel blade. The DNA was purified using QIAquick™ gel extraction kit (QIAGEN®) (Catalogue number 28706 q) as per manufacturer's instructions.

To optimise the ratio of insert to backbone, and thus the chances for correct ligation, for each ligation at least four different ligations were set up according to the table below. Ligation mixture was incubated for 2hr at room temperature.

Substance (µl)	Ligation 1	Ligation 2	Ligation 3	Ligation 4	Ligation 5	Ligation 6
Backbone	1	1 of 1:10 dilution	1	1 of 1:10 dilution	1	1 of 1:10 dilution
Insert	3	3	3 of 1:10 dilution	3 of 1:10 dilution	-	-
Ligase	1	1	1	1	1	1
Ligase buffer	1	1	1	1	1	1
H ₂ O	4	4	4	4	7	7

JM109 cells were then transformed with the ligation mixtures prior to selective overnight culture and miniprep purification as previously described. The insertion of D2R into the pIRES2-DsRed vector was confirmed by a test digestion. Followed by transformation of D2R pIRES2-DsRed

vector as described in section 6 followed by DNA purification using Maxiprep kit (according to QIAprep™ Maxiprep kit QIAGEN).

2.9 *Statistical analysis*

Statistical analysis was performed using commercially available software GraphPad Prism 6 (GraphPad Software Inc.). Non-parametric statistical tests (Kruskal–Wallis tests) were used for continuous distributions, and χ^2 or Fisher's exact tests were used for nominal data.

CHAPTER 3 - Autoantibodies in paediatric infective and inflammatory encephalopathies

3.1 Introduction

Autoimmune forms of encephalopathies are now well recognised in children as well as adults, but there are few comparative studies of their clinical and investigative features or outcomes. The precise proportion of patients that have antibodies to the CNS targets remain to be determined in robust studies, as these tests have only been more widely available in the last few years and mostly restricted to children with well-defined clinical syndromes. N-Methyl-D-Aspartate receptor (NMDAR) antibodies were the first to be reported in children (Florance et al., 2009, Dale et al., 2009, Irani et al., 2010b), alongside antibodies to the voltage-gated potassium channel (VGKC)-complex proteins (Suleiman et al., 2011a, Suleiman et al., 2011b, Haberlandt et al., 2011, Iyer et al., 2012, Illingworth et al., 2011, Dhamija et al., 2011, Hacohen et al., 2012) or other CNS antigens such as glutamic acid decarboxylase (GAD)(Haberlandt et al., 2011). In a large US cohort (Florance et al., 2009, Titulaer et al., 2013) 40% of NMDAR antibody encephalitis presented in childhood or adolescence, and in a smaller cohort from the UK, 23% of patients were under the age of 18 (Irani et al., 2010b). The results from two cohorts are presented in this chapter; a cohort of children with a presumed diagnosis of autoimmune encephalopathy recruited from London and Birmingham over a three-year period, and a second Australian based cohort of all children with encephalitis presenting to one hospital over 15 years.

3.2 Paediatric autoimmune encephalopathies: UK cohort.

The clinical phenotypes associated with autoimmune encephalopathies are increasingly recognised but many patients with presumed autoimmune disease are negative for the available

antibody tests. Figure 3.1 describe a 16 year old with clinical and radiological evidence of limbic encephalitis, with no detectable antibodies (Figure 3.1). In order to assess the clinical and investigative features, immunotherapy responses and outcomes, in patients with specific antibodies, and to compare with those in whom no antibodies were detected, patients whose sera were referred from five paediatric neurology centres were identified and those in whom the final diagnosis was of a probable autoimmune encephalopathy were studied.

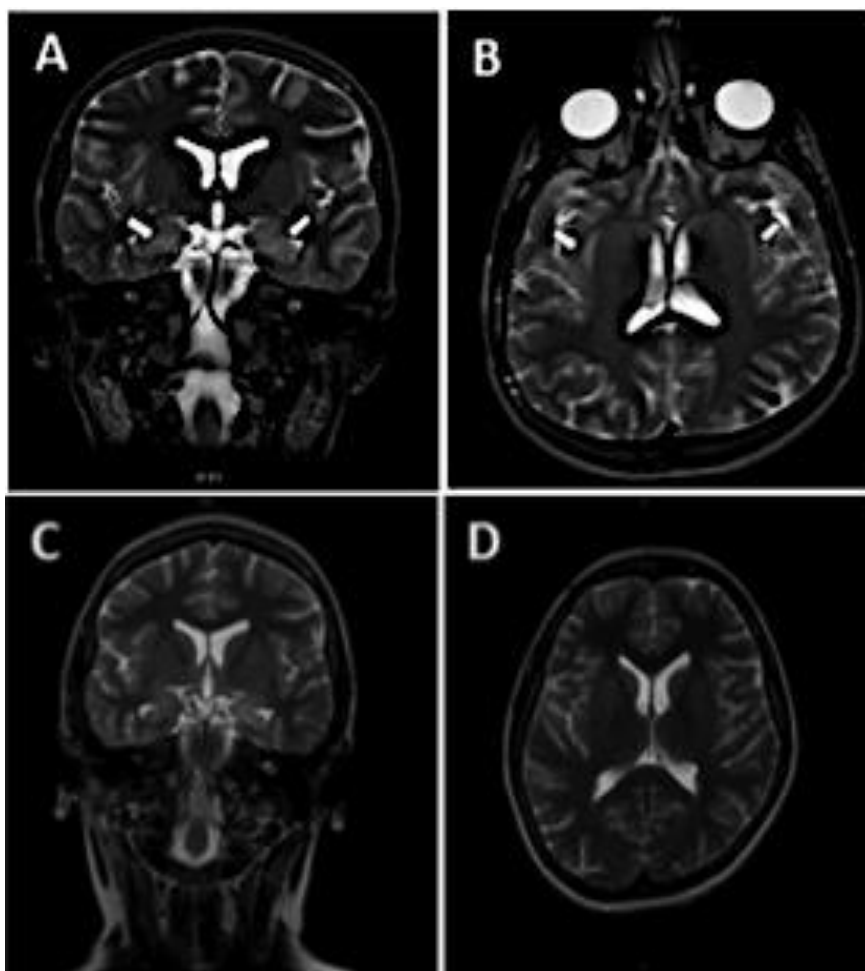


Figure 3.1 Brain MRI of a girl with seronegative limbic encephalitis.

A 15-year-old girl who presented with encephalopathy seizures and psychiatric features requiring recurrent IVIG infusion for symptom control. (A-D). Coronal T2 demonstrates signal change involving both hippocampi (arrows) and amygdala with swelling of the medial temporal structures (A). Axial T2 demonstrates additional high signal involving the external capsule bilaterally (arrows; B). Repeat imaging at 6 months shows subtle residual signal change in the mesial temporal lobes bilaterally with mild hippocampi volume loss (C) with resolution of changes in the external capsule (D)

3.2.1 Patient selection

Between 2007 and 2010, serum samples from 111 patients had been sent to the Oxford laboratory for testing for CNS autoantibodies. Patients' clinical data were anonymously presented to a panel of four paediatric neurologists, without revealing the results of the antibody tests, who identified patients with a diagnosis of autoimmune encephalopathy. Details for the patients' selection are discussed in Chapter 2.1.1 and illustrated in figure 3.2

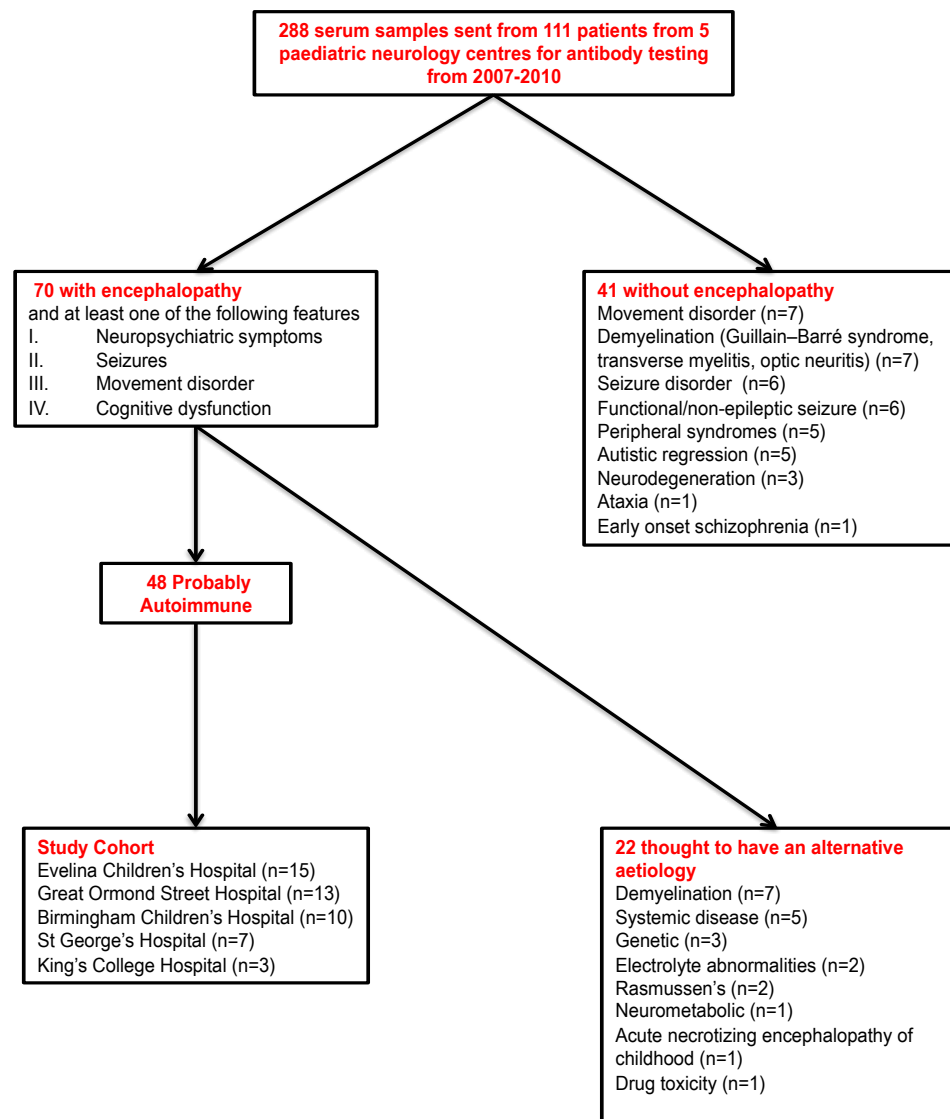


Figure 3.2 Study flow chart showing expert review and record linkage outcome

Study flow chart showing expert review and record linkage outcome. Four patients who were excluded from the study because they were not encephalopathic were positive for autoantibodies in the serum; two had VGKC-complex antibodies (one Guillain-Barré syndrome and one myositis) and two had NMDAR antibodies (one movement disorder, one optic neuritis). Patients with demyelinating conditions, Rasmussen encephalitis, or with neurological symptoms secondary to systemic diseases could have an autoimmune mechanism for their disorder but were excluded from this study. Modified from Hacoen et al. J Neurol Neurosurg Psychiatry. 2013.

3.2.2 Autoantibody testing

All samples were originally tested for VGKC-complex or NMDAR antibodies as requested by the referring clinicians. Retrieved sera were retested for all available antibodies (Chapter 2.4).

Briefly, antibodies to NMDAR, leucine-rich glioma inactivated 1 (LGI1), contactin-associated protein 2, contactin-2, glycine receptor (GlyR), and the dopamine receptors 1 and 2 (D1R and D2R) were tested using cell-based assays in routine clinical use. The semi quantities scoring scale is illustrated in Figure 3-3. AMPA receptor (AMPA) and GABA (B) R antibodies were tested using a commercial assay (Euroimmun AG, Luebeck, Germany). VGKC-complex antibodies were measured using immunoprecipitation of rabbit brain VGKC-complexes labelled with radioactive dendrotoxin (normal <100 pM, low positive 100-150 pM, positive 150-400 pM and high positive >400pM). GAD antibodies were measured by immunoprecipitation of ¹²⁵I-recombinant GAD.

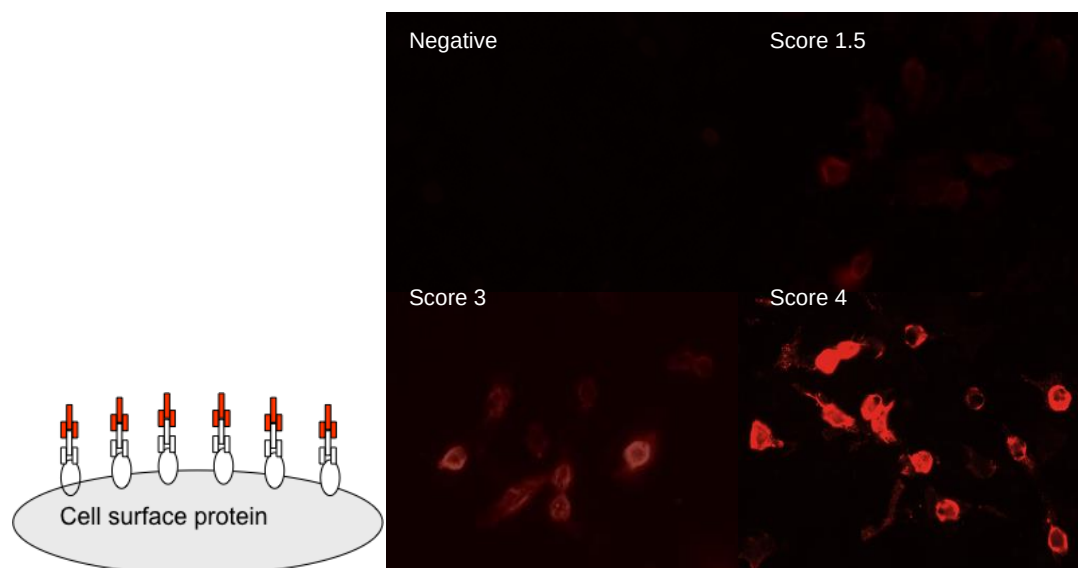


Figure 3.3 NMDAR-Ab cell-based assay.

Binding to NMDAR-expressing HEK cells was scored from 0 – 4 by visual observation (two independent observers). Binding is scored as 0 (control serum), 1.5, 3 and 4 (NMDAR-Ab positive patients). Red = anti-human IgG (magnification x 40).

3.2.3 Patients' demographics

Following the multicentre expert review panel, 48/111 patients were considered to have a diagnosis of autoimmune encephalopathy (Figure 3.2). Of the 48 patients, 26 (54%) were females (mean 8.7 years, range 1.83-17) and 22 were males (mean 9 years, range 0.16-16). The ages of onset and patients' demographics are shown in Figure 3.4. Non-Caucasians represented 24/48 (50%) of the cohort.

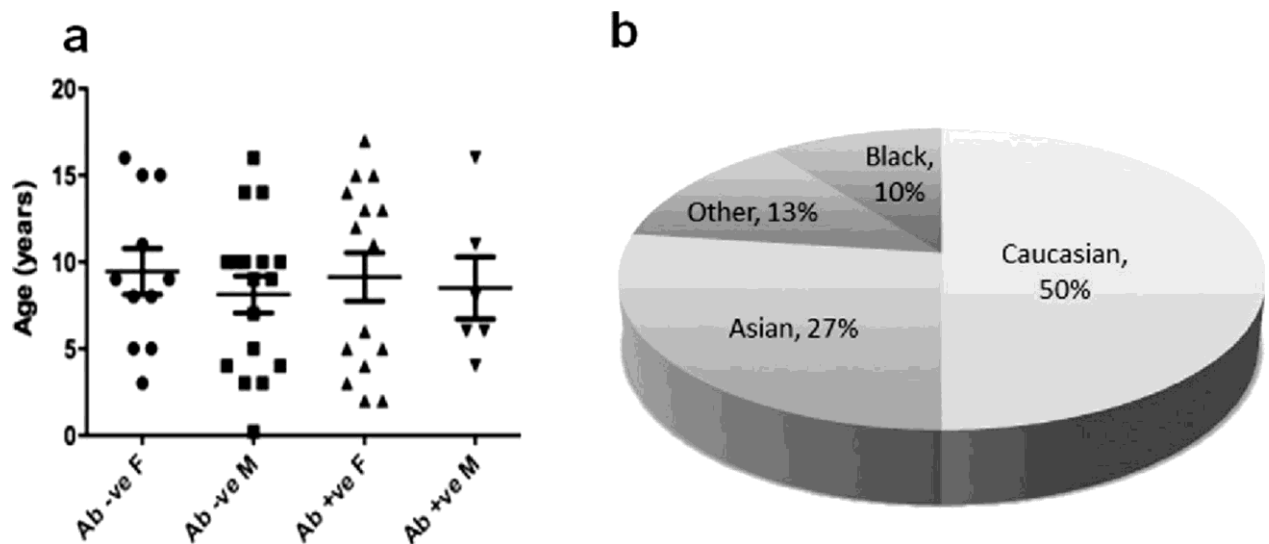


Figure 3.4 Demographics of patients with autoimmune encephalopathy.

(A) Age at disease onset of the 48-paediatric patients with autoimmune encephalopathy divided between antibody positive and antibody negative, male and female patients. (B) Ethnicity of the 48-paediatric patients with autoimmune encephalopathy; 24 patients (50%) were non-Caucasian: Asian (13), Black (5) and other (6). Modified from Hacoen et al. J Neurol Neurosurg Psychiatry. 2013.

3.2.4 Clinical features of the patients with autoimmune encephalopathies

Prodromal symptoms either with fever and/or associated infectious episodes were noted in 27(56%) patients. Three patients received a recent vaccination (Pandemrix for H1N1, HPV and Men C booster; one month, 4 weeks and one week prior to onset respectively). The most common presenting features included seizures (83%; generalised seizures 71%, focal seizures 56%), behavioural changes (63%) and confusion (50%). Twenty-seven patients had neuropsychiatric symptoms (56%; agitation 38%, hallucinations 25%) and 18 developed a movement disorder (38%; choreoathetosis 29%, myoclonus 2%, startle 4%, tremor 4%). 25 patients (52%) required intensive care admission for seizure control or reduction in conscious level.

Forty-five patients had NMDAR antibodies requested and 32 patients had VGKC-complex requested initially; positive results were confirmed on retesting at the time of the study. Overall, twenty-one patients (44%) were antibody positive. Thirteen patients (27%, mean age 8.9, range 1.83-17) were positive for NMDAR-Abs with cell-based assay scores ranging from 2.5-4 (controls<1.5). Sera from seven different patients (17%, mean age 9, range 4-15) immunoprecipitated VGKC-complexes with results ranging from 105-1360 pM. Two of the VGKC-complex antibody patients were also positive for GAD antibodies, one of them with Type 1 diabetes mellitus, but surprisingly none were positive for the VGKC-complex proteins LGI1, CASPR2 or contactin-2 antibodies. A 5-year-old girl who presented with explosive-onset epileptic encephalopathy (without rigidity and myoclonus) was found to have GlyR antibodies in her serum (score 2.5) and CSF (score 1) (See case 1 and Figure 3.5-6). None of the patients had antibodies to AMPAR, GABA(B)R, D1R or D2R. No apparent differences in clinical features were found between the antibody positive and negative groups (Table 3-1).

Case 1 - Autoimmune encephalopathy with GlyR antibodies

A previously well 5-year old girl presented with explosive onset generalized afebrile seizures with subsequent evolution to refractory focal epilepsy associated with cognitive and behavioural regression. Interictal EEG recordings at presentation and subsequent follow-up demonstrated diffuse background slowing with runs of high voltage slow waves and spikes (Figure 3-5). Serial MRI of her brain revealed mild global atrophy. An extensive infective, neurometabolic, genetic and inflammatory screen did not reveal an identifiable aetiology. Coeliac antibodies were positive, as was a subsequent jejunal biopsy but a gluten-free diet did not lead to an amelioration of her neurological symptoms. Following a recent re-evaluation of her case, she was reinvestigated for an underlying immune-mediated encephalitis. GlyR antibodies were found to be positive (Figure 3-6). Persistence of antibody positivity was confirmed in a new serum sample, and additionally measured and detected in CSF prior to commencing immunomodulation with IVIG. This was chosen in preference to corticosteroids, which had minimal efficacy with intolerable side effects in the early management of her epilepsy. She did not response to IVIG and was commenced on plasma exchange. On completion of 5 cycles, her antibodies were undetectable in her serum with apparent improvement in seizure control. At 3 weeks following her plasma exchange her seizures came back and her antibodies level in the serum returned to pre exchange levels. At this time, her GlyR antibodies were negative in the CSF and there were no other signs of CNS inflammation (unreactive CSF with no intrathecal oligoclonal bands and normal neopterin, and no contrast enhancement on neuroimaging). In view of the limited response and the potential side effects of escalating immune modulation, the parents have declined further treatment. She currently suffers from significant learning difficulties and is in special education.

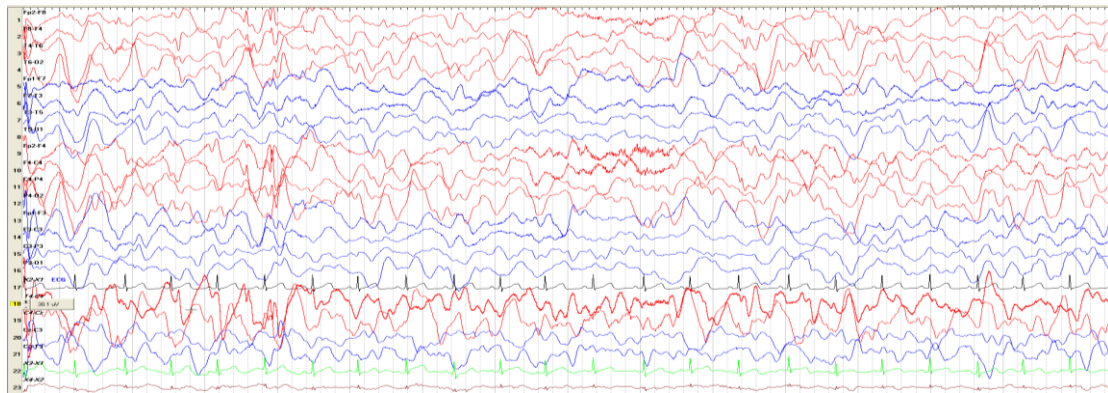


Figure 3-5 EEG finding in a patient with autoimmune encephalopathy and GlyR-Abs

EEG demonstrates generalised encephalopathy; asymmetrical background attenuated on the left; and generalised spike-and-wave discharges are more prevalent on the right hemisphere.

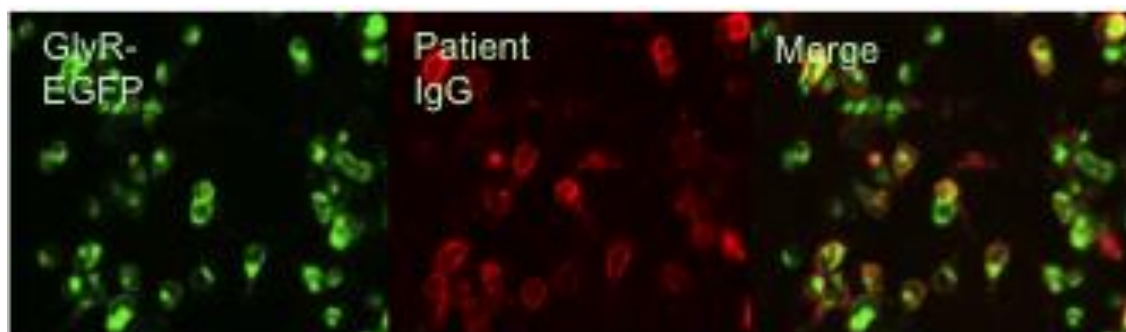


Figure 3-6 GlyR cell-based assay of a patient with autoimmune encephalopathy

Patient sera were tested for binding to HEK cells transfected with EGFP-tagged GlyR α 1 subunit. Control serum IgG did not bind the GlyR-EGFP expressing cell (not shown). Patient CSF was tested using the same method and demonstrated positivity (not shown).

3.2.5 CSF analysis, neuroimaging and electrophysiological studies.

CSF analysis revealed lymphocytosis in 8/47 (17%) patients and oligoclonal bands (OCB) in 9/13 (69%, Table 3.1). Interestingly, three patients had viral DNA detected by PCR in the CSF (2 HSV, 1 adenovirus); the two positive for HSV, who were additionally positive for NMDAR-Ab, became progressively worse despite adequate antiviral therapy.

Brain imaging was normal in 35/48 (73%) at presentation and the remaining patients had increased T2 changes in the cortex (n=6), hippocampus (n=3), white matter (n=3) or basal ganglia (n=1). These changes had normalised by three months but five other patients developed atrophy on a repeat scan. Reversible MRI changes, without atrophy, were common in the VGKC-complex antibody patients. By contrast, the majority of the NMDAR-Ab group had a normal MRI at the onset of the disease but 4/13 developed atrophy by six months. One 14-year-old girl, who was positive for NMDAR-Ab, had an ovarian teratoma identified by ultrasound giving an incidence in children of 8%.

Electroencephalography (EEG), at presentation, demonstrated slow background consistent with an encephalopathy in 33/47 (70%) patients. In 10 others, the EEG demonstrated epileptiform discharges early in the disease course; seven of these progressed to an encephalopathic pattern. In two patients there was slowing of alpha and increased beta activity probably due to benzodiazepine.

The clinical and paraclinical features of patients with NMDAR and VGKC-complex antibodies were consistent with the reported literature to date, but when the clinical features of patients with NMDAR antibodies and VGKC-complex antibodies were compared, there was a trend towards more frequent movement disorders and hallucinations with the NMDAR antibodies (Table 3-2).

	Antibody Positive (n = 21)	Antibody Negative (n = 27)
Cognitive dysfunction	18 (86%)	19 (70%)
Behavioural change	16	14
Confusion	11	13
Aphasia	7	7
Amnesia	1	2
Psychiatric disturbance	13 (62%)	14 (52%)
Hallucinations	9	3
Agitation	7	11
Mood disorders	2	1
Obsessive behaviour	0	1
Seizures	18 (86%)	22 (81%)
Generalised	14	20
Focal	15	12
Movement disorders	7 (33%)	11 (41%)
Choreoathetosis	7 (limb 7, face 4)	7 (limb 6, face 2)
Myoclonus	0	1
Startle	0	2
Tremor	0	2
Dysautonomia	2 (1%)	4 (7%)
Blood pressure instability	2	1
Tachy/bradycardia	2	3
Sleep disorders with disturbed sleep wake cycle	7 (33%)	11 (41%)
Fever at presentation	8 (38%)	9 (33%)
Associated infection	10 (47%)	17 (63%)
Mycoplasma	2	2
Anti-streptococcal antibody titre (ASOT)	3	3
Herpes simplex virus (HSV)	2 (PCR positive in CSF)	0
Epstein-Barr virus (EBV)	1	0
Human herpes virus 6 (HHV6)	0	1
Influenza	0	3 (2 H1N1, 1 Influenza A)
Adenovirus	0	1 (positive PCR in CSF)
Neoplasm	1 (5%)	0 (0%)
Screening for neoplasm	18 (86%)	9 (33%)
MRI abnormalities	11(52%)	7 (26%)
Normal at presentation	14	21
Reversible changes	7	6
Atrophy	4	1
EEG abnormalities	20 (95%)	23 (88%)
Generalised encephalopathy	12	12
Focal encephalopathy	2	3
Encephalopathy with epileptic discharges	2	2
Epileptic discharges only	3	2
CSF abnormalities		
Lymphocytosis (number of patients with wcc>5, range)	5 (WCC 8-73)	3 (WCC 16-57)
Intrathecal oligoclonal bands	6/10 (60%)	3/3 (100%)

Table 3-1 Comparison of the clinical and paraclinical features in children with antibody positive and antibody negative encephalopathy

No differences were observed in the clinical features, associated infections (co-existing or preceding), immunotherapy response or outcome in both groups. Screening for neoplasms was done less frequently in the antibody negative group and might reflect clinicians' views on the link between autoimmune encephalopathies and malignancies in patients without a known antigen.

	NMDAR this study (n = 13)	VGKC-complex this study (n =7)
Age at onset	8.9yr (Range 6-15)	9.6yr (Range 1.83-17)
Cognitive dysfunction	13 (100%)	4 (57%)
Psychiatric problems	10 (77%)	3 (43%)
Seizures	10 (77%)	7 (100%)
Movement disorders	7 (54%)	0 (0%)
Fever at presentation	4 (30%)	4 (57%)
Neoplasm	1 (8%)	0 (0%)
EEG abnormalities	12 (92%)	7 (100%)
MRI abnormalities (early)	2 (15%)	4 (57%)
MRI abnormalities (late)	4 (31%)	1 (14%)
CSF abnormalities	6 (46%)	2 (29%)
Lymphocytosis	3 (WCC 11-73)	2 (WCC 8-20)
CSF oligoclonal bands	6/9 (67%)	0/1(0%)
PCR positive	2/13 (15%)	0(0%)

Table 3-2 Comparison of clinical and investigatory findings of patients with VGKC-complex and NMDAR encephalitis.

Patients with NMDAR-Ab encephalitis were more likely to have cognitive dysfunction and movement disorders. Seizures were seen more commonly in VGKC-complex encephalitis, these patients were more likely to have abnormal cranial MRI and less likely to receive immunotherapies. Fisher exact's test were applied but differences were not significant.

3.2.6 Response to immunotherapy in patients with autoimmune encephalitis

An immunotherapy response was defined as “definite”, “probable” or “no response” (Chapter 2.2).

The outcomes, as measured by the range of difficulties the patients were experiencing (cognitive, behaviour, seizure, and motor), were retrieved from the patient's medical records or, if unavailable, were obtained directly from the patient's primary paediatric neurologist. Full recovery was defined by the absence of medical, educational and social concerns reported from parents, school and the patient's primary clinician at follow up (minimum follow up time of 1 year). The modified Rankin Scale (mRS) for children, as previously used by Bigi et al (Bigi et al., 2011) was applied to assess the degree of disability both during the acute admission and at follow up (Chapter 2.3).

Immunotherapy was given in 34/48 (73%) (See table 3.3). First-line immunotherapies with corticosteroids, intravenous immunoglobulins (IVIG) and plasma exchange (PLEX) were given in 29 patients and five required regular immunosuppressive treatments (azathioprine, cyclophosphamide, rituximab, mycophenolate mofetil). Two patients received immunotherapy

late in the disease (more than 6 months from the onset of symptoms) and did not respond to treatment.

Of the patients who did not receive immunotherapy, only 4/14 (29%) recovered completely compared to 18/34 (53%) who received immunotherapy, although this difference did not reach statistical significance ($p=0.2$, Fisher's exact). Both HSV/NMDAR antibody positive children showed a definite response to intensive immunotherapy with steroids, IVIG and PLEX. Overall 32/34 (94%) showed some response to immunotherapy; 23/34 (68%) "probably" responded (7 NMDAR, 3 VGKC-complex, 13 seronegative), with only 9/34 (28%) showing a "definite" response (5 NMDAR, 1 VGKC-complex, 3 seronegative) (see earlier definitions), but interestingly there was no apparent difference in treatment responses between the antibody positive and the antibody negative groups (Table 3-3).

3.2.7 Medium term outcome of patients

At the latest follow-up (12-60 months from onset, mean 24 months), 20/48 (42%) patients had made a complete recovery, 24 (50%) still had cognitive and behavioural problems, and 16 (33%) had on-going seizures with one child still severely disabled (Figure 3.7). The outcomes in the antibody positive group were slightly worse, with only 6/21 (29%) compared to 14/27 (52%) in the antibody negative group making a complete recovery. Nevertheless, the mRS scores demonstrated a significant change between nadir and final follow-up in the group as a whole, with trends in both the antibody positive and negative groups (Table 3-3 and Figure 3.8).

	Antibody Positive (n = 21)	Antibody Negative (n = 27)
Immunotherapy received	17 (80%)	17 (63%)
Corticosteroids only	6	11
IVIG only	0	2
Corticosteroids + IVIG	11	4
Additional PLEX	4	0
Disease modifying drugs	5	0
Immunotherapy response	16 (94%)	16 (94%)
Probable response	10	13
Definite response	6	3
mRS at nadir	4.5+0.60	4.5+0.58
mRS at follow up	1.8+0.75	1.6+0.84
On-going problems (Further details in Figure 3)	15 (71%)	13 (48%)
10/14 (71%) untreated	3/4(75%)	13 (48%)
18/34 (52%) treated	12/17 (70%)	6/17 (35%)

Table 3-3 Comparison of immunotherapy response and outcome in paediatric patients with antibody positive and antibody negative encephalopathy

No difference was seen in immunotherapy response and outcome (Fisher's exact).

Antibody positive patients were more likely to receive PLEX and second line immunotherapy.

IVIG intravenous immunoglobulins mRS modified Rankin scale score PLEX plasma exchange.

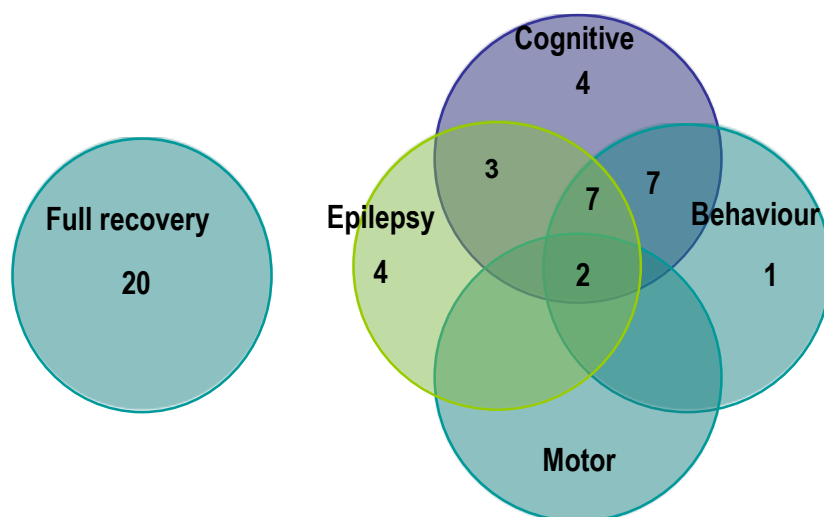


Figure 3.7 Outcome of 48 patients with autoimmune encephalopathy.

The problems encountered in children at follow-up of 1–5 years (mean=24 months). Twenty patients recovered completely. Cognitive problems were still present in 23, seizures in 16, behavioural problems in 17 and two patients had additional motor problems. Modified from Hachon et al. J Neurol Neurosurg Psychiatry. 2013.

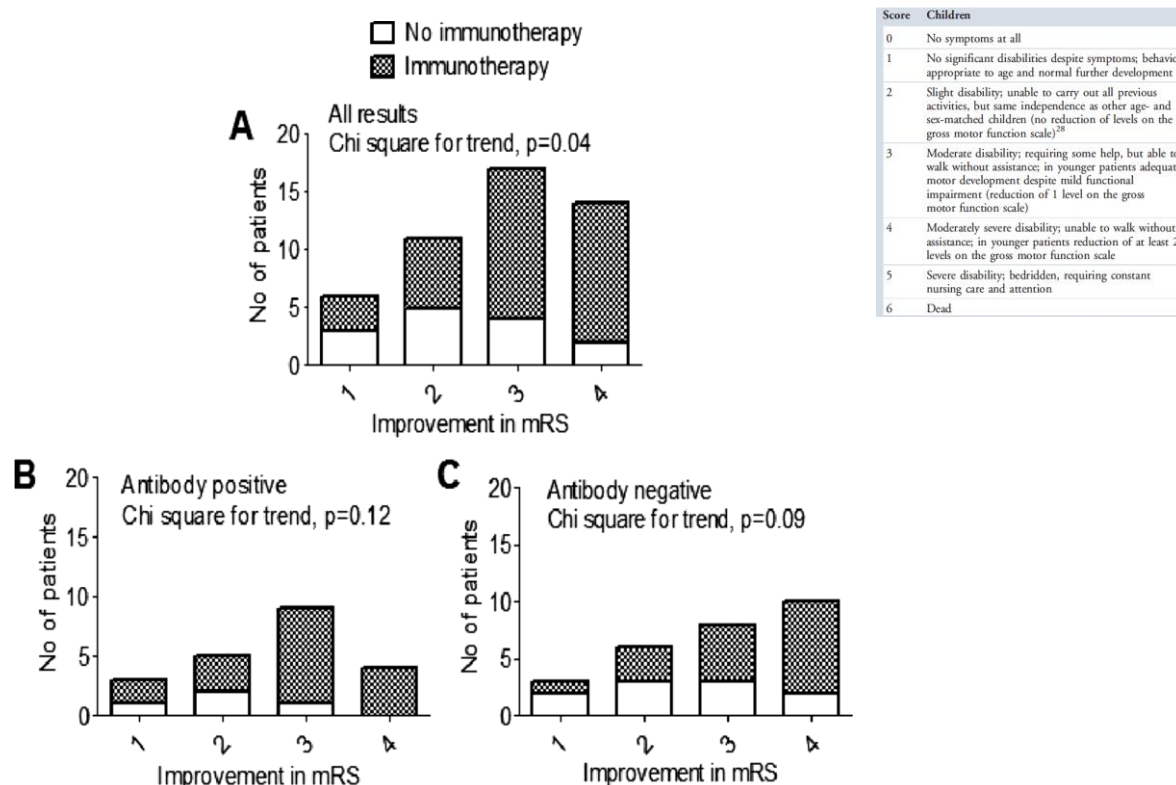


Figure 3.8 Modified Rankin scale (mRS) for children score reduction in total cohort and in the antibody positive and negative groups, stratified according to immunotherapies.

A significant improvement of the mRS score between nadir and final follow-up, as measured by the χ^2 test, was seen in the patients who received immunotherapies ($p=0.04$) (A), with a similar trend in the antibody positive (B) and antibody negative (C) groups.

Modified from Hacohen et al. J Neurol Neurosurg Psychiatry. 2013.

3.3 Neuronal antibodies as a cause of acute encephalitis – The Australian cohort

Infections have been considered to be the major cause of encephalitis and more than 100 different causative pathogens have been recognised. In a cohort of 203 patients with encephalitis from the UK (Granerod et al., 2010b), herpes simplex virus (HSV) is the most commonly implicated virus (19%) with varicella zoster virus (VZV; 5%), enteroviruses (1%), Epstein Barr virus (EBV; 0.5%), measles (0.5%) and human herpesvirus 6 (HHV 6; 0.5%) being the other most commonly identified (Granerod et al., 2010a). Autoimmune encephalitis can frequently mimic infectious encephalitis and in a multicentre UK encephalitis study (Granerod et al., 2010a), 16/75 (21%) of patients without a detected cause were found retrospectively to have a neuronal

autoantibody. This was higher than that of any individual virus identified. A similar observation regarding NMDAR antibodies was made in the Californian encephalitis project (Gable et al., 2012). To evaluate the contribution of neuronal antibodies to encephalitis in children, an Australian paediatric cohort presenting with a range of encephalitis was screened for the presence of these brain directed /neuronal surface antibodies (collaborative work with Dr Pillai in Prof Dale's laboratory)

3.3.1 Patient selection

A retrospective analysis of all cases of encephalitis (in immunocompetent children) presenting to The Children's Hospital at Westmead (Sydney, Australia), from January 1998 to October 2010 identified 164 patients with acute encephalitis (Chapter 2.1.5). The median age of presentation was 5.4 years. The male to female ratio was 1.4:1. Testing of up to 42 different microorganisms was carried out during this study period as clinically indicated. A CSF was obtained at median admission duration of 2.9 days (range; 1-48 days). Bacterial and viral cultures of the CSF were done on 155 (94.5%) and 152 (92.6%) patients respectively. CSF PCR testing was obtained in 131 (79.8%) of the total cohort, with ADEM patients (62.9%) less frequently tested. Nucleic acids were extracted using a MagnPure extraction kits (Roche Diagnostics) and the PCR product was analysed using the QIAxcel DNA Screening kit (Qiagen). Serological testing for one or more microorganisms was performed on 139 (84.8%) patients. Serological studies for microorganism used complement fixation testing (CFT) and IgM enzyme linked immunosorbent assay (ELISA). Testing of non-CNS specimens was performed as follows: blood culture (n=144, 87.8%), nasopharyngeal aspirate (n=67, 40.9%), stool/rectal swab (n=58, 35.4%), throat swab (n=41, 25%) and skin swab (n=5, 3%). Nasopharyngeal aspirate (NPA) testing was performed using immunofluorescent antigen detection for respiratory syncytial virus (RSV), influenza A, influenza B, parainfluenza 1-3 and adenovirus. Faeces were tested for rotavirus and adenovirus antigen

using immunochromatography. Serum samples taken acutely were available from 125 patients (patients with a preliminary diagnosis of ADEM were excluded from autoantibody testing, as the diagnosis is not dependent on autoantibody testing) and were tested blindly for antibodies to NMDAR, VGKC-complex and the associated proteins (LGI1, CASPR2 and Contactin-2), GlyR and GAD. D1R and D2R antibodies were measured in Sydney.

The final aetiological diagnosis was determined by stringent application of the consensus guidelines proposed by Granerod et al. Patients with an infectious aetiology were classified as confirmed, probable or possible. Confirmed cases had detection of the organism or autoantibody in CSF or brain. A probable diagnosis was based on serological evidence of acute infection or autoantibody, while a possible diagnosis was based on detection of the organism from a specimen sample outside the CNS such as stool or nasopharyngeal aspirate. When one or more aetiological agent was identified, the agent with the strongest hierarchical association (confirmed > probable > possible) was determined as the aetiology of encephalitis. As accepted in the literature, we used the term infection-associated encephalopathy rather than encephalitis to denote infections related to influenza virus and rotavirus. The hierarchical classification for each of the aetiological agents is shown in table 3-4.

Aetiology of encephalitis	Confirmed	Probable	Possible	Total (%)
Infectious (n=49)				
Enterovirus	17	-	3	20 (12)
<i>Mycoplasma pneumoniae</i>	-	11	-	11 (7)
Herpes simplex virus (HSV) ^	4	1	4	9 (5)
Cytomegalovirus	-	-	3	3 (2)
Other infections #	-	1	5	6 (4)
Infection-associated encephalopathy (n=13)				
Influenza virus	-	-	5	5 (3)
Rotavirus	-	-	5	5 (3)
Acute necrotizing encephalopathy * (<i>Mycoplasma pneumoniae</i> , parainfluenza 3, adenovirus, HSV-1)	3	-	-	3 (2)
ADEM	35	-	-	35 (20%)

Table 3-4 Hierarchical classification for the aetiology of encephalitis adapted from Granerod et al (Granerod, Cunningham et al. 2010)

^ HSV-1, n=4 CSF PCR confirmed, HSV-2, n=1 skin swab PCR possible, # Group A streptococcus (n=1, probable), adenovirus (n=1, possible), Epstein-Barr virus (n=1, possible), parainfluenza 3 virus (n=1, possible), parvovirus B19 (n=1, possible), varicella zoster virus (n=1, possible). * associated infections in 2 patients

3.3.2 Range of neuronal surface antibodies identified

Autoantibodies were identified in 21 children (17%) with NMDAR antibodies in nine and VGKC-complex antibodies in 13. None of the patients had antibodies to the VGKC-complex associated proteins; LGI1, CASPR2 and Contactin-2, and all patients were also negative for GlyR and GAD antibodies. Neuronal autoantibodies were found in patients with unknown aetiologies but also in patients with identified infective aetiologies (Figure 3-9). NMDAR-Ab and VGKC-complex Ab were found in 1 and 8 patients with infective aetiologies respectively and in 8 and 5 respectively with unknown aetiology ($p=0.03$, Fisher's exact). NMDAR-Ab were all scores 2-4, but the VGKC-complex Ab were low with only above 400pM in two (of which one was additionally mycoplasma positive)

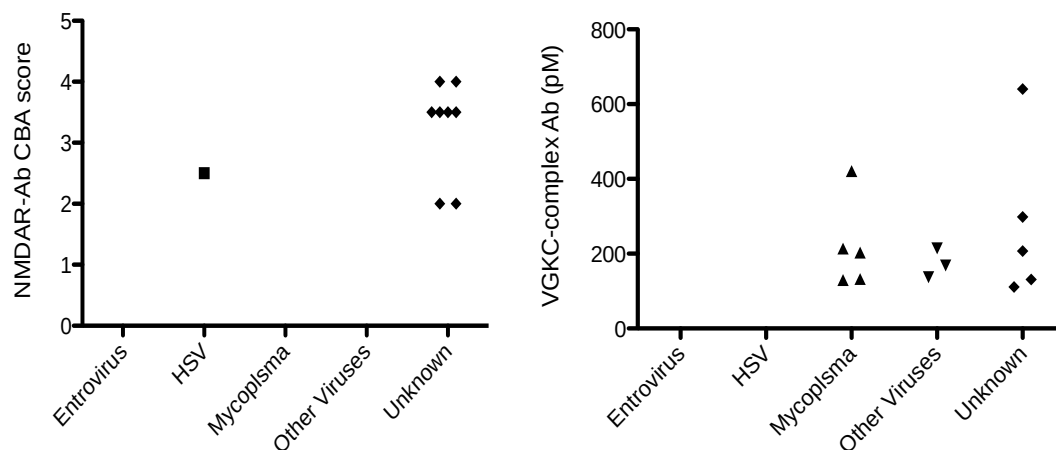


Figure 3.9 NMDAR-Ab and VGKC-complex Abs stratified to aetiology of encephalitis.

NMDAR-Ab were identified predominately in patients without an infective pathogens, whereas VGKC-complex Abs were identified both in the seronegative patients but also in association with positive infective pathogens particularly Mycoplasma. Patients were described as 'unknown' if they were negative for an infectious aetiology.

3.3.3 Clinical and paraclinical features

Clinical and paraclinical features of the most frequent aetiologies are shown in Table 3-4. Age at onset stratified to the different aetiology is shown in Figure 3.10. Overall, fever at presentation was seen in 38/61 (62%) patients with viral/ viral associated encephalitis compared to 2/9 (22%)

patients with NMDAR-Ab ($p=0.03$, Fisher's exact). Behavioural change and seizures were seen in both groups, but movement disorders and psychiatric features were seen more common in the NMDAR-Ab encephalitis group with 9/9 vs. 12/61 ($p<0.001$, Fisher's exact) and 8/9 vs. 4/61 ($p<0.001$, Fishers exact). Sleep abnormalities were seen in only 5/61 of patients with viral encephalitis compared to 7/9 patients with NMDAR-Ab encephalitis ($p<0.001$, Fisher's exact). Reactive CSF (WCC >5) was reported in 61% and 67% of viral and NMDAR-Ab patients at presentation respectively. MRI abnormalities were seen in 38/52 (73%) of viral patients compared to 5/9 (50%) patients with NMDAR-Ab.

Immunotherapy was given in 11/61 (18%) of patents in the viral group and 5/9 (56%) in the NMDAR-Ab group. Hospital stay was longer in the NMDAR-Ab group (Mean 100 SEM 21) compared to viral encephalitis (Mean 19 SEM 3.1) ($p<0.001$) (Figure 3.11). Nineteen children with viral encephalitis (31%) and 5/9 (56%) of patients with NMDAR-Ab encephalitis made a complete recovery.

VGKC-complex antibodies were identified in 13 patients (male 5, age range 1.7-15.1 years, median 7.3years); Eight had an infective encephalitis (mycoplasma identified in five; Influenza in two and one patient had multiple organisms identified), one patient was also positive for NMDAR-Ab, and only four had no known infective or autoimmune aetiology. Seizures were reported in 8 (61%), psychiatric features in 3 (23%) and movement disorder in one (8%) girl who was additionally positive for NMDAR-Ab. CSF OCB were identified in 1/3 (33%) patients tested and the MRI was abnormal in seven (54%), of which two presented with bilateral basal ganglia changes and were thought to have basal ganglia encephalitis. Only two VGKC-complex Ab positive patients received immunotherapy (steroids in both) but five patients made a full recovery.

	Enterovirus (n=16)	HSV (n=9)	<i>Mycoplasma pneumoniae</i> (n=13)	NMDAR (n=9)
Age (years) Range, Median	0.5-13.8, 3.4	0.1-4.2 0.7	1.3-13, 4.9	0.7-12.9, 7.3
Sex M:F	13:3	4:5	4:9	3:6
Prodrom	3/16 (19%)	1 (11%)	3 (23%)	1 (11%)
Fever	12 (75%)	5 (56%)	7 (54%)	2 (22%)
Confusion	4 (25%)	1(11%)	4 (31%)	2 (22%)
Behavioural change	15 (94%)	9 (100%)	13 (100%)	9 (100%)
Seizures	5 (31%)	8 (89%)	8 (62%)	6 (67%)
Psychiatric	2 (13%)	0 (0%)	2 (15%)	8 (89%)
Movement disorder	2(13%)	1 (11%)	4 (31%)	9 (100%)
Sleep	1 (6%)	0 (0%)	2(15%)	7 (78%)
Autonomic	4 (25%)	0 (0%)	4 (31%)	5 (56%)
CSF wcc >5	7/15 (47%)	7/8 (88%)	8/13 (62%)	5 (56%)
CSF prot>0.4 g/dL	6/15 (40%)	4/8 (50%)	8/13 (62%)	2 (22%)
CSF OCB	0/6 (0%)	0/1 (0%)	1/1 (100%)	3 Mirrored, 1 intrathecal, 2 negative
MRI abnormal	9/13 (69%)	8/9 (89%)	9/12(75%)	5 (56%)
PICU*	4 (25%)	3 (33%)	5 (39%)	5 (56%)
Immunotherapy	3 (19%)	0 (0%)	4 (31%)	5 (56%)
Full recovery	4/14 (29%)	1/9	6/12 (50%)	5 (56%)

Table 3-5 Clinical and preclinical features in patients with viral etiology Vs. NMDAR-Ab encephalitis

The most common infectious encephalitis was enterovirus (12%), and highlights a significant difference with predominantly adult cohorts in whom herpes simplex encephalitis is more common. *Mycoplasma pneumoniae* associated encephalitis occurred in 7%, although the diagnostic specificity of mycoplasma IgM in this context has been questioned. *Mycoplasma pneumoniae* can result in both direct and immune mediated encephalitis and a broad spectrum of clinical syndromes are described. Even with broad testing for HSV (84%), HSV encephalitis was only diagnosed in 5%. HSV encephalitis had the worst outcome despite early acyclovir therapy. Admission to the intensive care unit (ICU) was required in 66 (40%) patients the majority requiring management of encephalopathy, seizures or both. The NMDAR encephalitis patients in this study had typical clinical features, with dominant movement disorders and psychiatric features. As VGKC-complex antibodies were seen in patients with other possible aetiologies, they were not included in this table.

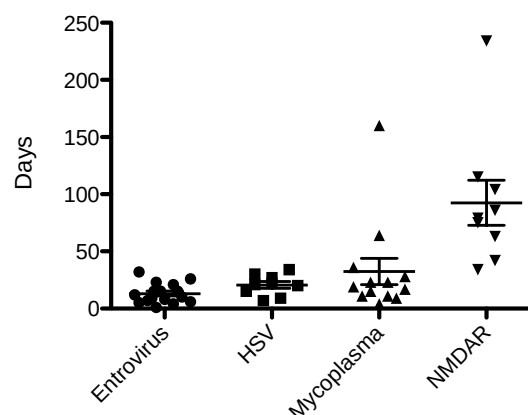


Figure 3.10 Hospital stay in patients with viral aetiologies and patients with NMDAR-Ab encephalitis.

The median length of hospital stay of the whole cohort was 13 days (range; 1-618 days). Patients with NMDAR-Ab encephalitis had a longer hospital admission than patients with viral encephalitis.

3.4 Discussion

Two cohorts of children with encephalopathies were studied in order to evaluate the frequency of neuronal autoantibodies in (i) patients diagnosed with autoimmune encephalopathies and (ii) children presenting with acute encephalitis (regardless of the primary aetiology). The cohort of patients with autoimmune encephalopathy was defined by their clinical features and exclusion of other known causes. Only 21 (46%) had antibodies to either the NMDAR (27%, one with an ovarian teratoma) or the VGKC-complex (17%), and one (2%) to GlyR antibodies. but no clinical differences were found between the antibody-positive and negative groups. Moreover, both antibody positive and negative patients responded to immunotherapies with improvements in the mRS scores and complete recovery in 20/48 (42%) overall. In a second study of patients with acute encephalitis, movement disorder, psychiatric features and sleep abnormalities were seen more frequently in patients with NMDAR-Ab encephalitis compared to viral encephalitis and, as only rarely seen in viral encephalitis, can suggest an autoimmune aetiology at the onset.

NMDAR-Ab and VGKC-complex antibodies were identified in a total of 17% of patients presenting with acute encephalitis; NMDAR-Ab were identified predominantly in the unknown group whereas VGKC-complex antibodies were found in both patients with unknown aetiologies but also in similar frequencies in patients with positive infective aetiologies.

Despite the VGKC-Ab positivity in 7/48 (15%) patients with autoimmune encephalopathy and in 13/125 (10%) of patients with acute encephalitis, all 173 children were negative for the VGKC-complex associated proteins LGI1, CASPR2 and Contactin-2. Antibody titers of more than 400pM were only reported in two, and since even these two were not associated with the cell-surface antigens, contrasting with most adults, there are questions concerning their significance (Paterson et al., 2013) (See Chapter 5). The association of VGKC-antibodies with respiratory pathogens particularly *Mycoplasma pneumonia*, suggests that they could be induced as part of the immune response to infections.

3.4.1 Clinical presentation of patients with autoimmune encephalopathy

The clinical presentations of patients with NMDAR-Ab (Table 3-1 and 3-2) in both cohorts were consistent with those previously described in the adults (Irani et al., 2010b, Dalmau et al., 2008, Dalmau et al., 2007) and paediatric literature (Dale et al., 2009, Florance et al., 2009) at the time, although recent observation suggests that the spectrum is expanding (Chapter 4). The existence of only one patient with a teratoma reflects the young age of the children studied (Dalmau et al., 2011). Children with VGKC-complex antibodies presented either with limbic encephalitis and prolonged seizures or with a less specific encephalopathy syndrome with varying degrees of seizures and psychiatric features, as previously reported in smaller studies (Suleiman et al., 2011a, Haberlandt et al., 2011, Dhamija et al., 2011). Only one child had an unexpected positive GlyR antibody which is more commonly associated with a progressive or relapsing remitting form of stiff person syndrome, often with additional brainstem and autonomic features and mainly, but not exclusively, in adults (Hutchinson et al., 2008, Turner et al., 2011, Piotrowicz et al., 2011). A 7 year old boy (from the Australian cohort) who presented with encephalopathy, prominent movement disorder, psychiatric features and seizures was positive for both NMDAR and VGKC-complex Ab with NMDAR CBA score of 4 and VGKC-complex level of 111pM. The clinical phenotype and the high NMDAR-Ab titres (compared to the low positive VGKC-complex Ab), suggest his encephalopathy was more likely to be mediated by NMDAR-Ab. Given that some of the antibody-negative autoimmune encephalopathy patients had movement disorders we looked for antibodies to the dopamine receptors D1R and D2R. Antibodies to the D2R have recently been reported in a paediatric cohort with basal ganglia encephalitis (Dale et al., 2012). These patients were phenotypically similar to some of the antibody-negative patients in the UK cohort, but D2R antibodies were not detected.

Both the UK cohort of children with autoimmune encephalitis and the Australian cohort of patients presenting with encephalitis only included patients with an encephalopathy and thus do not reflect the full range of antibody-mediated CNS disorders. A case of isolated hemidystonia was previously reported in a 19-year-old woman with positive NMDAR-Ab who made a good response to immunotherapy (Rubio-Agusti et al. 2011). To date, as the majority of the reported literature comprises of studies of specific antibody mediated encephalitis, the true incidence of presentation without encephalopathy may be under recognised.

3.4.2 Paraclinical features of patients with probable autoimmune encephalopathies

Generalised MRI atrophy was reported in 5/48 (10%) of the patients with presume autoimmune encephalopathy. The small sample size did not allow us to correlate the MRI findings with symptom severity or outcome. Post-mortem reports of some NMDAR-Ab encephalitis patients showed pronounced brain atrophy, although reversible atrophy was observed in others who survived (Iizuka et al., 2010). By contrast, a recent study of immunopathology of antibody-associated encephalopathies showed lack of neuro-axonal injury with minimal inflammation and no atrophy on imaging of patients with NMDAR encephalitis (Bien et al., 2012). The cause of the late atrophy despite the minimal changes on imaging is unclear but could be a result of low grade inflammation, recurrent seizures or medication (corticosteroids), all causing chronic neuronal dysfunction and subsequent neurodegeneration.

Bilateral basal ganglia changes were reported in four patients with VGKC-complex antibodies (See Figure 3.12 for two of the cases). Three of the four presented with explosive-onset seizure disorder without a movement disorder and made a complete clinical and radiological recovery, without immunotherapy. These findings have been reported in one other adult (Hiraga et al., 2006). The reason why this distinct clinico-radiological phenotype did not manifest as basal ganglia symptomatology in the children remains unclear. Localized MRI

changes in the basal ganglia have been reported in adult patients who were positive for both VGKC and the associated protein LGI1 (Irani et al., 2010a), but the striking neuroimaging changes with predominant bilateral basal ganglia involvement were not found in these patients. The fourth child was additionally positive for NMDAR-Ab; he presented with a movement disorder without seizures, received both steroids and IVIG and following a prolonged hospital admission (including 16 days in intensive care) he was discharged home with severe cognitive and behavioural problems.

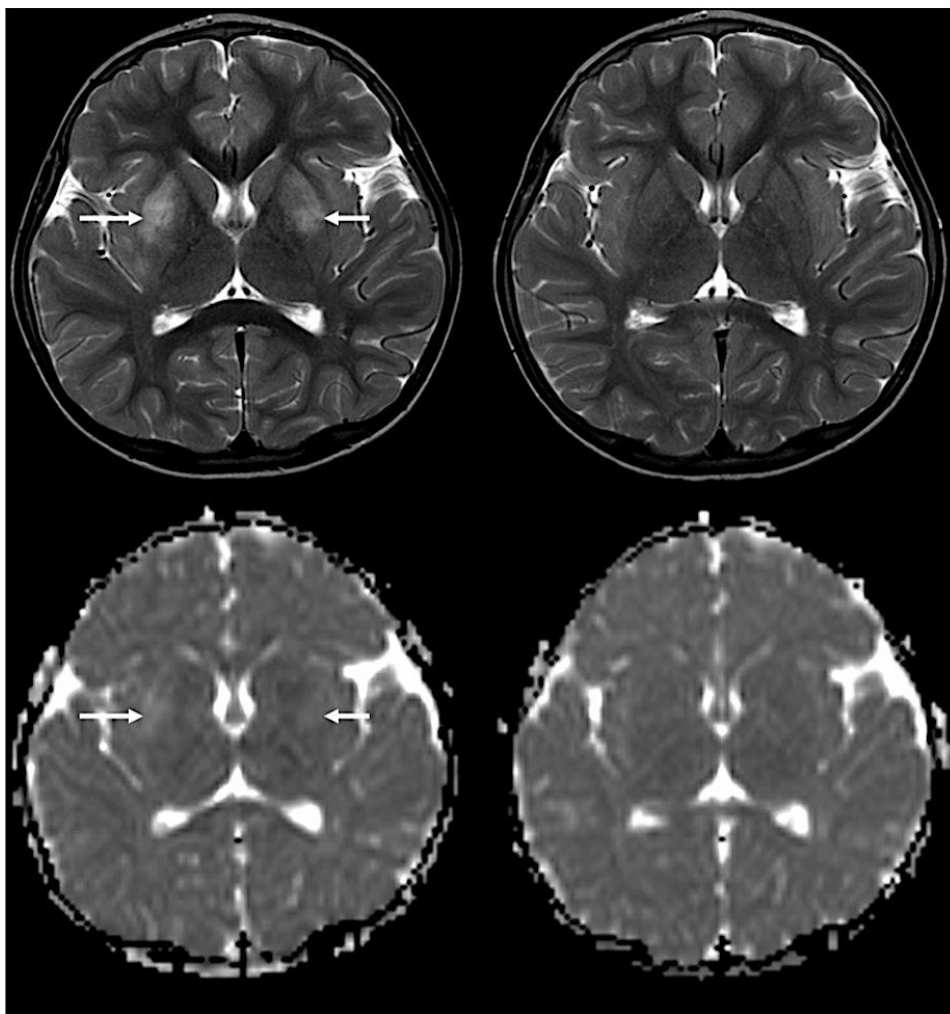


Figure 3.11 Basal ganglia changes on MRI in patients with VGKC-complex Abs.

MRI Case 1 (left). Axial T2-weighted images (top row) at presentation and follow up show increased signal intensity in the lentiform nuclei bilaterally on the initial scan (solid arrows), with minimal cortical changes in the posterior right insula (dashed arrow) which have resolved on the follow up scan. Additionally, corresponding ADC maps (bottom left) from diffusion weighted imaging revealed increased diffusivity (solid arrows), which resolved on the follow up imaging. MRI Case 2 (left) Similar findings as in Case 1, but more extensive involvement of the basal ganglia (solid arrows) on the T2-weighted images, and minimal cortical change in the left frontal lobe (dashed arrow).

3.4.3 Autoimmune Vs. infectious encephalitis

All recruiting centres undertook investigative studies to exclude infective aetiologies, often with the support from paediatric infectious disease teams. Nevertheless, an overlap was seen in both groups, with infective organisms identified in patients with autoimmune encephalopathies and neuronal antibodies in patients with known infective encephalitis. Even in patients presenting with the characteristic NMDAR-Ab encephalitis, acute infective organisms were reported in 5 (HSV (2), mycoplasma (2), EBV (1), and 3 patients had a raised ASOT, in keeping with previous reports of mycoplasma IgM positive serology in 50% of patients with NMDAR-Ab without a neoplasm (Gable et al., 2012). Moreover, the two NMDAR-Ab positive patients who were also positive for HSV PCR in the CSF continued to regress despite acyclovir treatment but improved following immunotherapy. Similarly, an eight months old boy presenting with encephalopathy, seizures and a dyskinetic movement disorder was found to have intrathecal HSV response and although HSV PCR was negative he was diagnosed with HSV encephalitis, made a full recovery following treatment with acyclovir, steroids and immunoglobulin and was retrospectively found to be NMDAR-Ab positive (Mohammad et al., 2014). It is possible that some patients have existing antibodies that gain access to the brain when there is a viral infection, or alternatively a para-infectious antibody-mediated process may begin during an acute infective process. Consistent with this, NMDAR-Abs were reported in 30% of adults with HSV encephalitis, possibly as a secondary epiphenomenon (Pruss et al., 2012).

Behavioural change and seizures were reported in most patients and were helpful clinically to differentiate between the different aetiologies at onset. By contrast movement disorders and psychiatric features were reported almost exclusively in patients with autoimmune encephalopathy, particularly NMDAR-Ab encephalitis. On rare occasions, children with infective encephalitis present with a movement disorder (chorea/dyskinesias) and although movement

disorder are seen frequently in patients with Japanese encephalitis and West Nile virus (Solomon et al., 2000, Sejvar et al., 2003) outside the regions endemic for these arboviral encephalitides, the diagnosis of NMDAR-Ab should be considered in all children presenting with encephalopathy and a movement disorder.

Although typically described in patients with NMDAR-Ab encephalitis, autonomic instability was also a feature in the seronegative autoimmune group and in patients with infective causes such as enterovirus and mycoplasma, which may simply reflect brainstem involvement. Autonomic instability with or without cranial neuropathies is frequently reported in patients with brainstem encephalitis secondary to both infective and inflammatory causes (Tan et al., 2013).

Recently, guidelines for the diagnosis of CNS neuronal surface antibody associated syndromes were proposed in adults (Zuliani et al., 2012), trying to aid in the diagnosis of autoimmune encephalopathy, particularly when no known antibody is identified. In contrast to these guidelines, evidence of CNS inflammation with CSF reactivity and MRI finding suggestive of inflammation would not be useful in identifying children with antibody mediated encephalitis. In fact, even when analysing patients with a well-established neuronal antibody such as NMDAR-Ab, only half had CSF and MRI abnormalities suggestive of inflammation, a lower rate than detected in the infective encephalitis group. The other features intended to help identify an immune aetiology were prodromal symptoms, preceding infections or occurrence of other autoimmune diseases but these also did not help in distinguishing between the different groups in the cohorts reported here.

3.4.4 Immunotherapy response and outcome

Immunosuppressive strategies with corticosteroids, IVIG and PLEX have been used extensively in both adults (Vincent et al., 2011) and children with autoimmune encephalopathies (Wong-Kisiel et al., 2012). Responses to immunotherapy, particularly for NMDAR-Ab encephalitis, can be slow

(over months) and variable. Furthermore, the latency to treatment is also very variable (months to years) as in previously reported childhood series (Dale et al., 2009, Florance et al., 2009, Suleiman et al., 2011a, Haberlandt et al., 2011, Dhamija et al., 2011), making interpretation and direct comparison of treatment efficacy difficult.

Complete recovery was seen in 20/48 (42%) of patients with autoimmune encephalopathies in the UK cohort and in 37/95 (39%) of all children with acute encephalitis in the Australian cohort, in whom follow-up data was available. It is important to note that learning, memory and behavioural problems were often not identified until the child was integrated back into the educational system. NMDAR-Ab encephalitis relapses are reported in approximately 30% of adults (Irani et al., 2010b, Dalmau et al., 2011) and 25% of children (Florance et al., 2009). The need for longer-term immunosuppression, currently used in only five of the patients in the UK cohort, will have to be evaluated when more data are available.

Within the autoimmune group, worse outcomes were seen in patients who did not receive any immunotherapy and delay to therapy was likely to have influenced outcome, as seen in the two patients who receive immunotherapy late and did not respond. Nevertheless, four patients who did not receive immunotherapy made a complete recovery. Spontaneous recoveries in immune-mediated encephalopathies have been described (Hacohen et al., 2012, Buckley et al., 2001, Suleiman et al., 2011a) suggesting that some of the clinical syndromes are monophasic and can resolve without treatment. Treatment decisions must balance the potential benefits of early and sustained immunotherapy in prevention of progression of disease and in facilitating recovery.

Of the patients presenting with acute encephalitis (Australian cohort), 28/125 (22%) received immunotherapies and of the patients with presumed autoimmune encephalitis (UK cohort) 34/48 (71%) received immunotherapies. Both patients with unknown and infective aetiologies had a trial of immunotherapy with a varying degree of success. The current strategy

for children with immune-mediated encephalitis is to commence treatment promptly with corticosteroids as first line. Although glucocorticoids may be anti-inflammatory, their use can be challenging in patients with CNS inflammation in whom the diagnosis of viral infection cannot be ruled out. Although traditionally thought to be contraindication in infective encephalitis, there is a growing interest in the use of steroids in HSV encephalitis as an adjuvant therapy to acyclovir (Ramos-Estebanez et al., 2014). Reports of clinical benefits and favourable outcome following treatment with IVIG in infective encephalitis such as Japanese encephalitis virus, West Nile virus, Coxsackie viruses and Mycoplasma pneumonia (Khetsuriani et al., 2002) suggest that IVIG may be an appropriate first line treatment in acute encephalitis irrespective of the primary aetiology.

3.5 *Summary and conclusion*

Autoimmune encephalopathies are a common cause of acute encephalitis in children. In many, no known CNS autoantibody can be identified. Seronegative patients can respond to immunotherapy resulting in both clinical improvements acutely but also in better long-term outcome. The similarities between patients with and without identified antibodies indicate a need for re-evaluation of the diagnostic and management strategies. Moreover, defining an antibody negative group without an antibody biomarker is difficult, particularly where there is phenotypic overlap with viral or other infective encephalitis. However, those patients, presenting with an unexplained encephalopathy with a movement disorder and psychiatric features were more likely to have an autoimmune aetiology. These patients were often afebrile at presentation, had low CSF cell counts and did not have MRI abnormalities that were characteristic of identifiable viral encephalitis. The results detailed in this chapter are published in (Hacohen et al., 2014b).

CHAPTER 4 - Paediatric N-methyl-D-aspartate receptor (NMDAR) antibodies associated neurological syndromes

4.1 Introduction

Antibodies to neuronal surface antigens have been shown to be pathogenic in a number of immune mediated conditions (Lancaster and Dalmau, 2012). N-methyl-D-aspartate receptor (NMDAR) antibody (Ab) is the most common antibody identified in children (Armangue et al., 2012). Originally reported as a paraneoplastic syndrome in young adult females with ovarian teratoma (Dalmau et al., 2007), patients typically present with a well-characterised encephalopathy syndrome with prominent psychiatric symptoms, movement disorder and seizures. The cell-surface target antigen is perturbed by the antibodies, resembling symptoms associated with NMDAR antagonists (Dalmau et al., 2011). Milder or incomplete phenotypes of the disease are less commonly reported (Dalmau et al., 2011) and in a large observational cohort of 577 patients with anti-NMDAR encephalitis, only 1% of patients remained monosymptomatic at 4 weeks from symptom onset. By contrast, in a study of paediatric onset movement disorders 5/52 were positive for NMDAR-Ab (Dale et al., 2010) and previously, a case of isolated hemidystonia was reported in a 19 year-old woman who made a good response to immunotherapy, suggesting that partial phenotypes may be under recognised (Rubio-Agusti et al., 2011). Nevertheless, in prospective studies looking into NMDAR-Ab in patients with new onset psychosis (Kayser et al., 2013) and new onset epilepsy (Suleiman et al., 2013) the incidence was indeed low.

Despite the severity of the disease in many patients, conventional MRI of the brain is normal in the majority of patients, often referred to as the clinical-radiological paradox (Dalmau et al., 2011). When abnormal, MRI usually reveals predominantly subtle, non-enhancing

lesions, in both the grey and the white matter, which often reverse spontaneously (Irani et al. 2010b), and are not usually restricted to the white matter. Good outcome is seen in over 75%, but over half of the patients do not respond to first line treatment (steroids, intravenous immunoglobulins and plasma exchange) and require escalation to second line immunosuppression (cyclophosphamide, rituximab) (Titulaer et al., 2013)

Recently, we observed that one adult, initially reported in the UK cohort of anti-NMDAR encephalitis (Irani et al., 2010b) was later diagnosed with multiple sclerosis (MS), and a further patient with MS subsequently developed NMDAR-Ab encephalitis.

Additionally, in a systematic evaluation of glial, myelin and neuronal-directed antibodies in children with a range of demyelination syndromes, we identified two patients with NMDAR-Ab (Hacohen et al., 2014b). These observations, together with existing case reports of NMDAR-Ab in patients with neuromyelitis optica spectrum disorder (NMOSD) (Kruer et al., 2010), acute demyelinating encephalomyelitis (ADEM) (Lekoubou et al., 2012) or MS (Takeda et al., 2013), raise questions regarding the clinical relevance, potential pathogenic effect, and direct contribution of NMDAR-Ab to neurological syndromes that involve the white matter.

Finally, there is a growing interest in the relationship between viral infections and autoimmunity of the central nervous system particularly; the movement disorder seen in NMDAR-Ab encephalitis has phenotypic similarities to the choreoathetosis in relapsing herpes simplex encephalitis (HSVE). NMDAR-Ab have been detected in a proportion of adult patients with acute HSVE (Pruss et al., 2012) and one of 20 Spanish children with anti-NMDAR encephalitis had presented as choreoathetosis post HSVE (Armangue et al., 2013). These and other evidence of viral infections coincident with NMDAR-Ab in adults (Irani et al., 2010b) and children (Hacohen et al., 2013), raise the possibility that induction of NMDAR-Ab during the virus infection may be responsible for a second phase of immune-mediated encephalopathy.

In this chapter the range of NMDAR-Ab mediated CNS diseases will be discussed in particular the non-encephalopathic partial phenotypes, the white matter syndromes and the HSVE neurological relapses.

4.2 *Patients*

Between 2009-2013, 662 children including 50 of 102 NMDAR-Ab positive children (under 18 years old) identified in the UK, were referred for serum and/or cerebrospinal fluid (CSF) NMDAR-Ab testing from 6 paediatric neurology centres (Chapter 2.1.2 and figure 4.1). Clinical data, comprising demographic information, clinical features at presentation, discharge and follow up, and results of laboratory, electrophysiological and neuroimaging testing, were reviewed.

4.3 *NMDAR-Ab positive patients*

Of the 50 patients identified consecutively from these 6 centres, 31 had typical NMDAR-Ab encephalitis (Table 4-1), eight had a partial phenotype without the encephalopathy (Table 4-2), five presented with acquired demyelinating syndromes (ADS), three with brainstem encephalitis and three with a neurological relapse following HSVE. NMDAR-Ab were positive in the CSF in 10 out of 11 patients tested with typical NMDAR-Ab encephalitis, in two of the three tested with the partial phenotype, and in all three tested with white matter syndromes; serum levels were higher than CSF in all paired samples (Figure 4.2). Testing of all patients' sera did not reveal the presence of antibodies to voltage gated potassium channel (VGKC)-complex or the associated proteins (leucine-rich glioma-inactivated 1, LGI1; contactin associated protein-like 2, CASPR2; and contactin 2), glycine receptor, glutamic acid decarboxylase (GAD), the dopamine receptors (D1R and D2R) or aquaporin-4 (AQP4). All patients had screening for occult malignancy (ultrasound scan of abdomen +/- ovaries and paraneoplastic antibodies).

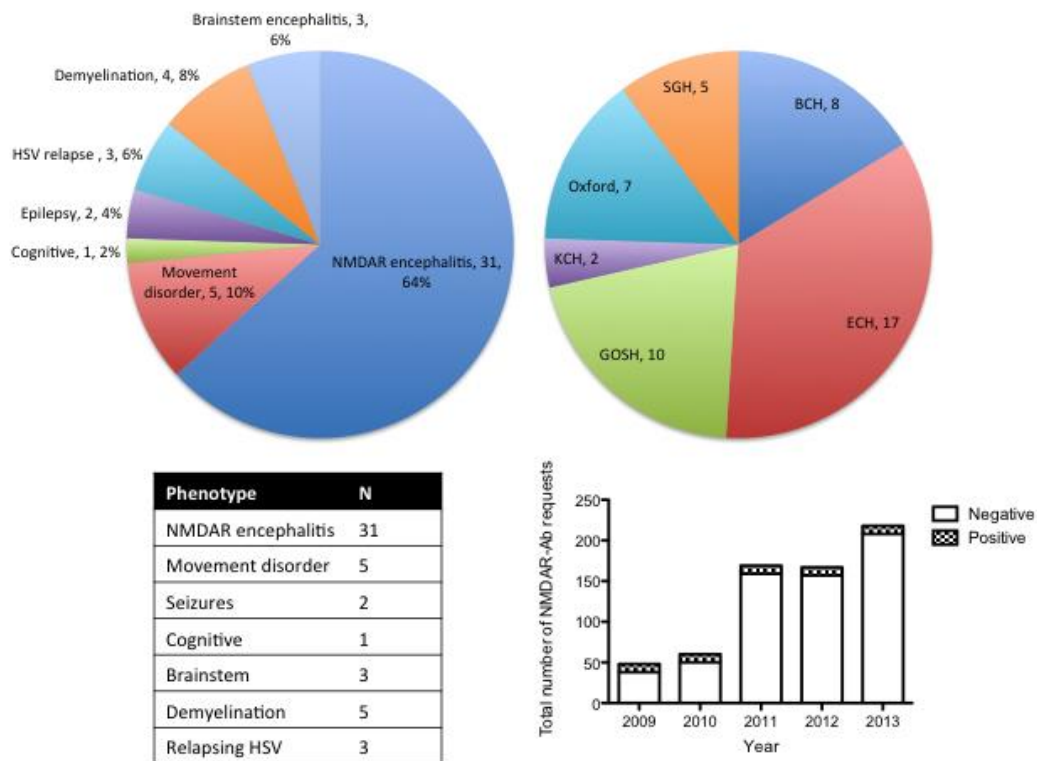


Figure 4.1 NMDAR-Ab spectrum disorder.

Fifty children were positive for NMDAR-Ab; Evelina Children's Hospital, London (n=17), Great Ormond Street Children's Hospital, London (n=10), Birmingham Children's Hospital, Birmingham (n=8), Oxford University Hospital (n=7), St George's Hospital, London (n=5), and King's College Hospital, London (n=2). Despite the increase in antibody testing a total of 10 new cases were diagnosed every year. Of a total of 50 patients with NMDAR-Ab 62% had NMDAR-Ab encephalitis. An additional 4 patients had the characteristic encephalitis following brainstem encephalitis (n=2) and HSVE (n=2).

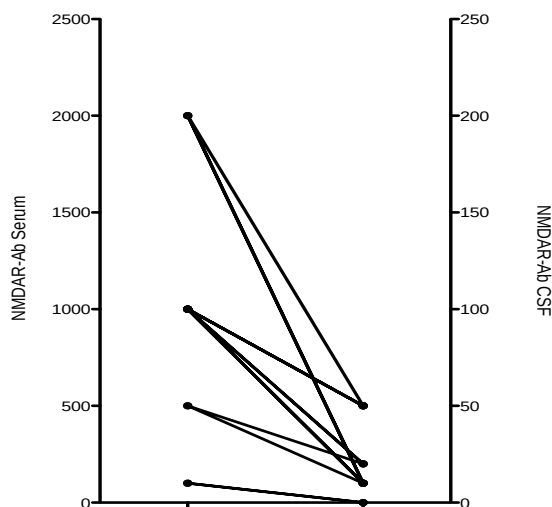


Figure 4.2 Paired serum and CSF titres

Antibody levels were higher in the serum than in the CSF in all paired samples. NMDAR-Ab were positive in the CSF in 10 out of 11 patients tested with typical NMDAR-Ab encephalitis, in 2 of the 3 tested with the partial phenotype, and in all three tested with white matter syndromes.

	Demographics	Presentation	Relapse	MRI	Treatment	Outcome
1	14yr F Asian	E, C, P, N	No	Normal	Steroids, IVIG, PLEX	F
2	2yr F Asian	E, C, S, M	No	Normal	Steroids, IVIG, PLEX, Rituximab	C
3	13yr F Caucasian	E, C, P, S, M, A	No	Normal	Steroids, IVIG, MMF	B
4	11yr F Caucasian	E, C, P, A	No	Normal	Steroids, IVIG	CB
5	11yr F Caucasian	E, P, S, M, A	No	Normal	Steroids, IVIG, PLEX, MMF	F
6	16yr F Caucasian	E, C, P, S, M	No	Normal	Steroids, PLEX, Cyclophosphamide, Rituximab	C, P, S
7	4yr F Black	E, C, P, S, M	No	Normal	Steroids	C, B
8	8yr M Black	E, C, P, S, M	No	Normal	Steroids, IVIG	C, B
9	2yr F Black	E, C, P, S, M	No	Normal	Steroids	C, S
10	13yr M Other	E, P, S, M	No	Normal	Steroids, PLEX	F
11	14yr F Caucasian	E, C, P, S, M, A	No	Normal	Steroids, IVIG, PLEX, Cyclophosphamide, Rituximab	C, B, S
12	5yr F Asian	E, C, P, S, M	No	Normal	Steroids, IVIG, Rituximab	C, B
13	3yr F Caucasian	E, C, P, S	No	Normal	Steroids	F
14	6yr M Other	E, P, S	No	Normal	Steroids, IVIG	S
15	2yr F Caucasian	E, S, P, M	No	Normal	Steroids, IVIG	C, B
16	5yr F Black	E, S, P, M	No	Normal	Steroids	C, B
17	3yr M Black	E, C, P, S	No	Normal	Steroids	F
18	15yr F Asian	E, C, P	No	Normal	No immunotherapy	C, P
19	14yr F Caucasian	E, C, P, M	No	Left cerebellar cortical signal change	Steroids, IVIG	C, B
20	5yr F Other	E, C, S, M	No	Mild signal change hippocampus and middle temporal gyrus.	Steroids	F
21	16yr M Caucasian	E, C, P, S, A	No	Transient cortical signal change	Steroids, IVIG, PLEX	F
22	17yr F Other	E, P, M, A	No	Transient cortical and subcortical signal changes	Steroids, IVIG, PLEX	F
23	16yr M Caucasian	E, C, P	Yes	Transient bilateral hippocampi and insular high signal	Steroids, IVIG, MMF	F
24	17yr F Caucasian	E, C, P, S, M	Yes	Periventricular leukomalacia (Old)	Steroids	F
25	7yr M Asian	E, P, S	Yes	Normal	Steroids, IVIG	C, S
26	16yr F Caucasian	E, C, S	Yes	Normal	Steroids, IVIG, PLEX, Azathioprine	S
27	4yr M Caucasian	E, C, P, S, M, A	Yes	Normal	Steroids, IVIG, PLEX, Rituximab, MMF	C, B, S
28	13yr F Black	E, C, P, S, M, A	Yes	Normal	Steroids, IVIG, PLEX, MMF	F
29	5yr F Caucasian	E, C, P, S, M	Yes	Normal	Steroids, IVIG, PLEX	C, B, S
30	2yr F Caucasian	E, C, P, S, M	Yes	Normal	Steroids, IVIG, PLEX	C, S
31	6yr M Asian	E, C, S, M	No	Normal	Steroids, IVIG	S
32*	1yr F Caucasian	E, C, S, M	Yes	Changes consistent with HSVE (old)	IVIG (10yr later)	C, B, S, M

Table 4-1 Patients with typical NMDAR-Ab encephalitis.

Clinical symptoms were classified as; A autonomic, B behavioural, C cognitive, E encephalopathy, F full recovery, M movement disorder, N neoplasm, P psychiatric, S seizures. Abbreviations: IVIG intravenous immunoglobulins, MMF Mycophenolate mofetil, MRI magnetic resonance imaging, PLEX plasma exchange. * Patient presented following relapse of HSVE.

4.3.1 Clinical and paraclinical features of patients with NMDAR-Ab encephalitis.

Thirty-one patients (age range 2-17years; median 8years) presented with typical NMDAR-Ab encephalitis (Table 4-1). All patients were encephalopathic with psychiatric features in 28 (90%), seizures in 25 (81%), cognitive in 23 (74%), movement disorder in 20 (65%) and autonomic dysfunction in 7 (23%). All patients were screened for malignancies but only one 15 year-old girl (patient 1) had an ovarian teratoma (3%).

Electroencephalography (EEG) was abnormal in 30/31 (97%) showing changes consistent with encephalopathy with or without epileptiform discharges. The finding of extreme delta brush was not reported. Brain MRI was abnormal in 6 (19%) of which one had long standing changes (patient 24). Oligoclonal bands were present in the CSF in 14/18 (78%) patients tested.

Immunotherapy was given in 30/31 (97%); 30 Steroids/IVIG; 13 PLEX and 10 received 2nd line immunotherapy. Eight patients relapsed (26%) and 11 patients made a full-recovery (34%).

4.3.2 Clinical and paraclinical features of patients presenting with partial phenotype without an encephalopathy

Eight patients (Age range 5-18 years; median 11.5 years) presented with partial phenotypes without an encephalopathy (Case 33-40; Table 4-2).

Five patients had predominant movement disorder. A 5 year old and a 12-year-old female with acute onset hemichorea, a 10-year-old female and an 18-year-old male with generalised chorea, and a 12-year-old male with abdominal myoclonus. None of these patients developed the characteristic encephalopathy syndrome seen in NMDAR encephalitis, but all had other associated subtle cognitive deficits with seizures in two and psychiatric features in

two (Table 4-2). These patients had normal brain MRI and normal CSF studies. NMDAR-Ab were tested in the CSF in four patients (Case 33-36) and all were positive. Two patients (Cases 33 and 34) had other features of autoimmunity with positive IgG anti-cardiolipin antibody, with subtle functional abnormalities identified when testing for anticoagulant activity (Case 33) and anti-neutrophil antibodies and granulocyte specific IgM antibodies (with prolonged but asymptomatic neutropenia), rheumatoid factor anti-double stranded DNA (transiently positive and normalised within the acute admission) (Case 34). Immunotherapy was given in all five patients with good response and resolution of the movement disorder. One patient (case 33) has relapsed and required repeated courses of IVIG. Full recovery was seen in three but in two, although the movement disorder resolved, the psychiatric features persisted.

Two had predominant seizures; an 11-year-old girl with learning difficulties and focal seizures who was only treated with antiepileptic medication and has never received immunotherapy and, an 8-year-old girl with history of encephalitis with HSV2 positive skin lesions (negative CSF PCR) and recurrent seizures with associated flair-ups of skin vasculitis; she improved on IVIG.

One 15-year-old girl presented with acute onset cognitive decline with word finding difficulties only. She was NMDAR-Ab positive both in the serum (1:1000) and in the CSF (1:50). Her brain MRI was normal and EEG demonstrated evidence of slowing over the left hemisphere with sharp components also spreading to the right. She was not treated with immunotherapy and her symptoms persisted.

	Demographics	Presentation	Relapsing neurological events	Conventional brain MRI	Immunotherapy	Outcome
33	12yr M Caucasian	M, Cog, Psych	No	Normal	Steroids	Psych
34	10yr F Caucasian/Black	M, Cog	Yes	Normal	Steroids, IVIG	Full recovery
35	5yr F Black	M, Cog, S	No	Normal	Steroids, IVIG	Full recovery
36	18yr M Caucasian	M, Cog, P	No	Normal	Steroids	Psych
37	12yr F Asian	M, Cog, S	No	Normal	Steroids, PLEX	Full recovery
38	11yr F Caucasian	S, Cog	Yes	Normal	NT	S
39	8yr F Caucasian	S, Cog	Yes	Right mesial temporal sclerosis	NT	S, B
40	15yr F Asian	Cog	No	Normal	NT	Cog

Table 4-2 Patients with NMDAR-Ab and a partial phenotype without an encephalopathy. Clinical symptoms were classified as; B behavioural, C cognitive, M movement disorder, Psych psychiatric, S seizures. Abbreviations: IVIG intravenous immunoglobulins, MRI magnetic resonance imaging, PLEX plasma exchange.

4.3.3 NMDAR-Ab associated with distinct clinico-radiological white matter syndromes.

A third group identified were patients with white matter involvement on conventional neuroimaging. Ten patients (20%) (Cases 41-50; 5 male, age range 1.3-18yr; median 8.5yr) were identified. They were classified as having three neurological syndromes; brainstem encephalitis (n=3), HSVE relapsing with a distinct leukoencephalopathy (n=2) and ADS (n=5). Their clinical features, imaging and treatment responses are described below and summarized in Table 4-3 and Figures 4.3-5.

Brainstem encephalitis (n=3): three patients had encephalopathy, predominant brainstem signs (cranial neuropathies and ataxia), and neuroimaging findings of brainstem abnormalities, or a clinical syndrome unequivocally localised to the brainstem (Tan et al. 2013). Two boys aged 5 and 18-year (Cases 41 and 43 respectively) and a 10-year-old girl (Case 42), presented

with a clinical syndrome consistent with brainstem encephalitis. Cases 41 and 43 were treated with steroids and plasma exchange, and both demonstrated significant improvement with resolution of brainstem signs at discharge. Case 42 only presented to hospital three weeks later, when her encephalopathy worsened with intractable seizures, movement disorder and hyperpyrexia. Case 42 presented eight weeks following the first presentation with a poly symptomatic encephalopathy. NMDAR-Ab were identified confirming the clinical suspicion of anti-NMDAR encephalitis, raised after the relapse in Case 42, and in Case 43, following recurrent periods of encephalopathy that ensued (retrospective diagnosis five years from initial encephalopathic presentation). In the most contemporary patient (Case 41), NMDAR-Ab positivity in sera and CSF was confirmed at onset of brainstem encephalitis (Figure 4-3).

HSVE relapse (n=2): we identified two patients, Cases 44 and 44 (M2y, F16m) with NMDAR-Abs in serum and CSF (Table 4-3) during neurological relapses with substantial leukoencephalopathy on imaging (Figure 4-4) occurring within 2 months of HSVE (headache, fever, seizures, typical imaging and CSF HSV PCR positivity). Case 44 presented with cognitive, behavioural and motor decline, and Case 45 presented with a further encephalopathic illness with new onset movement disorder. Both patients had negative HSV PCRs in the CSF at relapse. Both were treated with intravenous immunoglobulins (IVIG) or plasma exchange (PLEX), which resulted in clinical improvement and reduction of NMDAR-Ab titres (Figure 4-4D, E). A significant radiological improvement was observed in Case 44 (Figure 4-4G). Other potential causes of leukoencephalopathies, such as neurometabolic conditions, were excluded.

Acquired demyelination syndrome (ADS) (n=5): five patients presented with ADS classified according to the IPMSSG criteria (Krupp et al., 2013). Three initially had optic neuritis: Case 46

(F6y) and Case 47 (M9y) with bilateral optic neuritis were treated with intravenous methylprednisolone (IVMP) followed by a weaning course of oral prednisolone. Case 48 (F11y) presented with right optic neuritis and poor visual recovery; she only received a course of oral steroids one year later and relapsed 3 years later with left optic neuritis and new brain lesions (Figure 4-5). Case 49 (M8y) presented with ADEM, relapsed at one month with a second encephalopathic illness and imaging evidence of new brainstem lesions (Figure 4-5); at 12 and 13 months he developed right and left optic neuritis respectively. At this stage, he was treated with mycophenolate mofetil (MMF) with good visual recovery, no further relapse, and a reduction in NMDAR-Ab levels (Figure 4-5). Case 50 (F10y) had an unusual presentation of recurrent episodes of hyperventilation, dizziness and double vision lasting from 2 days to over 2 weeks (Figure 4-5). Her brain imaging revealed periventricular white matter changes (Figure 4-5). She has not been treated and remains clinically unchanged. As antibodies to myelin MOG and AQP4 have been associated with demyelinating disorders (Derfuss and Meinl, 2012), sera from all 10 cases and the other 36 NMDAR-Ab positive patients were tested. MOG antibodies were only found in 3 of the 5 ADS patients (Cases 46, 47 and 49; Table 4-3). All patients were negative for AQP4 antibodies.

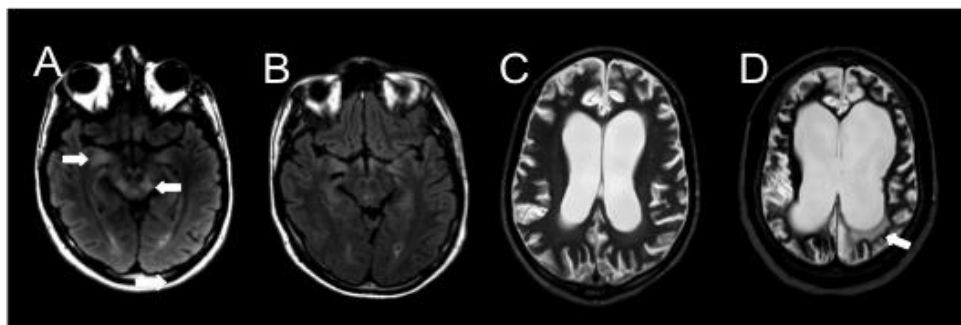
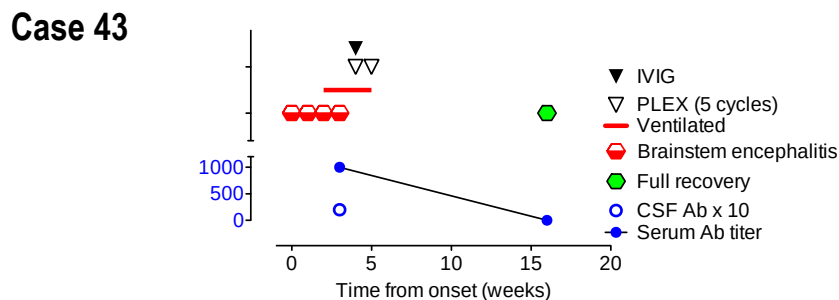
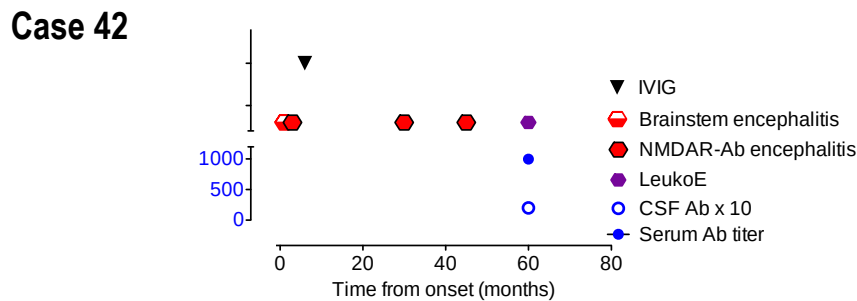
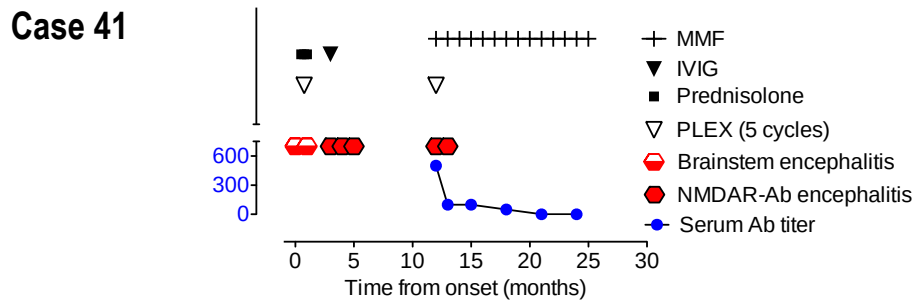
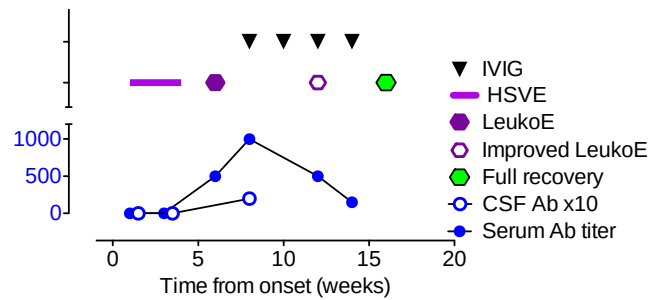


Figure 4.3 NMDAR-Ab in patients with brainstem encephalitis.

Cases 41-43 respectively presented initially with brainstem encephalitis; Cases 41 and 42 relapsed with encephalopathy, psychiatric features, movement disorder and dysautonomia. Case 42 had additional seizures. In both, the diagnosis of NMDAR-Ab encephalitis was made at the time of relapse. Case 43 had a monophasic illness and did not have any of the clinical characteristics of NMDAR-Ab encephalitis. A-B Case 41: Axial T2 fluid-attenuated inversion recovery (FLAIR) images showing hyperintensity in the midbrain and mesial temporal lobe (arrows in A) which subsequently resolved on follow up imaging performed at 2 months when patient had relapsed with a polysymptomatic encephalopathy (B). C-D Case 42: Axial T2-weighted images 6 months after her initial brainstem syndrome revealed diffuse global cortical atrophy (C). Imaging during an encephalopathic episode 5 years later revealed new white matter changes (arrow in D) and further atrophy. This radiological feature of leukoencephalopathy progressed on subsequent imaging (not shown).). Modified from Hacohen et al., Neurol Neuroimmunol Neuroinflamm. 2014

Case 44



Case 45

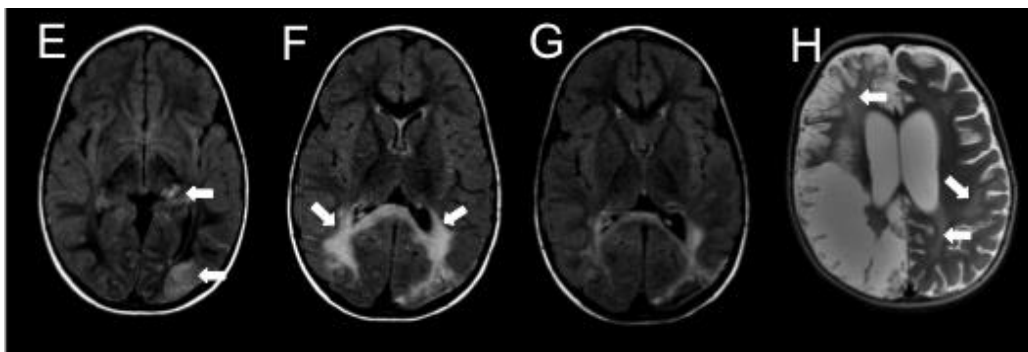
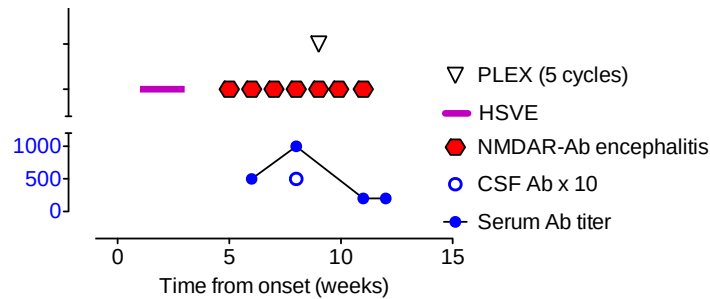
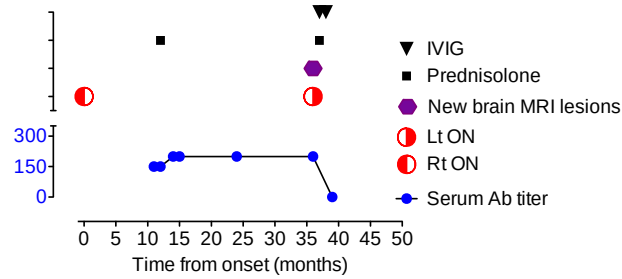


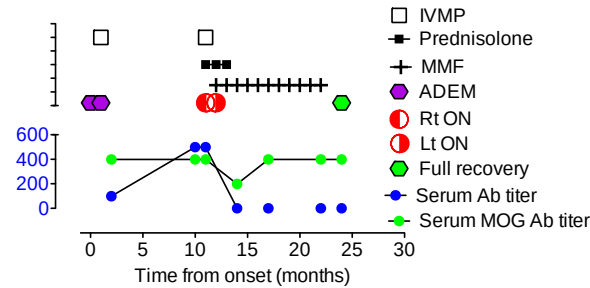
Figure 4.4 NMDAR-Ab in patients with leukoencephalopathy following HSVE.

Cases 44 and 45 presented with HSVE, then suffered a neurological relapse, which correlated with raised NMDAR-Ab in both serum and CSF and demonstrated a clinical response to immunotherapy with reduction with antibody levels. E-H Serial axial T2 FLAIR MRI images of a Case 44 (E-G) showing localized cortical changes in the left thalamus and occipital lobe (arrows in E) on initial imaging which progressed demonstrating significant bilateral white matter signal changes in the parieto-occipital region 2 months later (arrows in F) when the patient presented with significant worsening of cognitive, behavioural and motor regression. Following treatment and improvement of symptoms, follow up neuroimaging 2 months later demonstrated a significant resolution of this white matter change (G). Axial T2 weighted image of Case 45 at relapse 2 months after HSVE showing extensive bilateral but asymmetrical cystic encephalomalacia centred on the temporo-insular regions (only parietal changes shown) characteristic of HSVE, but in addition demonstrated the characteristic leukoencephalopathic changes seen globally (arrows in H). Modified from Hacohen et al., *Neurol Neuroimmunol Neuroinflamm.* 2014

Case 48



Case 49



Case 50

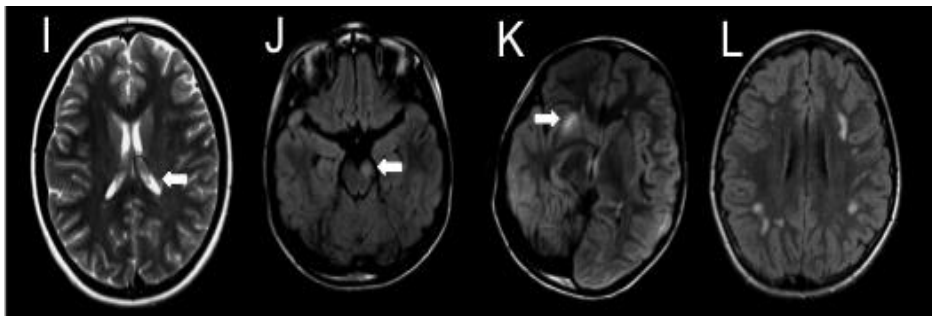
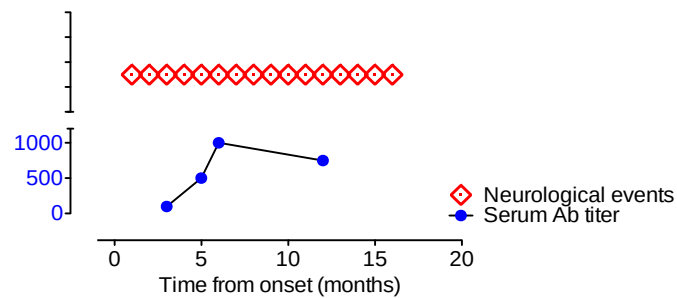


Figure 4.5 NMDAR-Ab in patients with ADS.

Case 46 and 47 presented with bilateral ON (not shown). Case 48 presented with optic neuritis and poor visual recovery, which prompted a neuroinflammatory screen and the identification of the antibody positivity. She only received a course of steroids a year into her illness and three years later had a neurological relapse. Case 49 presented initially with two episodes of ADEM and relapsed at 1 year with optic neuritis. He was additionally MOG-Ab positive, which remain detectable even when NMDAR-Abs are no longer detectable and when patient had clinically recovered. Case 50 suffered from recurrent episodes of hyperventilation, dizziness and double vision, did not receive any treatment and both her clinical and radiological features remained unchanged). I-L At time of relapse with left optic neuritis brain imaging in Case 48 showed subtle periventricular white matter signal change on a T2-weighted image (arrow demonstrating one periventricular lesion in I). Imaging of Case 49 showing midbrain (arrow in J), capsular (arrow in K) and thalamic changes (not shown) on axial T2 FLAIR. Axial T2 FLAIR image of Case 50 demonstrating patchy subcortical white matter changes (L). Modified from Hacohen et al., *Neurol Neuroimmunol Neuroinflamm*. 2014

Cases	Demo-graphics	Neurological syndrome at onset	NMDAR antibody*	Subsequent neurological event	Brain MRI	OCB	Treatment	Outcome/ Follow-up** (months)
41	M, 18yr Caucasian	Brainstem encephalitis	Onset: ND R1: ND R2: S (1:500); CSF: ND	R1 (D60): Encephalopathy, psychiatric, movement disorder and dysautonomia. R2 (1yr): Encephalopathy, psychiatric, movement disorder, and dysautonomia.	Onset: Brainstem signal change R1: Resolution of brainstem changes R2: New midbrain, mesial temporal lobe and posterior periventricular diffuse white matter changes.	ND	Onset: PLEX, OP R1: PLEX, IVIG R3: PLEX, MMF	Relapse free (oT) Cognitive and behavioural problems Antibody negative (30)
42	F, 10yr African Caribbean	Brainstem encephalitis	Onset: ND R1: ND R2: S (1:1000); CSF (1:20)	R1 (D21): Encephalopathy, seizures, movement disorder, hyperpyrexia R2 (5yr): Encephalopathy, worsening seizures.	Onset: No imaging R1: Widespread cortical signal change R2: Diffuse posterior white matter changes	ND	Onset: NT 6month: IVIG R1: NT	Quadruparesis Visual failure Seizures (29)
43	M, 5yr Caucasian	Brainstem encephalitis	Onset: S (1:1000); CSF (1:20)	Monophasic	Multiple cranial nerve enhancement	Negative	PLEX	Full recovery Antibody negative (8)
44	M, 2yr African Caribbean	HSV Encephalitis	Onset: S (-); CSF (-) R1: S (1:500); CSF (1:20)	R1 (D60): Cognitive, behavioural and motor regression.	Onset: Focal parenchymal changes. Relapse: Diffuse posterior bilateral white matter changes	Onset: Negative R1: Positive	R1: IVIG (Long term)	Full recovery Reduction in antibody titres. (11)
45	F, 16month Asian	HSV Encephalitis	Onset: ND R1: S (1:1000); CSF (1:50)	R1 (D60): Encephalopathy, movement disorder	Onset: changes consistent with HSV Relapse: posterior bilateral white matter changes	ND	R1: PLEX	Resolution of encephalopathy Residual motor deficit Reduction in antibody titres (6)
46	F, 6yr African Caribbean	Bilateral ON (Right>Left)	Onset: S (1:150); CSF ND Additional MOG S (1:1620)	Monophasic	Normal	ND	IVMP, OP	Incomplete visual recovery (10)

47	M, 9yr Caucasian	Bilateral ON	Onset: S (1:150); CSF ND Additional MOG S (1:1620)	Monophasic	Normal	ND	IVMP, OP	Full visual recovery (30)
48	F, 11yr Caucasian	Right ON	Onset: ND Interval: S (1:150) R1: S (1:200); CSF ND	R1 (3yr): Left ON	Onset: Normal. R1: Periventricular white matter lesions	ND	Onset: NT OP (1 year from onset) R1: OP, IVIG	Poor visual recovery Antibody negative (42)
49	M, 8yr Asian	ADEM	Onset: ND R1: ND Interval: S (1:150) R2: S (1:500); CSF ND Additional MOG S (1:400)	R1 (D30): ADEM R2 (1yr): Right ON R3 (13m): Left ON	Onset: Bilateral deep grey and white matter lesions. R1: Similar to onset R2: New brainstem lesions	ND	Onset: IVMP, OP R1: IVMP, OP R2: MMF	Full recovery (NoT) Antibody negative (30)
50	F, 11yr Caucasian	Recurrent episodes of hyperventilation, dizziness and double vision.	Onset: S (1:100); CSF ND Serum levels increasing to 1:1000	Recurrent episodes of initial presenting syndrome. One episode with loss of mobility for 2 weeks	Onset: Numerous lesions in subcortical white matter and body of the corpus callosum. No significant change on repeat imaging.	Negative	NT	Unchanged (24)

Table 4-3 Clinical and paraclinical features of 10 NMDAR antibody positive patients with distinct clinico-radiological white matter syndrome

Brainstem encephalitis (BE) was defined by encephalopathy with predominant brainstem signs (cranial neuropathies and ataxia). Neuroimaging findings of brainstem abnormalities during the admission were also required unless the clinical syndrome was unequivocally localized to the brainstem¹². **Herpes simplex virus encephalitis (HSVE)** with clinical, laboratory, and radiological findings consistent with HSVE and a significant clinical improvement with IV acyclovir.

Acquired demyelinating syndromes (ADS) were classified based on the IPMSSG criteria to acute disseminated encephalomyelitis (ADEM), clinically isolated syndrome (CIS), optic neuritis (ON).¹⁵ *Serial antibody titres are shown in Figure 4-3,4,5. **Median follow-up time of 30 months (Range 8-54) **Abbreviations:**

CSF cerebrospinal fluid; **HSV** herpes simplex virus; **IVIG** intravenous immunoglobulins; **IVMP** intravenous methylprednisolone; **MMF** mycophenolate mofetil; **MRI** magnetic resonance imaging; **ND** not done; **NoT** not on treatment; **NT** no treatment; **OCB** oligoclonal bands; **OP** oral prednisolone; **oT** on treatment; **PLEX** plasma exchange; **S** serum; and **R** relapse.

4.3.4 General features of Cases 41-50 and differences from the remaining 40 patients with NMDAR-Abs

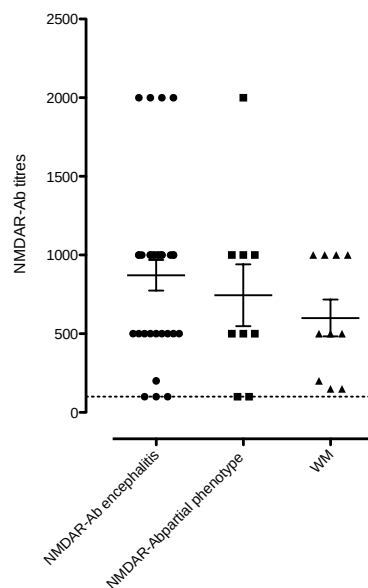
Table 4-4 summarise the key differences reported in each phenotype. Overall, six of the seven patients (85%) who were treated acutely improved following immunotherapy with steroids, IVIG and PLEX, either individually or in combination. At relapse, two patients had escalation of immunotherapy resulting in improvement of the clinical syndrome. Patients who were treated late (n=2) or untreated (n=1) had either a poorer outcome or persistence of symptoms. In 6 out of the 7 patients with serial samples available, there were relationships between NMDAR antibody titres and severity of clinical syndrome (Figure 4.6). Interestingly, MOG-Ab did not disappear after treatment in Case 49 even when NMDAR-Abs became undetectable, and after clinical recovery (Figure 4.5).

	Encephalitis (n=32)	Partial phenotype (n=8)	WM syndromes
Age (range; median)	2-17yr; 8yr	5-18yr; 11.5yr	1.3-18yr; 8.5yr
Sex (male: female)	9:23	2:6	5:5
Ethnicity (white: other)	16:16	4:4	5:5
Serum NMDAR- Ab (range; median)	1:100-1:2000; 1:1000	1:100- 1:200; 1:500	1:150- 1:1000; 1- 500
Serum MOG-Ab	0 (0%)	0 (0%)	3 (30%)
Positive CSF NMDAR-Ab	10/11 (91%)	2/3 (67%)	3/3 (100%)
CSF OCB	14/18 (78%)	1/4 (25%)	1/3 (33%)
Abnormal Brain MRI- at onset	6 (19%)	0 (0%)	7/10 (70%)
Abnormal EEG- at onset	31(97%)	5/7 (71%)	4/4 (100%)
Immunotherapy	31 (97%)	5(63%)	9 (90%)
2 ND line immunotherapy	10(31%)	0 (0%)	2 (20%)
Relapse	9 (29%)	3 (38%)	7 (70%)
Full recovery	11 (34%)	3 (38%)	4 (40%)

Table 4-4 Comparison of the different NMDAR-Ab CNS phenotypes.

Patients' demographics were similar in all groups although there female predominance was seen in patients with typical encephalitis and in the partial phenotype. Antibody levels both in the serum and CSF were similar in all groups (see also Figure 4.6). Patients with white matter involvement were more likely to have MOG-Ab. These patients were also more likely to have a second clinical relapse.

Serum levels of NMDAR-Abs ranged from 1:100 to 1:1000 at the earliest sample available, with CSF levels (Cases 42-45) from 1:20 to 1:50. Serum antibody titres across the different phenotypes are demonstrated in Figure 4-6. Importantly, both serum and CSF NMDAR-Ab titres were negative during the HSVE that preceded NMDAR-Ab relapse in Case 44 (Figure 4.4); the relevant samples were not available for Case 45. After immunotherapies, antibody levels fell in all patients and became undetectable in Cases 41, 43 and 49. Follow-up CSFs were not available.



Cases 41-50 presented with distinct white matter changes, either radiological changes compatible with the respective clinical syndromes (brainstem encephalitis and ADS group), or a leukoencephalopathy in addition to the radiological changes commonly observed within the relevant phenotype (brainstem encephalitis and HSVE). These patients were clearly distinct from the NMDAR-Ab encephalitis cases (Table 4-1), where only 6 out of 32 (19%) had abnormal neuroimaging, all sparing the white matter ($p < 0.0001$, Fisher's exact test). Cases 41-50 were also distinct clinically. Although three patients had classical NMDAR-Ab encephalitis,

in Cases 41 and 42, these episodes were remote from the first neurological syndrome (brainstem encephalitis) and in Case 45, the NMDAR-Ab encephalitis occurred following HSVE. In addition, 7/10 cases (70%) suffered from recurrent neurological episodes compared to only 8/31 (26%) patients with typical NMDAR-Ab encephalitis, or 11/39 (28%) cases without white matter involvement ($p=0.02$ and 0.03 respectively; Fisher's exact test). Finally, those patients with white matter disorders (3/10, 30%) were more likely to have MOG antibodies (30% vs. 0%; $P=0.008$ Fisher's exact test) than those without white matter involvement.

4.4 *NMDAR-Ab in post HSVE neurological relapse*

Three patients presented with neurological relapses following HSVE (of which two were reported in the leukoencephalopathy group above). Two relapsed with encephalopathy and movement disorder and the third relapsed with subtle behavioural, cognitive and motor decline.

At the identification of the first case (Case 32), the reports of post-HSVE relapses in adults and children were systematically reviewed. To delineate the aetiology and outcome of HSVE relapses, a review of the literature (Medline 1950-present) using the search terms "encephalopathy", "encephalitis", "relapse", "relapsing", "Herpes Simplex Virus", "HSV" and "Herpes" in various combinations was performed (Table 4-5). Sixty-five cases were identified from 32 manuscripts. Patients presented initially with clinical phenotype, consistent with HSVE, but at relapse a significant proportion of adults (25%) and children (22%) did not have evidence of viral reactivation. Relapse with a movement disorder was frequent (75%) in children, whereas it was not reported in any of the adults. Only eight of the childhood cases received immunotherapy and benefit was reported in four who either received more intensive (steroids and/or intravenous immunoglobulins with additional cyclophosphamide and/or Rituximab and/or extended duration (at least two months of oral steroids) of treatment, in comparison to the other four who received only immunoglobulins or short courses (<10 days) of steroids. Although an

immune mediated aetiology was likely, and some have phenotypic similarities to anti-NMDAR encephalitis, none of these cases were initially tested for NMDAR-Ab.

	Patients with relapse	Virological evidence at initial diagnosis	Time of relapse following stopping treatment	Number of cases with HSV PCR detected at relapse/number tested	Movement disorder at relapse
Case Reports (Paediatrics)	15 (7 months-18 yr)	PCR positive (n=4) Clinical diagnosis (n=11)	Day 17 - month 20	1/6	10/15
De Tiège et al 2003	6/42 (14%) (7 months-13 yr)	Intrathecal synthesis of HSV (n=5) HSV seroconversion (n=1)	Day 5 – month 26	0/6	2/6
Ito et al 2000	7 /27 (26%) (3 months-16 yr)	All CSF PCR positive	Day 1 – day 14	2/7	No data
Barthez-Carpentier et al 1995	5 (5-17 months)	4/4 tested for HSV PCR positive.	Day 23 – day 32	0/4	5/5
Wang et al 1994	3/12 (25%)	Clinical and raised serum HSV antibodies	Day 20 – day 36	No data	3/3
Gascon et al 1993	4 (3-16 months)	PCR not done. 2 out of 4 rising serum HSV titres.	Day 2 – day 14	1/2	4/4
Kimura et al 1992	4/15 (21%) (3 months-16 yr)	All had initial positive HSV PCR in the CSF.	Day 2 – day 16	2/4	No data
Pike et al 1991	3 (9-11 months)	PCR not done. HSV titres in CSF positive in 1.	Day 9 – day 33	No data	2/3
Children Total	47*	PCR positive in all tested (n=24)	Day 2 – month 26	PCR positive in 6/29** (21%)	27/36(75%)
Case Reports (Adults)	9 (28-64 yr)	PCR positive (n=3) PCR negative but HSV titres increase (n=1) Clinical diagnosis (n=5)	Day 27 – month 18	3/3	0
Sköldenberg et al 2006	4/32 (13%)	PCR positive in all	1 - 4 months from acute infection	0/4	No data
Dennett C et al 1996	5 (16-67yr)	PCR in the CSF and intrathecal synthesis of HSV antibodies	Day 56 - day 90	0/5	No data
Adult total	18	PCR positive in all tested (n=12)	Day 27 – month 18	PCR positive in 3/12 (25%)	0

Table 4-5 Summary of HSV encephalitis with neurological relapse reported in the literature.

References for adult studies (Dennett et al., 1996, Skoldenberg et al., 2006). Reference for paediatric studies (Barthez-Carpentier et al., 1995, Gascon et al., 1993, Ito et al., 2000, Kimura et al., 1992, Wang et al., 1994, De Tiege et al., 2006, Pike et al., 1991). References for adult case reports (Davis and McLaren, 1983, Dix et al., 1983, Duarte et al., 1994, Koenig et al., 1979, Kwiatkowski et al., 2012, Landau et al., 2005, Peters et al., 2010, Tyler et al., 1995, Yamada et al., 2003). References for paediatric case reports (Armangue et al., 2013, Abramson et al., 1984, Barthez et al., 1987, De Tiege et al., 2003, Baxter et al., 1997, Devrim et al., 2008, Fowler et al., 1992, Hargrave and Webb, 1998, Joos et al., 2003, Nicolaidou et al., 1993, Preiser et al., 1996, Rathi and Rathi, 2010, Shanks et al., 1991)

To evaluate the role of NMDAR-Ab in neurological relapse following HSVE, we studied seven children who relapsed following their initial presentation out of the 20 cases of HSVE studied at the Evelina Children's Hospital, ECH (London, UK; n=9) and Bicêtre Hospital, BH (Paris, France; n=11), between 2000-2012 and 2006-2012 respectively. Clinical information and neuroimaging were reviewed.

The five available sera and one cerebrospinal fluid (CSF) were tested for IgG, IgA, IgM and IgE antibodies to the NMDAR using a cell-based assay, with subtype-specific goat anti-human secondary (Chapter 2.4.1). Antibody binding to primary cultures of rat hippocampal neurons (sera 1:200) and rat brain sections (sera at 1:200, CSF at 1:10) were used to support serum antibody positivity, particularly in the absence of CSF availability. The clinical and immunological features of the seven patients with a second encephalopathic episode following HSVE are summarized in Table 4-6

The children initially presented with clinical, laboratory and radiological findings consistent with HSVE and had made a significant clinical improvement with intravenous (IV) acyclovir at 30 mg/kg (500 mg/m²) per dose 8 hourly for 21 days. Three cases (ECH Cases 2, 3 and 4) were then given long-term valaciclovir. At relapse (16-36 days after initial presentation), all patients were encephalopathic, with additional choreoathetoid movement disorder in five (71%), seizure disorders in four (57%) and cognitive regression in two (29%). Viral reactivation was identified in only one patient (14%). For the remaining six patients with no evidence of viral reactivation, a range of infective and alternative inflammatory causes were sought but not identified at the time.

Overall, serum IgG NMDAR antibodies, but not the IgA, IgM or IgE subtype, were detected in both of the two patient samples tested at relapse (BH Case 1 and BH Case 2), and in one (of three) patients tested 10 years from her initial relapse during the course of her

relapsing remitting disease (ECH Case 3); with endpoint titers of 1:540 or greater (Table 4-6). One additional CSF (ECH Case 2) was negative after relapse and no samples were available from BH Case 3. All three patients with positive NMDAR-Ab were acutely or sub-acutely symptomatic at the time of sampling, in contrast to one of three who were antibody negative. The three NMDAR-Ab positive sera showed the typical neuropil NMDAR-Ab staining of the hippocampus on rat brain tissue sections (see Figure 4-6G, H for BH Case 2) and sera from two patients (BH Case 2; and ECH Case 3) bound to the surface of live hippocampal neurons; insufficient serum was available for testing from BH Case 1. Where sera and/or CSF were sufficient, further testing of 5 patients (no samples for ECH Case 2 and BH Case 3) did not reveal antibodies to VGKC-complex (and the associated proteins LGI1, CASPR2), glycine or dopamine receptors (D1R and D2R).

Only children presenting to BH were treated empirically with immunotherapy at relapse, reflecting institutional differences in management. In all three patients with NMDAR-Ab, a convincing beneficial response was observed in BH Cases 1 and 2, and a probable early response following delayed treatment 12 years later in ECH Case 3.

	Age	Ab at onset	*Relapse (day)	Neurological syndrome at relapse	HSV PCR at relapse	NMDAR Ab at relapse	Current NMDAR Ab; time since relapse	Immunotherapy and response	Current neurological condition
ECH Case 1 (2000)	7 months M	N/A	27	Encephalopathy, seizures	Negative	N/A	Negative serum 11 years	Not treated	Residual motor and cognitive deficit
ECH Case 2 (2001)	20 months F	N/A	35	Encephalopathy, movement disorder (OFD, C, A)	Negative	N/A	Negative CSF 10 years	Not treated	Residual motor and cognitive deficit
ECH Case 3 (2001)	10 months F	N/A	40**	Encephalopathy, movement disorder (OFD, C, A), cognitive regression	Negative	N/A	Positive serum (1:1620); 10 years	IVIG – improvement in cognition, speech and seizure control	Residual motor and cognitive deficit ***
ECH Case 4 (2008)	6 months M	N/A	16**	Encephalopathy, movement disorder (OFD, C), seizures	Positive	N/A	Negative serum 4 years	Not treated	Residual motor deficit
BH Case 1 (2007)	15 years F	N/A	36	Encephalopathy, cognitive regression	Negative	Positive serum (CBA 1:540, IHC) ****	N/A	IVMP, cyclosporine – improvement with resolution of encephalopathy	Residual cognitive deficit
BH Case 2 (2010)	3 years F	Negative Serum	31	Encephalopathy, movement disorder (OFD, C, A, S), seizures	Negative	Positive serum (CBA 1:1620, HN, IHC)	Positive serum (1:1000)	IVMP, MMF, rituximab – resolution of encephalopathy and movement disorder	Residual cognitive deficit
BH Case 3 (2012)	9 months M	N/A	36	Encephalopathy, movement disorder (C, A), seizures	Negative	N/A	N/A	IVMP, PLEX, MMF – full recovery	Age appropriate development

Table 4-6 Children with relapsing neurological syndromes following HSV encephalitis.

All patients had positive HSV PCR in the CSF at onset. * Patients treated with intravenous acyclovir regime at both institutions; 30 mg/kg (500 mg/m²)/dose 8 hourly for 21 days. **Patients relapsed whilst on antiviral therapy. *** Patient continues to have intermittent episodes of cognitive regression unrelated to seizure disorder. ****Patient serum taken 12 months after relapse whilst on immunotherapy. A Athetosis Ab Antibody, C Chorea CBA Cell based assay, CSF Cerebrospinal fluid, HN Live hippocampal neuronal assay, IHC Rat brain section immunohistochemistry, OFD Orofacial dyskinesia and S stereotyped non purposeful movements. N/A denotes not available. IVIG, intravenous immunoglobulins; IVMP, intravenous methyl prednisolone; PLEX, plasma exchange, MMF, mycophenolate mofetil.

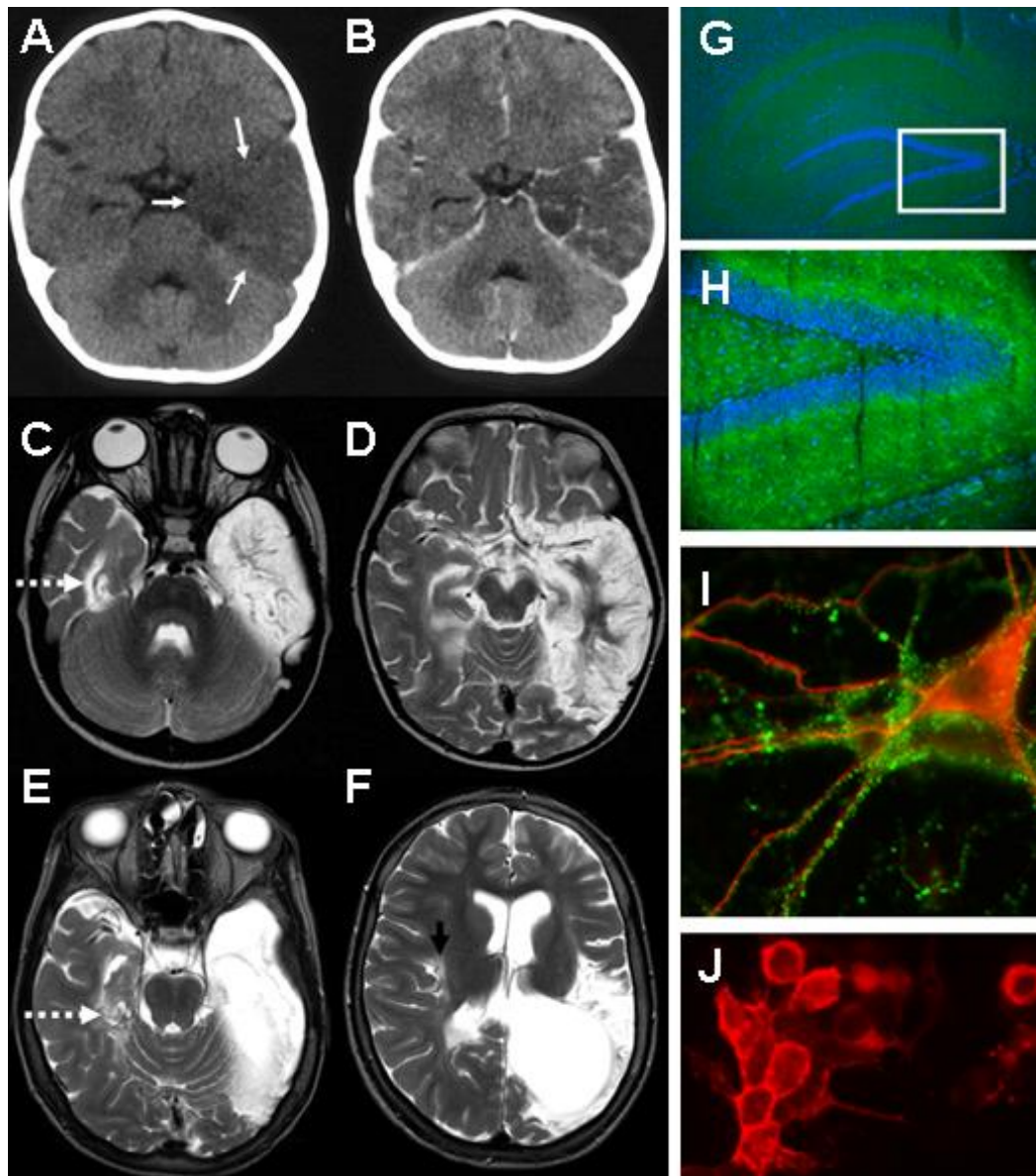


Figure 4.7 Radiological and immunological finding in HSVE neurological relapse. Neuroimaging of BH case 2 (A-F). Axial FLAIR images showing an area of parenchymal hyperintensity involving the right temporal lobe (dashed arrow, A) extending into the adjacent frontoparietal regions (dashed arrow, B) and insula (dashed arrow, C), with some early involvement in the contralateral temporal lobe. Repeat MRI on day 30 demonstrates progression of hyperintensity to involve larger regions of the left and right temporal lobes (white solid arrows, D), frontoparietal white matter (white arrows, E), and insula (white arrows, F) with ex vacuo enlargement of the left lateral ventricle and less marked encephalomalacia of the mesial temporal lobe (D, E). This characteristic limbic involvement with apparently unilateral onset and subsequent less marked contralateral limbic involvement (“sequential bilaterality”) is consistent with HSVE. Detection and characterization of NMDAR antibodies in BH case 2 (G-I). Hippocampal “neuropil” binding pattern on sagittal rat brain sections (G, $\times 10$) and the dentate gyrus shown in higher magnification (H, $\times 20$). IgG NMDAR-antibody-positive serum (green) showing surface binding to two superimposed neurons and their processes in primary cultures of live hippocampal neurons identified by the neuronal marker microtubule-associated protein 2 (MAP2, in red) (I, $\times 100$). Cell-based assay for NR1/NR2B (NMDAR) antibodies using transfected human embryonic kidney (HEK293) cells shows surface binding (red) (J, $\times 40$). Modified from Hacohen et al., *Mov Disord*.

4.5 Discussion

In this chapter a cohort of children with NMDAR-Ab and CNS disorders was described. A key observation was that 34% do not present with a polysymptomatic neurological syndrome with encephalopathy, but instead with a range of dominant symptoms often with milder additional symptoms, or a neurological syndrome with white matter involvement. This cohort had similar demographics to previous reported cohorts with female predominance and comprising only 50% Caucasians (Titulaer et al., 2013, Irani et al., 2010b). There was no significant family history of autoimmune disease. Co-occurrence of either neuronal or systemic antibodies was seen in three of the patients and positive serology for infectious organism were reported in 13; ASOT (n=5), HSV (n=3), EBV (n=2), Mycoplasma (n=2) and VZV (n=1). Only one girl had an ovarian teratoma. These observations substantially extend the range of clinical presentations and associated paraclinical findings in children with NMDAR-Ab

4.5.1 NMDAR-Ab encephalitis

The presentation of NMDAR-Ab encephalitis described here is similar to previous reports in both adults (Titulaer et al., 2013) and children (Titulaer et al., 2013, Armangue et al., 2013) with psychiatric features, seizures, cognitive decline and movement disorder most commonly seen. Although patients were identified using serum NMDAR-Ab testing, CSFs were positive in 10/11 tested. Recent reports suggesting CSF positivity in patients with negative serum antibodies, but using different methods (Gresa-Arribas et al., 2014), could have resulted in some cases not being identified, but given the serum/CSF results (Figure 4.2), this seems unlikely.

One 13 year old girl (patient 29) who was diagnosed with NMDAR-Ab encephalitis at the time of relapse, presented 7 years earlier with encephalopathy with movement disorder, psychiatric features and seizures and known to have common variable immunodeficiency

(CVID). In this condition up to 25% of patients have been reported with autoimmune complications that are often difficult to manage as the underlying immune dysregulation and the risk of overwhelming infections need to be balanced against the benefit of immunotherapies (Baldovino et al., 2013). The pathogenesis of autoimmunity in CVID is a paradox in which autoantibodies and autoreactive B-cells can be detected in patients even if serum immunoglobulins are very low and specific antigen responses are impaired (Baldovino et al., 2013).

4.5.2 Partial phenotypes

Although 66% presented with NMDAR-Ab encephalitis it is becoming clear that some patients only present with the partial/incomplete phenotype. These findings are different to a recent Spanish paediatric cohort reporting that all children (n=20) developed the “Full-blown” syndrome with movement disorder, psychiatric symptoms and language dysfunction (Armangue et al., 2013) and might reflect differences in patient selection. Amongst those who presented with a partial syndrome, five patients presented with predominantly movement disorder. These patients have similarities to other immune-mediated movement disorders such as Sydenham’s chorea and movement disorders associated with systemic autoimmune diseases. Interestingly, two of the patients (case 33 and 34) had features suggestive of lupus/antiphospholipid syndrome associated movement disorders; patients with these conditions were previously shown to have neuronal surface antibodies (Dale et al., 2011). Antibodies to the NR2 subunit of the NMDAR have been reported in some patients with SLE. Although their exact pathogenic role remains unclear, the possibility of cross-reactivity of the NR2 and the anti-DNA antibodies has also been suggested (Lapteva et al., 2006). The relatively high expression of NR1/NR2B receptors in basal ganglia (Paoletti et al., 2013) potentially explains this regional targeting of patients’ autoantibodies and could explain why

movement disorders are seen with NMDAR antibodies CNS diseases. Additionally, it is possible that the involuntary movement seen is not generated in the basal ganglia but in fact by the loss of cortical and brainstem inhibition (due to the NMDAR internalisation) resulting in release of primitive patterns of bulbar and limb movements as previously suggested (Kleinig et al., 2008).

Atypical presentation with acute cognitive decline and even “autistic-like” regression was observed in a 15 year old girl who had positive NMDAR-Ab in the serum and CSF (Case 40). At the time of her presentation the clinical relevance of the antibodies was questionable and she was not treated. Two boys aged 3 year (Scott et al., 2013) and a 9 year (Creten et al., 2011) presenting with acute autistic regression and NMDAR-Ab, without the typical characteristics of anti-NMDAR encephalitis, were treated with immunotherapy resulting in reacquisition of language and social skills. Expressive dysphasia and mutism are typically seen in patients with the full-blown encephalopathy and indeed word-finding difficulties can be a useful clue to the diagnosis as seen in five of the six patients with the partial phenotype who had a formal neuropsychology assessment. The exact mechanism is still unknown but as reduction in verbal fluency is frequently seen with NMDAR antagonists, this phenomenon is likely to be secondary to NMDAR loss of function.

4.5.3 NMDAR-Ab and white matter syndromes

Ten children were identified with NMDAR-Ab and white matter syndromes. An association between NMDAR-Ab and CNS demyelination has been recognized in individual cases with both monophasic (Hacohen et al., 2014b, Lekoubou et al., 2012) and relapsing syndromes (Kruer et al., 2010, Takeda et al., 2013) but it is unknown whether these antibodies are causative or secondary to the demyelination. As NMDARs are activated on oligodendrocytes following ischemia (Karadottir et al., 2005), the white matter may be a target for antibodies following prior or recurrent neurological insults; this could be the case in our patients who had

either a distinct previous neurological syndrome (HSVE and brainstem encephalitis) or frequent relapses of demyelination. Furthermore, upregulation of NMDAR subunits, in particular, highly calcium permeable NR2B-containing and magnesium-insensitive NR3A, appear to increase the vulnerability of the white matter to injuries in the developing brain (Jantzie et al., 2013). Age-specific NMDAR subunit composition, could also predispose children with an actively myelinating CNS to developing leukoencephalopathies; this might explain the high rate of white matter involvement in this paediatric NMDAR-Ab positive cohort. Experimental studies looking at the role of NMDARs in white matter suggest their importance in remyelination (Li et al., 2013) and the regenerating optic nerve (Kolls and Meyer, 2013), which could possibly explain the slow visual recovery seen in Cases 46 and 47; if they are induced secondary to the demyelination, the NMDAR-Ab may cause a loss of function and reduce this beneficial response to nerve injury.

NMDAR-Ab appeared to mediate an acquired white matter disorder in 3/50 patients. In these patients the neuroimaging changes were of a striking leukoencephalopathy. Two patients presented with neurological relapses following HSVE (cases 42 and 43) and these distinct leukoencephalopathic changes were also evident in BCH case 2 and in another patient in the brainstem encephalitis group (Case 44, Figure 4.3). Following immunotherapy, both HSVE patients improved clinically and, in the one patient imaged after treatment, significant radiological improvement was demonstrated. The relevance of leukoencephalopathy in these syndromes requires further evaluation; its detection by MRI could be a useful biomarker for patients at risk of relapses after HSVE or brainstem encephalitis, with obvious treatment implications.

Distinct episodes of classical NMDAR-Ab encephalitis were observed in two of the three brainstem encephalitis patients. Although the NMDAR-Ab positivity in both cases (Cases 41 and 42) could only be determined in sera taken during relapses, when they were poly-

symptomatic, the identification of NMDAR-Ab (serum and CSF) in Case 43 during the acute phase of brainstem encephalitis, suggests that the initial brainstem event in all three patients could represent a localised form of NMDAR-Ab-mediated brainstem inflammation. The early and optimal treatment in Case 43, may have prevented the subsequent evolution to a more generalised NMDAR-Ab encephalitis. Brainstem encephalitis does share clinical features with NMDAR-Ab encephalitis, and it was suggested that one of the original reported cases of Bickerstaff's brainstem encephalitis had features of NMDAR-Ab encephalitis (Merwick et al., 2013). Hypoventilation, probably due to disruption of the medullary-pontine respiratory network (Dalmau et al., 2011) and leading to ventilator dependency despite the improvement of the encephalopathy, was seen in Case 43. Typically, however, brainstem involvement in NMDAR-Ab encephalitis manifests as autonomic symptoms such as hyperthermia, blood pressure lability, arrhythmias, hypersalivation, and urinary incontinence (Dalmau et al., 2011) and is usually seen more frequently in adults than in children (Dalmau et al., 2011).

The brainstem parenchyma could be particularly susceptible to systemic autoantibodies. It is well known that antibodies can diffuse into the area postrema, as in neuromyelitis optica (Ratelade et al., 2011), and other brainstem regions may also be permeable as inferred recently from rodent studies (Chang et al., 2013). However, although brainstem encephalitis is assumed to be due to autoimmune and inflammatory aetiologies (Tan et al., 2013), no disease-defining autoantibodies have been defined to date.

There is a general interest in the role of CNS antibodies in demyelination stemming from the discovery of AQP4 antibodies in NMO (Lennon et al., 2004), and detection of MOG-Abs in a range of acquired demyelinating syndromes (Reindl et al., 2013). The clinical use of MOG-Abs as a marker of disease activity and prognosis is still being evaluated but interestingly, three of the five patients in the ADS group, two with bilateral optic neuritis (Cases 6 and 7) and one (Case 9) who presented with ADEM followed by recurrent optic neuritis

(ADEM-ON), had antibodies to MOG as well as NMDAR. Both these demyelinating phenotypes are recognized in children with MOG-Abs (Rostasy et al., 2012, Huppke et al., 2013) suggesting that these antibodies may have contributed to the clinical picture in addition to the NMDAR-Abs.

It was the leukoencephalopathy, and the less severe but distinct white matter changes, that drew our attention to these patients. In fact, advanced neuroimaging techniques are starting to identify white matter abnormalities in patients with anti-NMDAR encephalitis with no apparent abnormality on conventional imaging (Finke et al., 2013), suggesting that perturbations in white matter tracts are more widespread than previously thought. As detailed above, these could be mediated by direct antibody binding to NMDAR on oligodendrocytes and/or secondary to neuronal glutamate-mediated excitotoxicity (Paoletti et al., 2013). Although the improvement following immunotherapy in our patients with white matter disorders could simply reflect the natural history of these conditions, the poorer outcomes of those untreated or treated later, and the parallel reduction or resolution of antibody levels, provides evidence that whatever the origins and roles of the NMDAR-Abs, their removal can be helpful.

4.5.4 NMDAR-Ab as a cause of HSVE relapse

The third novel observation was the identification of NMDAR-Ab in patients who relapse following HSVE. Relapses in HSVE are not uncommon and occurred in seven of the 20 cases of HSVE in our two paediatric centres, reflecting the incidence (23%) in reviewed cases (Table 4-5). Although in some cases there is reactivation of HSV, no infective cause is identified in the majority of patients and empirical immunotherapies are often effective. Here we found NMDAR-Ab in 3/6 children who relapsed with an encephalopathic syndrome characterised variably by choreoathetosis, seizures and /or cognitive regression. The significant

improvement following immunotherapy suggests that NMDAR antibody mediates these relapses.

Identification of NMDAR-Ab post HSVE were also reported by an Australian group who additionally found D2R antibodies (with NMDAR-Ab in one child and in isolation in another) (Mohammad et al., 2014). A further prospective study (Armangue et al., 2014b) has supported our observation that NMDAR-Ab are negative at the initial presentation with HSVE.

NMDAR-Ab of immunoglobulin subtypes IgA, IgG or IgM were recently detected using a fixed CBA, in the acute samples of 13 of 44 (30%) adult patients with HSVE (Pruss et al., 2012) who responded to acyclovir and did not relapse. The phenotype in the patients was consistent with HSVE and none of the patients had a movement disorder.

The relationship between viral infections and autoimmunity in children is interesting. Inborn errors of innate immunity have been shown to be a susceptibility factor for childhood HSVE (Sancho-Shimizu et al., 2011) but have not been linked to neurological relapses or to NMDAR antibody encephalitis. In the three children with HSVE relapses who did have the antibodies, it seems very likely that the viral infection induced NMDAR-Ab which then led to a second phase of immune-mediated encephalopathy with features typical of anti-NMDAR encephalitis. Importantly, in the handful of previous cases of relapsing HSVE, when immunotherapy used was of adequate intensity and duration, a benefit was reported (Table 4-6). Although the eventual outcome may be confounded by the neurodevelopmental sequelae of the initial infective encephalitis, rather than that of the relapse, when patients relapse following HSVE in the absence of viral reactivation, an autoimmune (particularly NMDAR- Ab mediated) aetiology is an important differential, with investigative and treatment strategies needing to be directed appropriately.

4.5.5 Treatment and outcome in paediatric patients with NMDAR-Ab encephalitis

Of the patients with NMDAR-Ab encephalitis in this reported cohort (Table 4-1), nearly all patients (97%) received first-line immunotherapy but only 10/31 (32%) received 2nd line immunosuppression. A study looking at cognitive deficits following NMDAR-Ab encephalitis with comprehensive neuropsychological assessments (Finke et al., 2012), found that patients who received early immunotherapy with normalisation of antibody levels performed significantly better suggesting that good cognitive long-term outcome may depend on early and aggressive treatment. A recent large observational study of 577 patients with NMDAR-Ab encephalitis has highlighted that first line immunotherapy is often insufficient and the addition of second line immunotherapy is associated with better outcomes (Titulaer et al., 2013) which may explain the worse outcome seen in our cohort with only 35% of children recovering completely and 26% having a clinical relapse. The risk of immunosuppression particularly in children needs to be evaluated against the added benefits. Identifying individuals with a more severe disease at the onset might be an indication for a more aggressive treatment and indeed both clinical (intensive care admission) (Titulaer et al., 2013) and serological (higher Ab titres) (Gresa-Arribas et al., 2014) markers of disease severity have been suggested.

4.6 Summary and conclusion

NMDAR-Ab have been reported in both adults and children presenting with a distinct clinic-immunological syndromes. In this chapter, three new observations associated with these antibodies in children were described; partial phenotype without encephalopathy, neurological relapse following HSVE and NMDAR-Ab associated with disorders affecting the white matter specifically. Early identification of these patients and appropriate management may result in better long-term outcome and reduction of clinical relapses. Further studies are required to understand the triggers of the autoimmune response, whether the pathogenic mechanisms are

different in the new phenotypes and most importantly, whether these patients should be treated differently. The results described in this chapter were published in Hacoheh et al., 2014a, Hacoheh et al., 2014c and Hacoheh et al., 2014d.

CHAPTER 5 - Voltage Gated Potassium Channel (VGKC)-

complex antibodies in children: what do they mean?

5.1 Introduction

Voltage gated potassium channel (VGKC)-complex antibody associated CNS diseases have been described both in adults and children. In adults the most commonly associated phenotype is limbic encephalitis (Irani et al., 2010a). Patients present with seizures, amnesia, limbic changes on imaging and frequently low sodium levels. Associated neoplasm has been reported in less than 10% (usually thymomas) (Irani et al., 2010a) and patients respond well to immunotherapy, with substantial and sometimes complete recovery (Vincent et al., 2004, Irani et al., 2010a).

The voltage-gated potassium channel family include Kv1 subunits numbered through to Kv1.12 (Shaker family). The radioimmunoprecipitation assay (RIA) for VGKC-complex antibodies uses ^{125}I α -dendrotoxin (αDTX) to tag the VGKC-complexes. Three Kv1 channels (Kv1.1, 1.2 and 1.6) bind αDTX and thus the RIA only measures antibodies that immunoprecipitate one or more of these VGKC subunits. All three subunits have been shown to have CNS expression profiles, and Kv1.1 and 1.2 are also described in the peripheral nervous system (PNS) (Coleman et al., 1999, Irani et al., 2010a). These channels, which are critical in regulating neuronal excitability, form complexes with cell adhesion molecules, particularly the membrane-associated guanylate kinases (MAGUK), cytoskeletal scaffold, disintegrin and metalloproteinase 22 and 23 (ADAM22 and 23), and a soluble binding partner of ADAM22, leucine-rich, glioma inactivated 1 (LGI1) protein (Ogawa et al., 2010).

It is now recognized that the antibodies do not bind, to the VGKC subunits themselves, but to the complexed proteins (Figure 5.1). To date, many of the patients' antibodies, were shown to be directed towards three of these accessory proteins; LGI1, contactin-associated

protein-like 2 (CASPR2) and contactin-2; rather than the VGKC themselves (Figure 5.1). As a result, the term VGKC-complex was introduced to describe better the antibodies detected by radioimmunoassay (RIA), which include additional antigenic targets.

Three seminal papers described the antigenic targets in adult patients presenting with limbic encephalitis, neuromyotonia and/or Morvan syndrome with VGKC-complex Ab detected by the ²⁵I- α -dendrotoxin RIA (Irani et al., 2010a, Lai et al., 2010, Lancaster et al., 2011a). Irani et al (Irani et al., 2010a) identified an antigenic target in 78 out of 96 (82%) of patients with LGI1 seen most frequently (n= 55), followed by CASPR2 and contactin-2 but antibodies that immunoprecipitated the subunit of the Kv1s themselves were also reported in 3 patients (Irani et al., 2010a). Binding of patient's serum/CSF to LGI1 co-transfected with its receptors, ADAM22 or ADAM23, was reported in 57 patients with limbic encephalitis but antibodies from patients did not bind cells transfected with ADAM22 or ADAM23 alone (Lai et al. 2010).

The antibodies to the complexed proteins are detected by cell-based assays, similar to that in use for NMDAR-Abs (Chapter 2.4.1). Antibodies to the secretory protein LGI1 are associated with limbic encephalitis and faciobrachial dystonic seizures (often preceding the limbic encephalitis) (Irani et al., 2011), whereas CASPR2 antibodies can be found in a wider spectrum of diseases, often with peripheral phenotypes such as neuromyotonia. In rarer cases, patients' antibodies bind to more than one of the associated proteins; in Morvan's syndrome out of 29 patients, 3 had antibodies to all 3 antigens (LGI1, CASPR2, Contactin-2) and 12 patients had antibodies to both LGI1 and CASPR2 (Irani et al., 2012).

In general, in adults with neurological diseases, RIA levels >400 pM have been helpful in distinguishing autoimmune forms of encephalitis from peripheral diseases. But as more sera have been sent for testing, from a wide range of presentations, it has become clear that not all VGKC-complex RIA positivity can be accounted for by antibodies binding to the three proteins. Some patients may have antibodies targeting other extracellular proteins within the complex,

but some antibodies may bind to intracellular epitopes or intracellular proteins (Figure 5.1). A recent report of VGKC-complex antibodies, at high titre, in one patient with prion disease (Jones et al., 2014) highlights that their presence does not preclude the possibility of an alternative primary diagnosis. The aim here was to begin to explore further VGKC-complex epitopes.

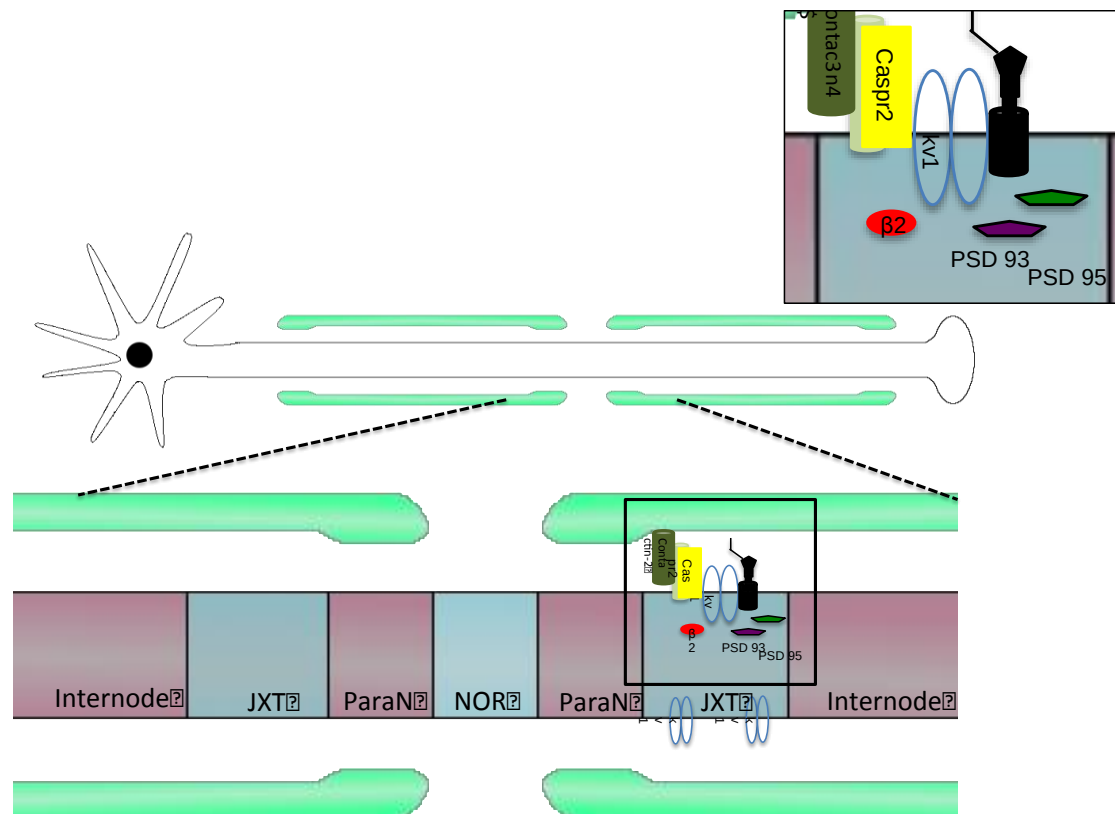


Figure 5.1 Kv1 channels protein complex at the juxtaparanodes of myelinated axons. At the juxtaparanode the cell adhesion molecules CASPR2 and contactin-2 are thought to initiate the assembly of the Kv1 channel protein complex. ADAM22 recruits PSD-95 and PSD-93.

5.2 Methods

5.2.1 Patients

From 2008-2013, 363 serum samples were sent from the Evelina Children's Hospital (241) and Great Ormond Street Children's Hospital (122) to the Clinical Neuroimmunology service at the Oxford Radcliffe Hospital Trust for VGKC-complex antibodies (Table 5-1). Patient case notes were retrospectively reviewed and patients were classified using the International Classification

of Diseases (ICD-10) into inflammatory diseases of the central and peripheral nervous system (n= 158) and non-inflammatory aetiology (n=205) (Chapter 2.1.3).

Diagnosis	Number (%)	VGKC-complex + (n=39)
Encephalopathy	88 (24%)	18 (20%)
Seizure disorder	80 (22%)	4 (5%)
Acquired demyelinating syndromes	52 (14%)	6 (12%)
Movement disorders	34 (9%)	3 (9%)
Neuroregression	32 (9%)	1 (3%)
Neuromuscular	18 (5%)	4 (22%)
Psychiatric	16 (4%)	0
Functional	13 (4%)	0
Vascular	6 (2%)	0
Pain syndromes	5 (1%)	1 (20%)
Complex motor disorders	4 (1%)	0
Sleep disorders	4 (1%)	0
Unknown	7 (2%)	1 (14%)
Other; Headaches, cranial neuropathies, cerebellar syndromes, neoplasm	8 (2%)	1 (13%)

Table 5-1 Clinical diagnosis of patients referred for VGKC-complex antibody testing. 363 requests for VGKC-complex abs testing from ECH (n=241) and GOSH (n=122) between 2007-2013

5.2.2 Autoantibody testing

VGKC-complex antibodies were measured by immunoprecipitation of rabbit brain digitonin-extracted VGKC-complexes labelled with radioactive dendrotoxin using 1µl and 5µl of serum, and the results presented as pM of ¹²⁵DTX precipitated as previously described (Vincent et al., 2004, Irani et al., 2010a) (normal values <100 pM). All sera positive for VGKC-complex Abs were tested for antibodies to LGI1 and Contactin-2 and CASPR2 using cell-based assays (CBAs, Chapter 2.4.1) as previously described (Irani et al., 2010a). Similar CBAs were used to look for binding to ADAM 22 and 23 (serum at 1:100) which are membrane proteins associated with VGKCs and LGI1 (Benarroch, 2012). Finally sera were tested for binding to the Kv subunits themselves by CBA, using cells transfected with Kv1.1, 1.2 and 1.6 (Irani et al., 2010a, Kleopa et al., 2006) and by radioimmunoprecipitation of the Kv1s subunits from digitonin extracts of these cells (described below). Monoclonal antibodies confirmed cell-surface expression of the antigens on their presence in the cell extracts in each case.

5.2.3 Protein extraction and labelling

To look for antibodies binding to the VGKCs themselves the KV1s subunit were extracted and labelled (Chapter 2.5). These extracts were then used in radioimmunoprecipitation assays as performed for the digitonin brain-extracted VGKC complexes.

5.2.4 Primary rat hippocampal and mixed neurons cultures.

To try to identify other possible antibodies within the VGKC-complex-Ab positive sera which bind the extracellular surface of live neurones, sera was tested on primary hippocampal and mix neuronal cultures (Chapter 2.7)

5.3 Results

5.3.1 Clinical diagnoses of all patients referred for testing

The clinical records for each patient sent for testing were examined and the diagnoses established. VGKC-complex Ab were identified in 39 patients (11%). Each phenotype with VGKC-complex Abs positivity is described in Table 5-1. A primary inflammatory condition was identified in 158 patients (67 males, age range 4 months- 18 years, median 8 years) and a non-inflammatory diagnosis in 205 patients (98 males, age range 2 months-18 years, median 9 years). Thirty-one of the 158 inflammatory patients had VGKC-complex antibodies compared to eight of the 204 non-inflammatory group ($p < 0.0001$, Fisher's exact) (Figure 5.2). The non-inflammatory group had significantly lower VGKC-complex titres at presentation (105-394 pM median 125 pM) compared to the inflammatory group (103-1385 pM median 169 pM) ($p = 0.02$, Man-Witney test), and whereas 11/158 inflammatory patients had titres > 400 pM, none of the 205 non-inflammatory group had titres above 400 pM ($p < 0.0001$, Fisher's exact) (Figure 5-3).

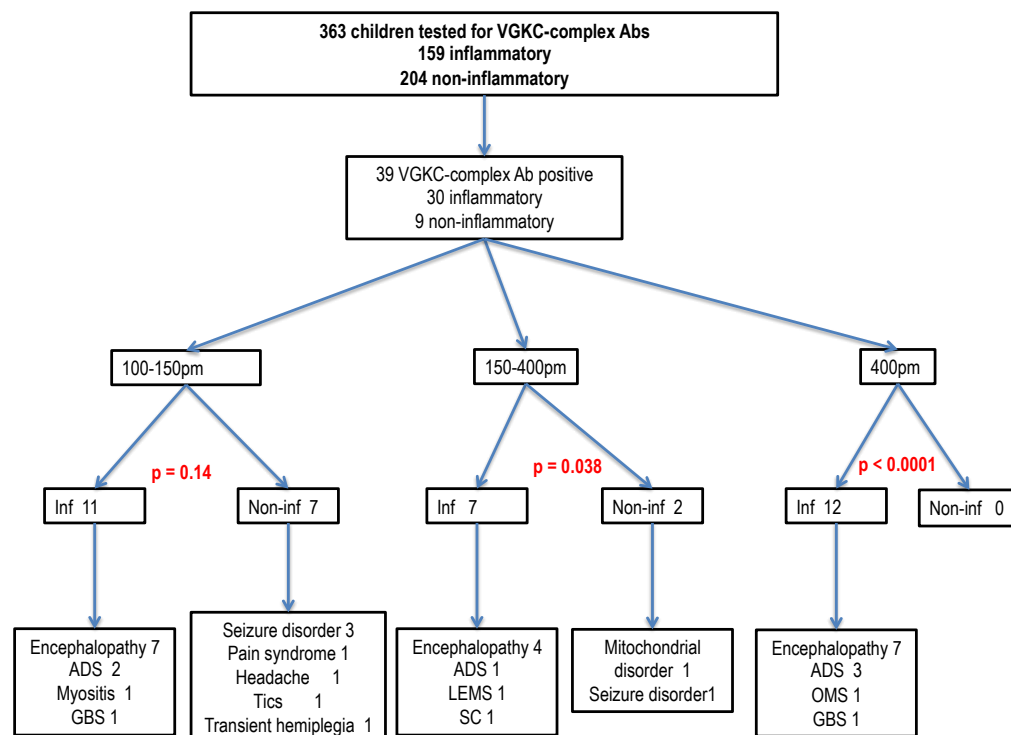


Figure 5.2 Flow chart of all patients who had serum sent for VGKC-complex Abs testing. VGKC-complex antibodies were positive in 31/159 patients with inflammatory conditions compared with only 8/204 in the non-inflammatory group. ($p < 0.0001$, Fisher's exact). This was most significant in patients with level $> 400\text{pM}$. **ADS** acquired demyelinating syndromes **GBS** Guillain-Barré syndrome **Inf** inflammatory **LEMS** Lambert–Eaton myasthenic syndrome **Non-inf** non-inflammatory **OMS** opsoclonus myoclonus syndrome **SC** Sydenham's chorea

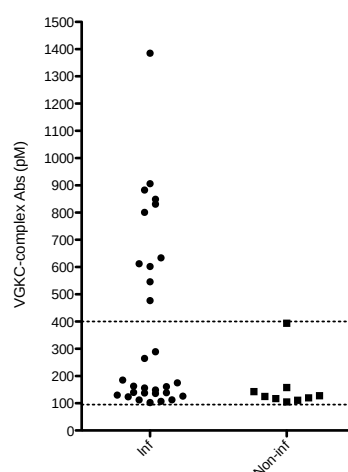


Figure 5.3 VGKC-complex Abs titres in patients with inflammatory and non-inflammatory CNS diseases.

Patients were classified using the International Classification of Diseases (ICD-10) into CNS inflammatory and non-inflammatory aetiology. 31/ 158 inflammatory patients had VGKC-complex antibodies compared to 8/204 non-inflammatory group ($p < 0.0001$, Fisher's exact). The non-inflammatory group had significantly lower VGKC-complex titres at presentation (105-394 pM median 125 pM) compared to the inflammatory group (103-1385 pM median 169 pM) ($p=0.02$, Man-Witney test).

5.3.2 Clinical phenotypes of patients with VGKC-complex antibodies

Eighteen children presented with encephalopathies (three with limbic encephalitis and one with NMDAR-Ab encephalitis). Six patients presented with acquired demyelinating syndromes (ADS). Four patients presented with autoimmune neuromuscular disorders, four with seizure disorders, three with movement disorder and four with other neurological syndromes; mitochondrial cytopathy (positive POLG), headaches, transient hemiplegia and chronic pain (Table 5-2 to 5-5). All patients were tested for antibodies in the serum and not in the CSF, which was not available. VGKC-complex antibody levels were seen at similar levels in the different phenotypes, or across the phenotypes (Figure 5.4).

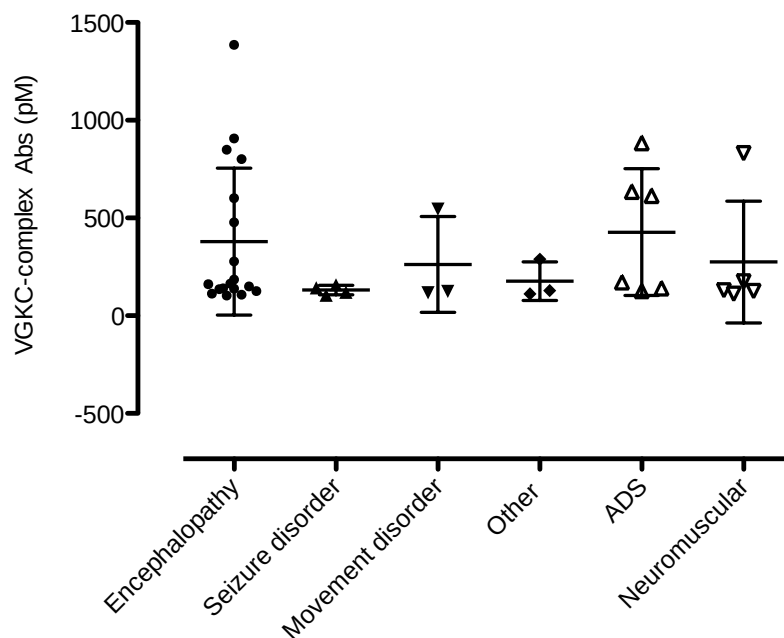


Figure 5.4 VGKC-complex Abs titres across the different clinical phenotypes.

Antibody titres were seen at similar levels in the different phenotypes, or across the phenotypes

Encephalopathy

Encephalopathy was the most frequent presentation seen in this cohort with 18 positive patients (5 Male, 9 Caucasians). Mean age at onset was 9 years (age range 2-17years; median 10 years). Antibody titres ranged from 103-1365pM (Median 162pM)(Fig 5-4). VGKC-complex

antibody positive patients represent 22% of patients with encephalopathy who had the test requested. All patients were encephalopathic with psychiatric features in 8 (44%), seizures 11 (61%) and movement disorder in 7 (39%). None of the patients had an underlying neoplasm. Brain MRI abnormalities were seen in 11/18 (61%) and CSF was normal in 14/17 (82%). Additional antibodies were present in five; NMDAR in one, GAD in one and thyroid peroxidase (TPO) in three. Three patients relapsed (one with NMDAR-Ab encephalitis). Immunotherapy was given in 11/18. Full recovery was seen in only 3/11 of the treated group and 3/7 untreated.

Acquired demyelination Syndromes (ADS)

Of the 52 patients with ADS [acute disseminated encephalomyelitis (ADEM, n=27) or clinically isolated syndromes (CIS, n=25)] who had VGKC-complex Ab tested, six patients (12%) were positive; three presented with ADEM, two with CIS-other and one patient with transverse myelitis (2 Male, 1 Caucasian). Mean age at onset was 6yr (1.3-15 years; median 4 years). Antibody titres ranged from 105-883pM (median 392pM). At follow-up (2-4 years; median 2.5 years), one patient, an 11-year-old girl who presented with CIS and VGKC-complex Ab titres of 105, was diagnosed with relapsing remitting multiple sclerosis (RRMS). Repeat testing at 3 months was available in three patients (all presented with ADEM) and was negative. This reduction in antibody titres correlated with patient's clinical recovery. Patients were managed according to their primary demyelinating phenotypes. Four patients received immunotherapy; steroids in four and one patient with ADEM received additional IVIG and PLEX.

Neuromuscular disorders

Eighteen patients with neuromuscular disorders had VGKC-complex antibodies tested; seven with demyelinating neuropathies [Guillain-Barré syndrome (GBS, n=7)], chronic inflammatory demyelinating polyneuropathy (CIDP, n=1), neuromyotonia (n=3, 2 with a genetic diagnosis), myokymia (n=2), disorders of the neuromuscular junction [myasthenia gravis (MG, n=1),

Lambert–Eaton myasthenic syndrome (LEMS, n=1)], myopathies (n=2) and one with acute myositis. Four patients (2 GBS, LEMS, myositis) were positive for VGKC-complex Ab (29%) with Ab titres of 112-881pM (median 130pM). All patients were managed with immunotherapy as indicated for their primary diagnosis. None of the patients had a second sample sent. Both patients with GBS made a complete clinical recovery. A 13-year-old girl, with known scleroderma presented with new onset leg weakness and raised creatine kinase, was diagnosed with myositis and treated with escalation of her background immunotherapy with improvement of her symptoms. The patient with LEMS had a clinical and neurophysiological phenotype consistent with LEMS and did not have an associated neoplasm.

Seizure disorders

Four children with seizure disorders had VGKC-complex antibodies identified from 78 tested (5%). Antibody titres were low, ranging from 105-158pM (Median 131pM). Patients presented with seizures and were subsequently diagnosed with focal epilepsy, idiopathic generalised epilepsy, pyridoxine dependent epilepsy and myoclonic astatic epilepsy (one for each presentation). All four patients continue to have on-going seizure.

Movement disorders

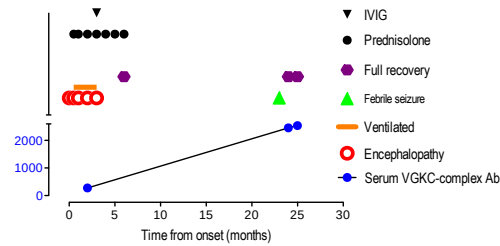
VGKC-complex antibodies were detected in 3/31 (10%) children with movement disorders tested. Antibody titres ranged from 117-546pM (Median 124pM). The patient with highest titres was a 2-year-old girl who presented with opsoclonus myoclonus (without a neuroblastoma), was also positive for NMDAR-Ab and her symptoms improved with steroids and rituximab. A 14 years old boy with motor tics and VGKC-complex antibodies of 117 was not treated and a 15 years old with known SLE (on immunotherapy) and new onset chorea, was diagnosed with

Sydenham's chorea, was treated with sodium valproate and oral antibiotics but continued to relapse on treatment.

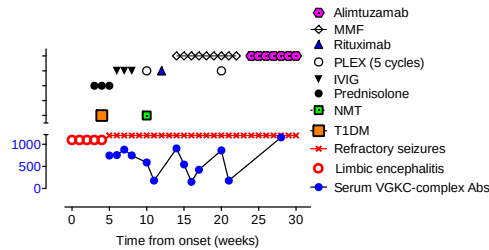
Clinical correlation with repeat samples

Repeat samples at follow-up were available in nine patients (6 encephalopathy, 3 ADS) (Figure 5.5) Serum antibodies became negative at three month follow-up in all three patients with ADEM but clinical correlation with titres was only seen in 3/6 patients with encephalopathies. There was clinical improvement with antibody reduction in two, but increase titers at relapsed in one, and persistence of symptoms and antibodies in a further patient. There were three patients in whom no temporal correlation with clinical picture and antibodies levels was seen; level increased when asymptomatic in two and persisted despite becoming asymptomatic in one.

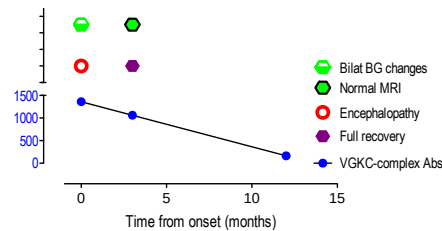
Case 19



Case 28



Case 29



Case 30

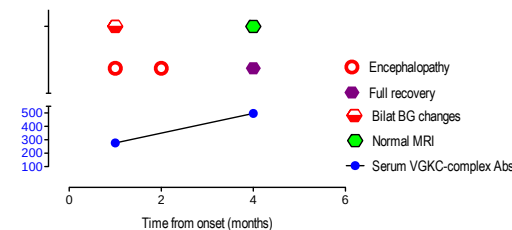


Figure 5.5 A series of graphs illustrating timeline-correlating antibody levels, clinical syndromes and therapy in informative patients with VGKC-complex Abs.

Case 19 and 29 (an 11 year old girl and a 6 year old boy) presented with encephalopathy, predominant seizures and bilateral basal ganglia changes on MRI. Both cases were not treated and made a complete clinical and radiological recovery (Hacohen et al., 2012). VGKC-complex Abs correlated with the clinical states and gradually reduced over time in Case 28 but increased at clinical recovery in Case 19. Case 28 (13-year-old female) presented with limbic encephalitis with auditory and visual hallucination and multiple seizure types. She developed neuromyotonia and insulin dependent diabetes. Despite aggressive immunotherapy she did not respond and continue to suffer from refractory seizures and gradual cognitive decline. Her VGKC-complex antibodies remained persistently elevated. Case 30 (an 11 year old male) presented with encephalopathy with prolonged seizures and was ventilated for seizure control and low GCS for 3 weeks. His VGKC-complex Abs were 277pM and he was treated with IVMP followed by oral prednisolone and made a complete recovery. Two years later he had a seizure with intercurrent illness, at the time, abs were measured at 2460pM. He was seen in clinic one month later and was completely well with no new concerns. Despite repeat Abs of 2500pM he was not treated. He has been well with no further seizures (2 years later).

5.3.3 Antibodies binding to defined components of the VGKC-complex

Only one child (Case 25) was positive for the known VGKC-complex associated proteins; the 2.8 year old with GBS without any encephalopathy who made a complete recovery. He was positive for both the associated proteins LGI1 and CASPR2 (Figure 5.6). Autoantibodies to ADAM 22 and 23 were not identified in his or any of the other 39 VGKC-complex Ab positive serum samples, and there was no detectable binding to live HEK cells expressing Kv1.1, 1.2, 1.6 either in isolation or when co-transfected with KV β 2

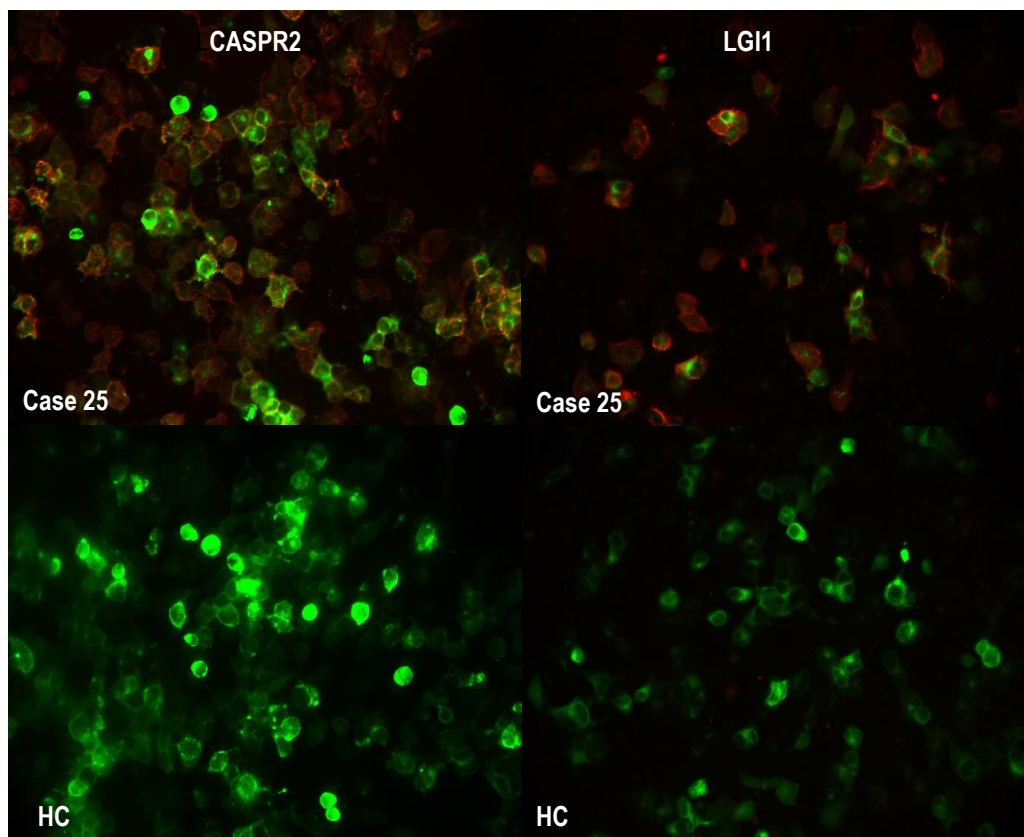


Figure 5.6 LGI1 and CASPR2 Cell based assays.

A 2-year-old boy with GBS and serum VGKC-complex Abs of 831pM. Cell-based assay for LGI1 and CASPR2 antibodies using transfected human embryonic kidney (HEK293) cells tagged with green eGFP shows surface binding (red) on both antigens.

5.3.4 Binding to Primary rat hippocampal and mixed neuronal cultures

Only four patients' serum showed binding to live neurons. Positive patients were identified with both hippocampal neurons and mixed neuronal culture. Of these four patients two were also positive for NMDAR-Ab (one with encephalopathy and one with OMS), One patient was positive for both LGI1 and CASPR2 (described above) and the fourth patient was the 13 year old girl with scleroderma who presented with acute myositis without any symptoms of CNS involvement. Binding to neurons of two positive patients is shown in Figure 5-7.

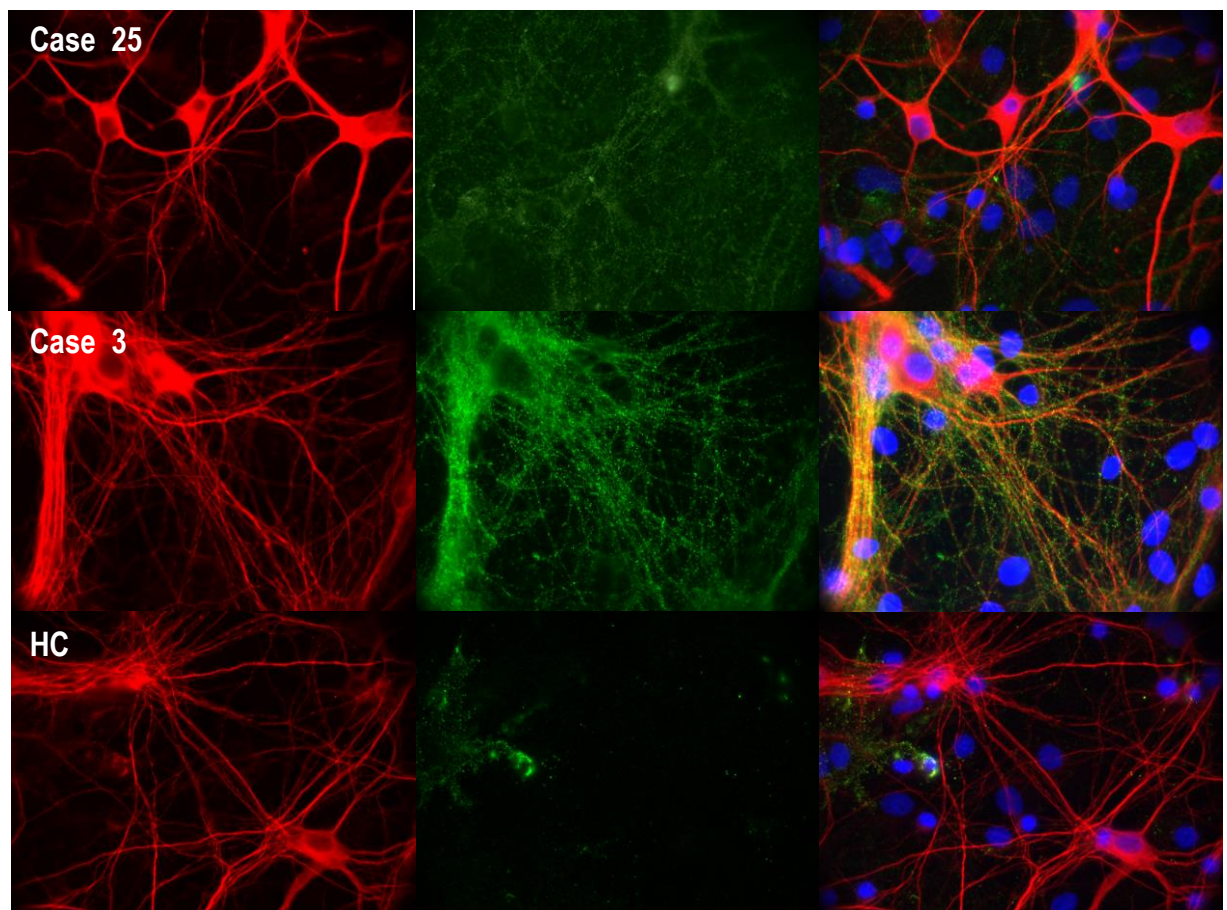


Figure 5.7 Hippocampal neurons.

Positive serum (green), but not healthy control (HC) showing surface binding to primary cultures of live hippocampal neurons identified by the neuronal marker microtubule associated protein 2 (MAP2, in red).

5.3.5 Binding to Kv1s and PSD 93 and 95 using protein labelling with ²⁵I- α -dendrotoxin measured using the radioimmunoassay

The lack of binding to Kv1 transfected cells could be explained by lack of sensitivity due to relatively poor surface expression of the Kv1 subunits, or because the antibodies that precipitate VGKC-complexes bind to intracellular determinants. Binding to fixed permeabilised cells was not informative (data not shown). To overcome this problem HEK cells were transfected with the Kv1s subunits with or without the PSD proteins. The Kv1-transfected HEK cells were solubilised in 2% digitonin and 50 μ l aliquots of the extracts labelled with ¹²⁵I- α DTX. These were then incubated with 5 or 2.5 μ l of patient sera. Commercial antibodies to Kv1.2 and β 2 immunoprecipitated ¹²⁵I-Kv1 subunits from extracts of cells expressing Kv1s plus PSD or Kv1s alone.

Seven patients were positive for Kv1s only, three were positive for the Kv1s plus PSDs and two were positive for both (both patients with ADS) (Figure 5.8). Of the 12 patients presenting with encephalopathy 5 (42%) were positive for Kv1 antibodies. The clinical and paraclinical features of patients with encephalopathy with or without KVP positivity were not different. The clinical, paraclinical and immunological phenotypes of all VGKC-complex antibodies stratified to levels below 150pM, 150-400pM and over 400pM, are summarised in Table 5-2 to 5-5.

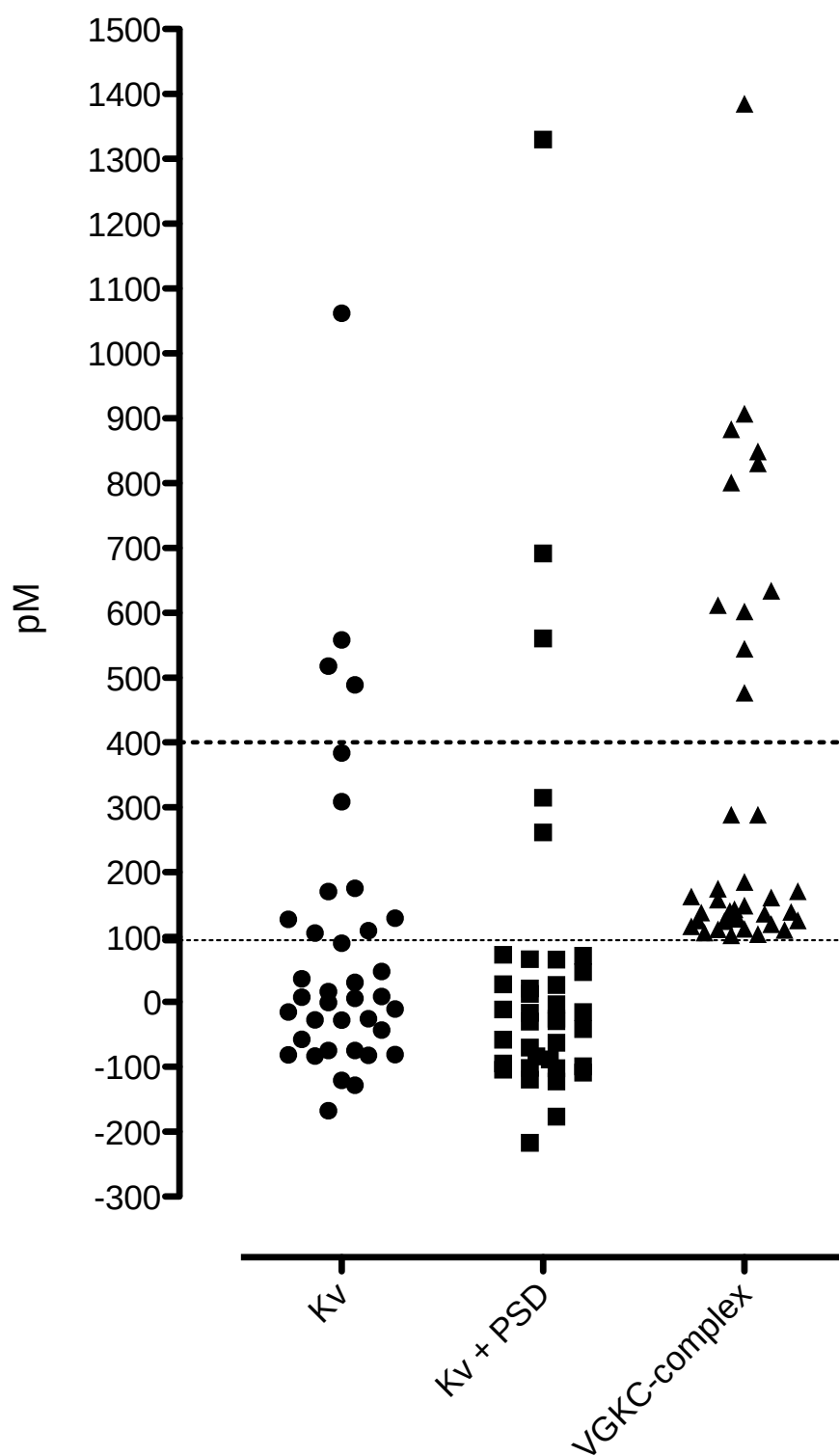


Figure 5.8 VGKC-complex Abs titres of patients with inflammatory and non-inflammatory aetiologies.

Twelve patients were positive on the KV1s extract (4 over 400pM) and 5 patients were positive on the Kv1s/PSD extract (three over 400pM).

	Demo- graphics	Presentation	Investigations	Diagnosis	Immunotherapy	Outcome	VGKC- complex Ab (pM)	
1	9yr F Asian	Encephalopathy, seizures	MRI: increase leptomeningeal enhancement CSF: WCC 15	Autoimmune encephalopathy	Not treated	Cognitive Behaviour Seizures	103	KV 558 KV+PSD negative
2	16yr F Caucasian	Encephalopathy, seizures, hallucinations	MRI: Signal change both hippocampi and amygdala, swelling of mesial temporal structures. CSF negative, TPO antibodies	Limbic encephalitis	Steroids, IVIG	Behavioural problems	107	Negative
3	13yr F Caucasian	Muscle weakness and leg cramps Background of scleroderma	Not done	Myositis.	Escalation of IT	Myositis resolved	112	KV 308 KV+PSD negative HN positive
4	2yr F Caucasian	Encephalopathy, seizures and movement disorder	MRI: cortical restricted diffusion. CSF Negative. TPO positive	Autoimmune encephalopathy	Steroid, PLEX, IVIG	Cognitive, behaviour	113	Negative
5	11yr F African Caribbean	Subacute onset weakness of both lower limbs with frequent falls	MRI: multiple high signal lesions in the brain and spine CSF: positive OCB	Clinically isolated syndrome	Steroids	Clinical relapse (relapsing remitting multiple sclerosis)	126	Negative
6	6yr M Asian	Ascending paralysis ophthalmoplegia, and ataxia	MRI brain: normal MRI spine: diffuse thickening and enhancement of ventral and dorsal roots within the cauda	GBS Miller Fisher variant	Steroids	Full recovery	130	KV 170 KV+PSD Negative
7	13yr M African Caribbean	Encephalopathy, aphasia, hallucination, parkinsonism	MRI: Normal. PET reduce uptake in the cortex CSF negative, ASOT 400, TPO Abs	Autoimmune encephalopathy	Steroids IVIG	Full recovery	136	Negative
8	10yr F African Caribbean	Encephalopathy, seizures, movement disorder	MRI: Widespread cortical signal change CSF negative NMDAR abs	NMDAR-Ab encephalitis	IVIG	Quadraparesis Visual failure Seizures	138	Negative HN positive
9	15yr F African Caribbean	Leg weakness and sensory loss	Multiple shot segment myelopathy	Transverse myelitis	Steroids	Prolonged recovery course	139	KV 262 KV+PSD 517
10	13yr F Asian	Acute confusional state with strange behaviour and hallucination and bradykinesia.	MRI: Normal CSF negative ASOT 400	Autoimmune encephalopathy	Steroids	Relapse at 8months with good response to steroids	140	KV 130 KV+PSD negative
11	3yr F Caucasian	Ataxia, urine incontinent, aphasia, seizure, hallucinations.	Reversible cerebellar signal change. OCB type 4.	Autoimmune encephalopathy	Steroids	Emotional liability, Behavioural problems	149	Negative

Table 5-2 Clinical, paraclinical and immunological features of patients with VGKC-complex Abs of 100-150pM and primary inflammatory diagnosis

ASOT antistreptococcal antibody titres **CSF** cerebrospinal fluid **GAD** Glutamate decarboxylase **HN** hippocampal neurones **IVIG** intravenous immunoglobulins **MRI** magnetic resonance imaging **NMDAR** N-methyl-D-aspartate receptor **OCB** oligoclonal bands **PET** positron emission tomography **PLEX** plasma exchange **TPO** thyroid peroxidase antibody **WCC** white cell count

	Demo- graphics	Presentation	Investigations	Diagnosis	Immunotherapy	Outcome	VGKC- complex Ab (pM)	
12	2yr F Caucasian	Encephalopathy, confusion, seizures	MRI: Bilateral hippocampi and amygdala, swelling of mesial temporal structures. CSF negative	Limbic encephalitis	Steroids	Full recovery	156	Negative
13	4yr M Caucasian	Encephalopathy, seizures, hallucinations	MRI normal CSF negative	Autoimmune encephalopathy	Steroids, IVIG, Azathioprine	On-going seizures. Cognitive	161	Negative
14	9yr F Caucasian	Confusion, seizures, nystagmus, dysmetria and ataxia	MRI normal CSF negative	Autoimmune encephalopathy	Not treated	Full recovery	163	Negative
15	15yr F Caucasian	Relapsing chorea despite treatment with Pen V and valproate Background of SLE	MRI normal CSF negative	Sydenham's chorea.	MMF for SLE	On going	175	Negative
16	16yr F Caucasian	Weakness, unsteadiness and double vision	EMG and nerve conduction study indicative of presynaptic neuromuscular junction defect	Lambert–Eaton myasthenic syndrome	Not treated	On going	175	Negative
17	12yr F Caucasian	Encephalopathy, confusional state and seizures	MRI: focal temporal cortical signal change CSF WCC 20	Limbic encephalitis	Not treated	On-going seizures and memory problems	185	Negative
18	2yr M African Caribbean	Encephalopathy with dysautonomia	MRI: bilateral signal change in the medulla and corticospinal tracts CSF negative	Acute disseminated encephalomyelitis	Steroids	Full recovery	265	KV negative KV+PSD 315

Table 5-3 Clinical, paraclinical and immunological features of patients with VGKC-complex Abs of 150-400pM and primary inflammatory diagnosis
CSF cerebrospinal fluid **IVIG** intravenous immunoglobulins **MMF** mycophenolate mofetil **MRI** magnetic resonance imaging **SLE** systemic lupus erythematosus **WCC** white cell count.

	Demo- graphics	Presentation	Investigations	Diagnosis	Immunotherapy	Outcome	VGKC- complex Ab (pM)	
19	11yr F Asian	Encephalopathy, seizures	MRI: bilateral reversible basal ganglia changes CSF negative	Autoimmune encephalopathy	Not treated	Full recovery	477	Negative
20	2yr F Caucasian	Truncal ataxia, opsoclonus, irritability, vomiting	MRI normal. OCB positive NMDAR-Ab positive	Opsoclonus myoclonus syndrome	Steroids, Rituximab	Symptoms improved	546	KV negative KV+PSD 560 HN positive
21	6yr F Caucasian	Encephalopathy seizures	MRI: left cortical, subcortical and WM changes with restricted diffusion. CSF not available	Presume viral encephalitis	Not treated	Mild right sided weakness	602	Negative
22	3yr M Caucasian	Transient episode of hemiparesis	MRI: patchy signal change within the frontoparietal deep white matter . CSF: negative	Clinically isolated syndrome	Not treated	Full recovery	612	Negative
23	14month F Asian	Encephalopathy, lethargy, sleepiness, right sided weakness of the arm and leg	MRI: left patchy pontine lesion with some minor haemorrhagic changes CSF negative	Acute disseminated encephalomyelitis	Not treated	Full recovery	634	Negative
24	12yr M African Caribbean	Acute confusional state with strange behaviour	MRI normal CSF negative	Autoimmune encephalopathy	Steroids	Full recovery	801*	KV 175 KV+PSD negative
25	3yr M Caucasian	Progressive weakness of the lower limbs with abnormal sensation, difficulty passing urine and constipation.	MRI brain: normal MRI spine: diffuse thickening and enhancement of ventral and dorsal roots within the cauda	Guillain-Barré syndrome	IVIG	Full recovery	831	CASPR2, LGI1 ar HN positive
26	7yr M Caucasian	Encephalopathy and seizures	MRI normal CSF WCC 125	Presume viral encephalitis	Not treated	Behaviour	849	Negative
27	5yr F African Caribbean	Encephalopathy, seizures, bulbar dysfunction	MRI: Widespread multifocal white matter lesions. CSF: positive OCB	Acute disseminated encephalomyelitis	Steroids, IVIG, PLEX	Full recovery	883	KV 489 KV+PSD 692
28	15yr F Asian	Encephalopathy, seizures, hallucinations	MRI: bilateral asymmetric signal change amygdala, hippocampus CSF negative. GAD Abs positive (CSF+Serum)	Limbic encephalitis	Steroids, IVIG, PLEX, MMF, rituximab	Progressive cognitive decline, uncontrolled seizures	906	KV 1062 KV+PSD negative
29	6yr M Caucasian	Encephalopathy, seizures and behavioural changes	MRI: bilateral reversible basal ganglia changes CSF negative	Autoimmune encephalopathy	Not treated	Full recovery	1385	Negative
30	11yr M Caucasian	Encephalopathy, seizures, psych	MRI normal CSF negative. TPO Abs	Autoimmune encephalopathy	Steroids	Full recovery. **	2500**	KV 107** KV+PSD negative

Table 5-4 Clinical, paraclinical and immunological features of patients with VGKC-complex Abs of > 400pM and primary inflammatory diagnosis

CSF cerebrospinal fluid **GAD** Glutamate decarboxylase **HN** hippocampal neurones **IVIG** intravenous immunoglobulins **MMF** mycophenolate mofetil **MRI** magnetic resonance imaging **NMDAR** N-methyl-D-aspartate receptor **OCB** oligoclonal bands **PLEX** plasma exchange **WCC** white cell count

	Demo- graphics	Presentation	Investigations	Diagnosis	Immunotherapy	Outcome	VGKC- complex (pM)	
1	9yr M Caucasian	Focal epilepsy with worsening seizures.	MRI: Left hippocampal sclerosis CSF not done	Focal epilepsy	Steroids	Good response to steroids	105	Negative
2	3yr M Caucasian	Transient Hemiplegia	MRI brain Normal	Undiagnosed	Not treated	Full recovery	111	Negative
3	14yr M Asian	Motor tics	Not investigated	Tic disorder	Not treated	On-going symptoms	117	Negative
4	8yr M Caucasian	Long standing seizure disorder	MRI normal CSF not done	Primary generalized epilepsy	Not treated	On-going seizures	120	Negative
5	16yr F Caucasian	Bilateral lower limb pain. Long standing Hypothyroidism	MRI brain and spine normal. NCS and EMG normal	Undiagnosed	Not treated	On-going symptoms	125	KV negative KV+PSD 1131
6	10yr M Caucasian	Headaches, history of allergies	MRI brain normal	Headaches	Not treated	Full recovery	128	Negative
7	4yr F African Caribbean	New onset myoclonic seizures	MRI normal CSF negative	Myoclonic astatic epilepsy	Not treated	On-going seizures. Developmental concerns	143	Negative
8	9yr M Asian	Encephalopathy with worsening seizures. Background learning difficulties	MRI: Bilateral grey matter heterotopia. CSF negative	Pyridoxine- dependent epilepsy (compound heterozygote mutation in ALDH7A1)	Not treated	On-going seizures	158	Negative
9	14month M Caucasian	Encephalopathy with prolonged seizures	MRI: Cortical swelling	Mitochondrial disorder with POLG mutation	Not treated	Patient died	394	Negative

Table 5-5 Clinical, paraclinical and immunological features of patients with VGKC-complex Abs and primary non- inflammatory diagnosis.
CSF cerebrospinal fluid **MRI** magnetic resonance imaging **POLG** polymerase gama

5.4 Discussion

In this large multicentre study of all children tested for VGKC-complex antibodies from two centres, we evaluated the relevance of these antibodies in clinical practice. We identified 39 positives from 363 (10.7%) patients samples, but only 11 had values >400 pM which has been established in previous studies (Vincent et al., 2004, Paterson et al., 2013) as a better cut-off for identifying CNS diseases. These VGKC-complex antibodies >400 pM were only present patients with inflammatory diseases as defined by ICD-10. Two of these 11 sera bound to hippocampal neurons; one with antibodies to both the VGKC-complex associated proteins LGI1 and CASPR2 and one with NMDAR-Ab encephalitis. However, seven additional sera Immunoprecipitated Kv1s subunits extracted from HEK cells, with or without the VGKC clustering proteins PSD 93 and 95. This suggests that even some of the high VGKC-complex antibodies may be binding to intracellular epitopes that are exposed only in detergent extracts, although given the different sensitivities of the RIA and CBAs, this needs to be confirmed in further studies.

The positivity was almost double than reported in a similar London based study done in adults patients which identified 5.2% positivity ($p=0.0005$, Fisher's exact) but these were not significantly different when comparing patients with high levels (>400pm) (2.1% vs. 3.3%) (Paterson et al., 2013). In contrast to the nine out of 52 (17%) patients who were found positive for either LGI1 or CASPR2 in the adult study (Paterson et al., 2013), we only identified 1/39 (3%) patient who was positive for both ($p=0.039$, Fisher's exact). The low rates of positivity to the associated proteins, seen both in our cohort and the adult UK cohort (Paterson et al., 2013), are consistent with a large US study, which only detected LGI1/CASPR2 in 26% of the patients (Klein et al., 2013) suggesting that other VGKC complex associated antigenic targets remain to be discovered. Nevertheless, as only four out of our 39 positive patients demonstrated binding to the surface of neurons (of which three were antigen positive), it is

possible that the VGKC-complex positivity reflects binding to intracellular proteins.

We identified serum binding to the potassium channel subunits themselves with or without the PSD proteins in 12 patients (31%). All patients were negative when tested on live CBAs transfected with the specific subunits, either in isolation or combined, suggesting that the RIA identifies binding to the intracellular domains of these proteins. It is not yet clear what the significance of these antibodies, and whether other components of the complex may also be involved. Five of the 18 patients with encephalopathies were positive for binding to the Kv1s subunits, although surprisingly these patients were negative for the Kv1s/PSD RIA. It is possible that the PSD proteins influence the Kv1s subunit composition and reduce the sensitivity of the assay or block the access of the antibodies to the target Kv1 subunit.

The differences in titres between the inflammatory and non-inflammatory groups raise questions regarding the sensitivity and specificity of the assay. Previous reports indicated that >400pM was helpful in distinguishing positive adults patients with CNS diseases, comparing with older controls. However, a study done by a different laboratory identified antigen positivity (LGI1+/CASPR2) in 22/109 patients with low values (Klein et al., 2013). A similar study on children has not been done.

The 39 positive patients presented with a range of clinical syndrome of which children with encephalopathy represented 46% of the cohort. These children presented with a range of presentations (vs. children with NMDAR who present with a well-characterised encephalopathy) (Table 5-2), not all showing a clear response to immunotherapy and antibody titres did not correlate with the clinical state in three out of the six patients in which serial samples were available.

In addition we identified VGKC-complex antibodies in both inflammatory CNS and PNS disorders such as central and peripheral demyelination. VGKC are found in the juxtaparanode of myelinated axons and it is possible that the antibodies simply reflect a secondary response

to demyelination. Interestingly, binding to the Kv1s subunits, only when transfected with the axoglial apparatus proteins PSD-93 and 95, was detected in 3/6 patients with demyelination but in only two of the other 33 positive patients ($p=0.019$, Fisher's exact). PSD-93 and 95 are part of the intracellular membrane-associated guanylate kinases (MAGUKs), bind the C-terminal consensus sequence known as the PDZ-binding domain of Kv1, and are important for clustering of Kv1 at the juxtaparanodes (Ogawa et al., 2010). In fact, axoglial proteins were enriched in the CSF of paediatric patients with acquired demyelination syndromes, 41 fold more than controls (Dhaunchak et al., 2012), which may represent immune targeting of these molecules.

Serum antibodies against axoglial proteins contactin-2 have been reported in 8% of patients with multiple sclerosis who were negative for the VGKC-complex Ab but their presence was not associated with a particular clinical-radiological phenotype (Boronat et al., 2012). Positivity of the associated proteins with negative VGKC-complex antibodies using the RIA was also reported in patients with cerebellar ataxia and CASPR2 antibodies (Becker et al., 2012). As the aim of this study was to identify the binding site of VGKC-complex antibodies, we did not screen all 363 for the complex associated proteins or live neurons. Nevertheless, when we previously tested children presenting with autoimmune encephalopathy ($n=48$)(Hacohen et al., 2013) and ADS ($n=64$)(Hacohen et al., 2014b) they were all negative for LGI1, CASPR2 and Contactin-2.

Owing to the wide range of paediatric clinical presentations associated with VGKC-complex antibodies they are now tested routinely as part of the investigation protocol for suspected inflammatory disorders. Particularly in paediatrics, the early neurological features of inflammatory disorders can be indistinguishable from a range of other “non-inflammatory” disorders and antibody testing was done on patients who were subsequently diagnosed with a range of these conditions, as reflected here. This has allowed us to also look at all patients who

were VGKC-complex negative. In very selected cohorts and isolated case reports these antibodies were reported in disorders such as status epilepticus (Suleiman et al., 2011a), fever-induced refractory epileptic encephalopathy in school-age children (FIREs), (Illingworth et al., 2011), seizures and basal ganglia changes (Hacohen et al., 2012) and focal epilepsies (Suleiman et al., 2013). Here only low-levels VGKC-complex antibodies (105-158pM) were identified in four of the 72 patients with seizure disorders (5.6%). We did not detect the antibodies in developmental and autistic regressions (n=27), primary psychiatric disorders (n=15) or functional illness (n=14).

Co-occurrence of other antibodies was seen in 6 of the patients (15%), with additional 2 patients suffering from long-term systemic autoimmune disease (SLE, scleroderma). This was also noticed in one paediatric (Dhamija et al., 2011) and one adult study (Klein et al., 2013). It remains unclear if this is sign of an underlying immune dysregulation and susceptibility to autoimmune diseases, or simply reflects an increased risk for detection of low positive antibodies using the RIA.

5.5 *Summary and conclusion*

In this chapter a cohort of children with VGKC-complex Abs was evaluated. Positivity of VGKC-complex antibodies did not indicate a specific clinical syndrome in this cohort and cannot be used reliably to monitor disease progression. The majority of the children were negative for the associated proteins (LGI1 and CASPR2) and did not bind the surface of live hippocampal neurones. In a proportion, the positivity using the radioimmunoassay was thought to indicate binding to the intracellular domains of channel proteins. Nevertheless, as these antibodies were detected predominately (but not exclusively) in primary inflammatory disorders they can be a useful biomarker of inflammation

CHAPTER 6 - Paediatric autoimmune encephalopathies: alternative antigenic targets in NMDAR antibody negative patients.

6.1 Introduction

Autoimmune encephalopathies in children are now well recognised with antibodies to the NMDAR most frequently seen (Armangue et al., 2012, Hacoheh et al., 2013). However, the majority of children with acquired encephalopathy are negative for known autoantibodies to neuronal surface receptors or synaptic proteins (Hacoheh et al., 2013). Nevertheless, an autoimmune aetiology is suspected, as outlined in chapter 3, since antibody positive and negative patients cannot be distinguished clinically and both seropositive and seronegative patients respond to immunotherapies. Specifically, 16 of 17(94%) seronegative patients and 16 of 17 (94%) seropositive children demonstrated clinical improvement with treatment. Therefore alternative antigenic targets should be explored in seronegative encephalopathy patients.

To identify patients for analysis, it is important to distinguish children with presumed autoimmune aetiology from those with infectious, para-infectious, metabolic, genetic, traumatic, malignant and toxic disorders. As shown in Chapter 3, the co-existence of encephalopathy, movement disorder and psychiatric features distinguished children with putative autoimmune encephalopathy from children with infectious encephalitis. Whilst response to immunotherapy, particularly the movement disorder component, added confidence to the diagnosis of autoimmune encephalopathy, an antibody biomarker would provide more concrete evidence.

Movement disorders implicate basal ganglia structures and psychiatric symptoms are a hallmark of disorders of dopaminergic pathways. The term basal ganglia encephalitis has been ascribed to these patients (Dale and Brilot, 2012). These patients have clinical and/or radiological features of basal ganglia involvement and present with a range of movement

disorders (either in isolation or in combination) such as parkinsonism, dystonia and chorea. They often resemble patients with the older description of encephalitis lethargica, still used frequently among paediatricians, and, of note, 10 of 20 patients with encephalitis lethargica were NMDAR-Ab positive (Dale et al. 2009).

In order to identify a pathogenic autoantibody that may cause basal ganglia encephalitis, a rational approach is to test proteins that are known to have an important function in the basal ganglia, and that have extracellular domains that would be accessible to antibodies present in the serum. As dopamine is a key neurotransmitter in the basal ganglia, identifying antibodies to the extracellular domain of the dopamine receptors D1R and D2R would be a natural choice. Indeed, Dale et al (2012) looked for serum antibodies to the dopamine receptors (D1R, D2R, D3R and D5R) and the dopamine transporter (DAT) and identified antibodies to the extracellular D2R in 12 of the 17 children with basal ganglia encephalitis and none of 67 paediatric controls. None of the patients had autoantibodies to the dopamine receptors 1, 3, or 5 or the dopamine transporter. Although the pathogenicity of these antibodies has not yet been demonstrated, immunolabelling using positive serum was markedly reduced in brain tissue of D2R knockout mice compared to mice with normal D2R expression, supporting D2R specificity.

Rather than searching for candidate antigens, an alternative approach for antigen identification is testing patient serum or CSF for binding to brain sections and/or neuronal cultures. These methods are useful in identifying potential novel autoantigens. If positive binding is observed, the antigen can then be immunoprecipitated from brain tissue or neuronal cultures by the patient's serum IgG. The immunoprecipitate can be analysed by SDS PAGE gel electrophoresis and mass spectrometry to identify one or more specific antigens. Both candidate and antigen discovery methods have been successfully used in the past (Becker et al. 2012; Lai et al. 2010; Littleton et al. 2009).

6.2 *Methods*

6.2.1 Patients - Basal ganglia encephalitis cohort

Basal ganglia encephalitis was defined as encephalopathy associated with predominant basal ganglia symptoms (movement, psychiatric or sleep disorders), and/or neuroimaging findings of basal ganglia abnormalities (Dale et al. 2012). In view of the phenotypic similarity between basal ganglia encephalitis and NMDAR-Ab encephalitis (Dale et al. 2009) patients were identified from the NMDAR-Ab referrals (Chapter 2.1.4). Patient case notes were retrospectively reviewed and 25 patients fulfilled the inclusion criteria proposed by Dale and Brilot (2012) (11 male, 12 Caucasians, age range 2-15, median 8 years). Of the 25, 22 had a movement disorder; 10 dyskinesia (seven orofacial dyskinesia), eight dystonia (two dystonic tremor), four parkinsonism, three chorea and two focal myoclonus. Psychiatric features were seen in 15 patients; 10 psychosis or hallucinations, six agitations, four emotional lability, one compulsive touching and one aggression. Sixteen patients had seizures (64%) and sleep disorders were seen in eight (32%) patients. MRI abnormalities were seen in 14 (56%), predominantly subtle, non-enhancing lesions, in both the grey and the white matter, which were typically transient. CSF lymphocytosis was seen in two (8%) with CSF OCB detected in seven of the 17 tested (41%). Immunotherapy was given in 17; five patients relapsed and nine patients made a full recovery. A comparison between patients with basal ganglia encephalitis and patients with NMDAR-Ab encephalitis (Chapter 4) is shown in Table 6-1. Clinical and paraclinical features of patients with basal ganglia encephalitis are summarised in Table 6-2

Previous testing had been negative for autoantibodies to GlyR, LGI1, contactin-2, AMPAR and GABA_bR. Seven patients were positive for the VGKC-complex antibodies, but as discussed in Chapter 5, VGKC-complex positivity (when the associated proteins are negative) suggests that another antigenic target might be clinically relevant. All patients were empirically investigated, to exclude a range of infective, alternative inflammatory and neurometabolic

aetiology, of which none were identified. Twenty-five patients with Tourette's syndrome, 25 healthy children, 25 NMDAR-Ab positive children, 25 adults with neuromyelitis optica (NMO) served as controls.

	NMDAR (n=31)	Basal ganglia (n=25)	p value (Fisher's exact)
Age Median (range)	8yr (2-17)	8yr (2-15)	NS
Sex M:F	9:22	11:14	NS
Caucasians	15	12	NS
Movement disorder	20 (65%)	22 (85%)	NS
Psychiatric	28 (90%),	15 (60%)	NS
Seizures	25 (81%),	17 (64%)	NS
Sleep	10 (32%)	8 (32%)	NS
MRI	6 (19%)	14 (56%)	0.006
CSF oligoclonal bands	14/18 (78%)	7/17 (41%)	0.04
Immunotherapy	30 (97%)	18 (72%)	0.02
Relapse	8 (26%)	5 (20%)	NS
Full recovery	11 (35%)	9 (36%)	NS

Table 6-1 Comparison between patients with NMDAR-Ab encephalitis and patients with basal ganglia encephalitis.

Both incidence and patients' demographics were similar in both groups. Patients with basal ganglia encephalitis were more likely to have abnormal MRI, less likely to have CSF oligoclonal bands. Immunotherapies were given to more patients with NMDAR-Ab encephalitis.

	Demo-graphics	Presentation	Other positives	Relapse	CSF	Brain MRI	IT	Outcome
1	10yr M Asian	E, S, C, M (chorea, OFD), Psych, sleep	H1N1 positive	No	WCC 16 No OCB	Normal	IVIG	C, S
2	5yr F Caucasian	E, C, M (OFD, dystonia), Sleep (insomnia)	H1N1 positive	No	No cells/org No OCB	Normal	IVIG	F
3	3yr M Middle eastern	E, S, M (dystonia)		No	No cells/org OCB type 2	Normal	NT	C, S
4	9yr F Afro Caribbean	E, S, C, M (chorea, dystonia), Psych, Sleep	ABGA	R1 (7yr): E, S, Psych	No cells/org OCB type 2	Acute: bilateral striatal necrosis. R1: Signal change, pons, midbrain and basal ganglia.	NT	C, B
5	7yr M Caucasian	E, S, C, M (dyskinesia), Sleep	ASOT	No	No cells/org	Reversible WM and subcortical changes	Steroids	B, S
6	3yr F Asian	E, C, Ataxia, M (Dystonic tremor)		No	No cells/org	Normal	Steroids IVIG, MMF	C
7	8yr F Caucasian	E, C, S, M (dystonic tremor)		No	No cells/org OCB type 2	Normal	Steroids IVIG	B, Tremor
8	13yr F Asian	E, S, Psych (OFD, dystonia)	VGKC-complex GAD ESR 157	Multiple relapses	No cells/org OCB type 2	Reversible limbic changes	Steroids IVIG, PLEX Ritux, MMF	C, B, S
9	10yr M Asian	E, S, M (OFD, dystonia)	Raised CK	No	No cells/org No OCB	D1 Normal. D14 limbic changes bilaterally	Steroids	C, S, M
10	5yr F Caucasian	E, S, Psych, M (dyskinesia),sleep		No	No cells/org	Normal	Steroids	F
11	10yr M Asian	E, S, M (OFD, startle), A	Mycoplasma	Yes	WCC 57 OCB type 3	Reversible WM and cortical signal changes	Steroids/IVIG	F
12	6yr M Caucasian	E, S, Psych		No	No cells/org	Reversible bilateral basal ganglia changes	NT	F
13	11yr M Caucasian	E, Psych, M (dystonia, parkinsonism)		No	No cells/org OCB type 2	Reversible WM and cortical signal changes	IVIG	B
14	3yr F Caucasian	E, S, C, Psych, M (OFD), sleep.	VGKC-complex	No	No cells/org OCB type 4	Reversible cerebellar signal change	Steroids	B
15	2yr F Caucasian	E, S, Psych, M (dyskinesia), sleep	VGKC-complex ESR 87	Yes	No cells/org No OCB	Reversible WM and cortical signal changes	Steroids PLEX MMF	C, S
16	13yr F Asian)	E, C, Psych, M (parkinsonism)	VGKC-complex	Yes	No cells/org No OCB	Non-specific WM changes	Steroids	F
17	13yr M	E, Psych, M (parkinsonism), A	TPO	No	No cells/org	Normal	Steroids	F

	Afro Caribbean		VGKC-complex				IVIG PLEX	
18	11yr F Asian	E, S	VGKC-complex	No	No cells/org	Reversible bilateral basal ganglia changes	NT	F
19	10yr F Caucasian	E, S, M (dystonia, focal myoclonus)		No	No cells/org	Reversible bithalamic signal changes	NT	B
20	3yr F Afro Caribbean	E, C, Psych, S, M (OFD), sleep		Yes	No cells/org No OCB	Reversible cerebellar signal changes	Steroids	C, B
21	4yr M Caucasian	E, C, M (bradykinesia)	Influenza B	No	No cells/org No OCB	Normal	NT	C, B
22	2yr M Caucasian	E, S		No	No cells/org No OCB	Reversible bilateral basal ganglia changes	NT	F
23	6yr F Asian	E, S, Psych, M (OFD)		No	No cells/org	Normal	Steroids	C, B
24	5yr F Caucasian	E, M (chorea)		Yes	No cells/org OCB type 2	Normal	Steroids	C, B
25	15yr F Asian	E, Psych, M (Parkinsonism) Known SLE	ANA, dsDNA	No	No cells/org OCB type 2	Normal	Steroids*	F

Table 6-2 Clinical and paraclinical features in patients with basal ganglia encephalitis.

Symptoms at presentation in addition to encephalopathy, and at final follow-up were classified as; A autonomic, B behavioural, Cog cognitive, M movement disorder, Psych psychiatric, S seizures. Abbreviations: ANA Antinuclear antibodies; AZA Azathioprine; C cerebrospinal fluid; CYC Cyclophosphamide; dsDNA double stranded DNA; IVIG intravenous immunoglobulins; MMF mycophenolate mofetil, MRI magnetic resonance imaging; OCB oligoclonal bands; OFD orofacial dyskinesia PLEX plasma exchange; PVL periventricular leukomalacia; ND not done; NT not treated; RTX Rituximab; S serum.

6.3 Cloning and expression of human D1R and D2R

Both full-length human dopamine receptors were individually subcloned from image clones into the mammalian expression vector pcDNA-DEST40. Monoclonal antibodies (anti-D1 dopamine receptor produced in rat, Sigma-Aldrich D2944; anti-DRD2 antibody produced in mouse, WH0001813M1 SIGMA), confirmed good surface expression of the antigens. (Figure 6.1). In order to create a vector suitable for a FACS analysis of antibody binding, D2R was subcloned into the pIRES2-dsRed2 vector (supervised by Dr Patrick Waters, Chapter 2.8). Figure 6.2 highlights the key steps during the cloning process.

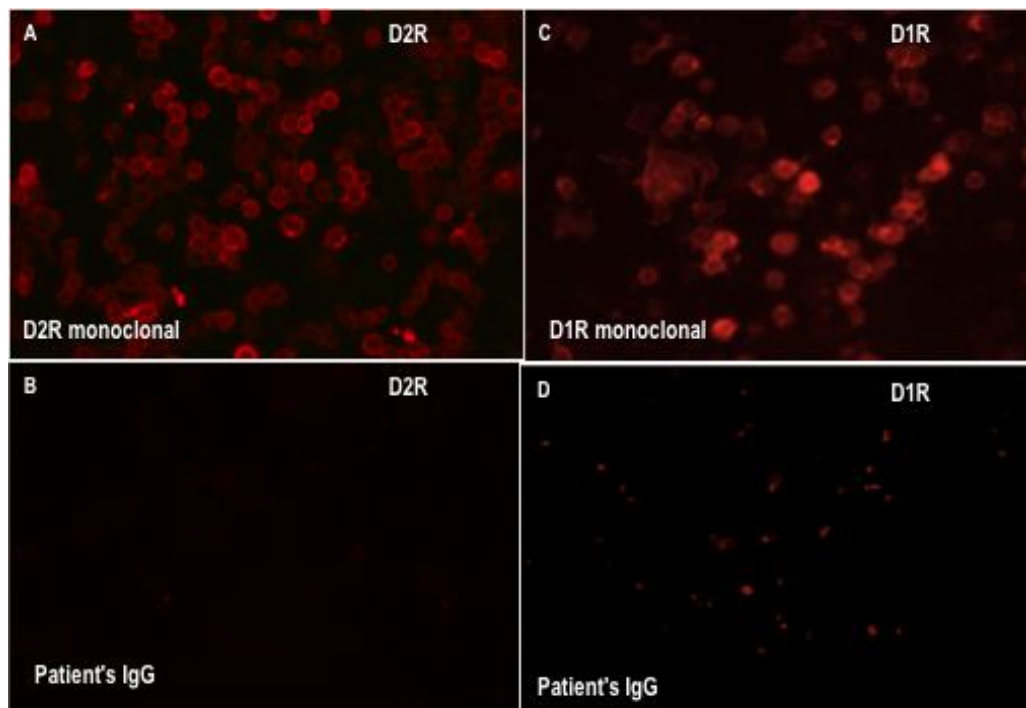


Figure 6.1 Dopamine receptor antibodies; D1R, D2R cell-based assays.

Monoclonal antibody Anti-DRD2 antibody bind to the cell surface (A) but not the patient's serum (B). The live D1R expressing HEK cells did not bind to the monoclonal anti-D1R, despite being raised against an extracellular epitope (not shown). However, when cells were permeabilised binding was seen with the monoclonal antibody (C) but not with patient's serum (D).

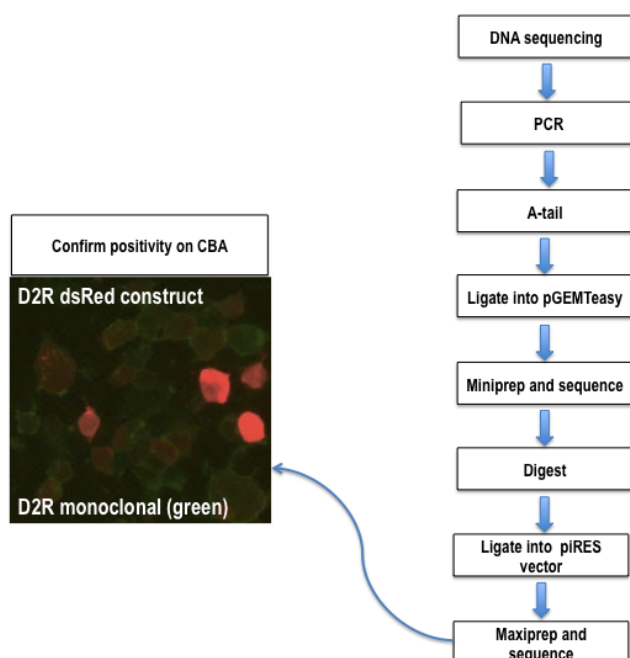


Figure 6.2 Flow diagram with molecular genetics steps undertaken for D2R cloning.

6.4 Cell-based assays for detection of antibodies to cell surface D1R and D2R.

For these cell-based assays, the binding of serum IgG to the surface of human embryonic kidney cells, transfected with cDNA encoding D1R and D2R, was followed by fluorescence-labelled secondary antibody. Binding can be detected using quantitative flow cytometry (FACS, see methods) or visual observation of the binding (Chapter 2.4). In addition to the construct, D2R subcloned into pIRES2-GFP vector (RD-D2R) with a D2R positive serum sample (Dale et al. 2012) (kind gift from Prof R. Dale, Sydney, Australia) was used. Comparison between the D2R constructs (PW-D2R vs. RD-D2R) did not reveal obvious differences in surface expression using the commercial D2R antibody (Figure 6.3). Although the D2R monoclonal antibody confirmed good surface expression (Figure 6.3), the positive patient control (positive patient from Prof Dale) did not appear positive with either construct (Figure 6.4)- nor could we confirm D2R positivity using the FACS analysis (Figure 6.5 and 6).

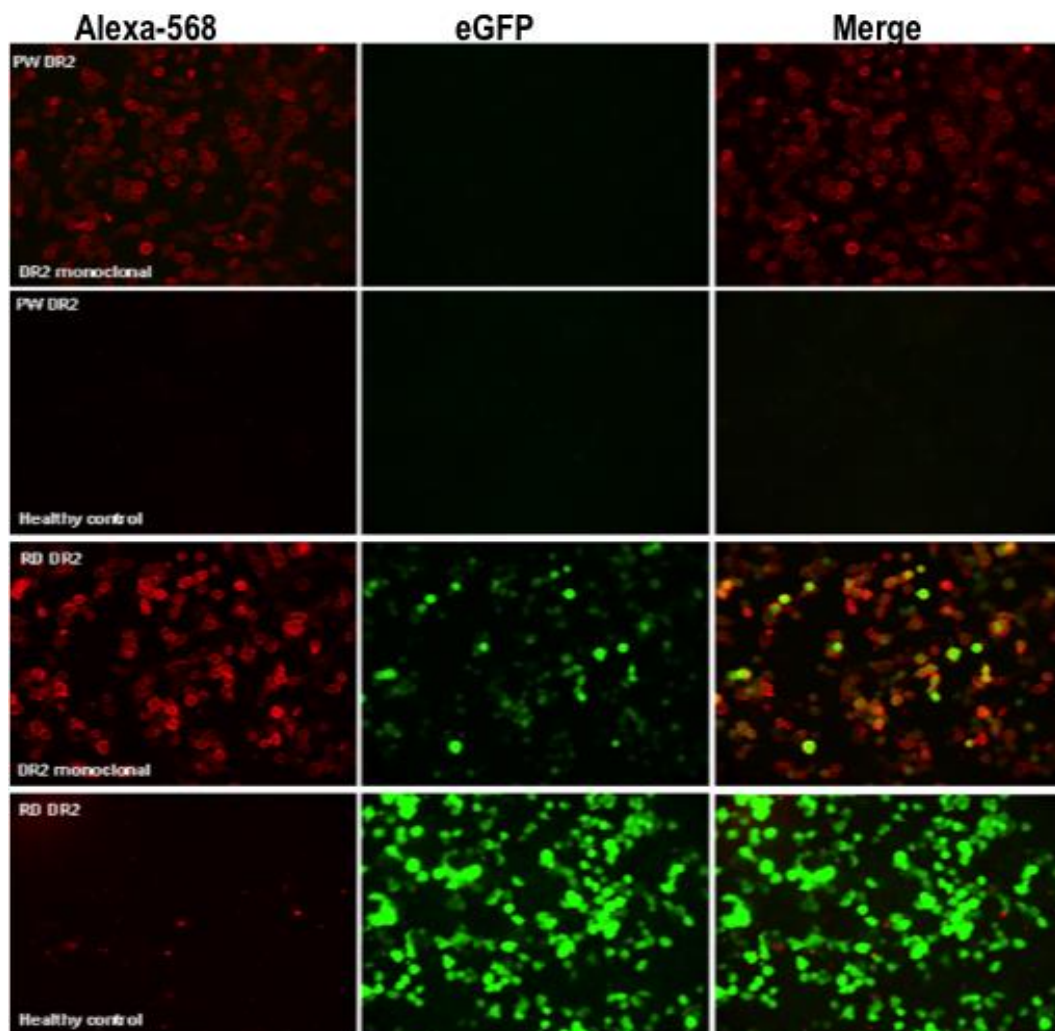


Figure 6.3 Comparison between Australian and UK D2R CBA using commercial monoclonal anti-D2R antibody

Top set panel; monoclonal anti-D2R antibodies binding extracellular surface of live HEK cells expressing UK D2R contract (DR2 PW) but not healthy control serum. Bottom set panel; monoclonal anti-D2R antibodies binding extracellular surface of live HEK cells expressing Australian D2R contract (DR2 RD) tagged with green EGFP, but not healthy control serum.

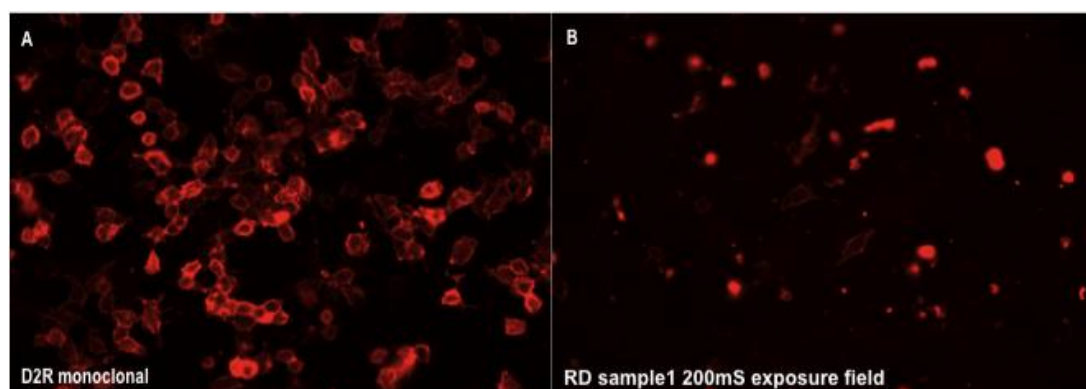


Figure 6.4 D2R Cell based assays of positive serum identified by Dale et al. Brain 2012

Serum from a patient with basal ganglia encephalitis, who was previously found to have D2R antibodies by the Australian group, was only showing weak binding. This was thought to be non-specific binding to the HEK cells.

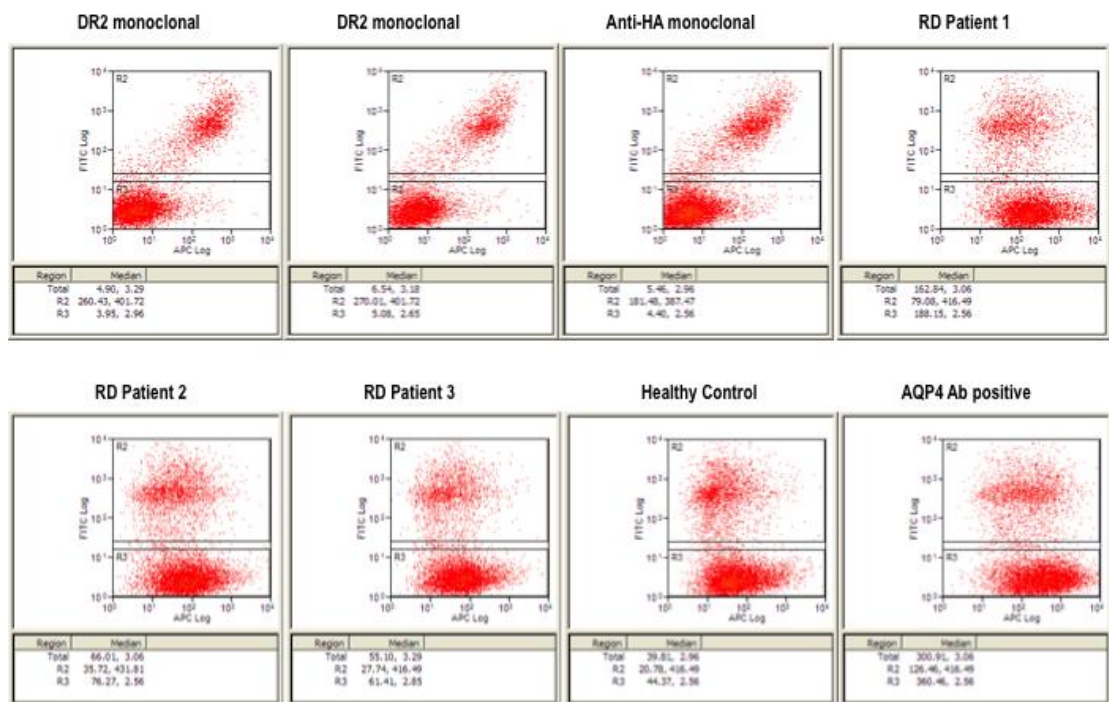


Figure 6.5 D2R FACS assay using DR2-IRES-eGFP construct

D2R CBA using FACS analysis demonstrating positivity of commercial D2R antibody and Anti-HA monoclonal with a shift of the transfected cell to the right. Dale et al positive patients, healthy control and AQP4 positive serum were negative (no shift to the right of the transfected cells).

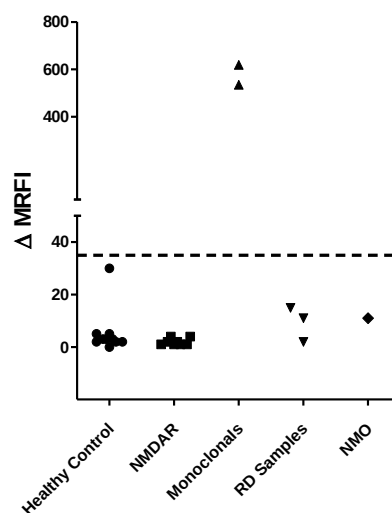


Figure 6.6 ΔMRFI scores of serum tested on D2R FACS assay using DR2-IRES-eGFP construct

Transient transfection. The δ median red fluorescence intensities, as measured by the difference between the red fluorescence intensities of D2R-eGFP transfected HEK cells and eGFP only transfected cells, are represented on the Y axis for healthy controls, NMDAR positive patients, DRD2 monoclonal antibodies, AQP4 positive neuromyelitis optica patients and patients with basal ganglia encephalitis and D2R-Ab sent as positive controls (RD samples)

6.5 *Detection of antibodies to the D1R and D2R in basal ganglia encephalitis cohort.*

Serum samples were tested using both D2R constructs (RD and PW). All 25 patients were negative for both D1R and D2R as were the 100 controls. Serum samples were tested using both D2R constructs (RD and PW). An additional 300 paediatric samples, from a range of neurological diseases, were also negative. Testing 2500 patients who were referred for NMDAR-Ab testing (control antigen for the routine NMDAR-Ab assay, cell-based assay performed by Dr Leslie Jacobson and scored by both Leslie Jacobson and myself) identified one positive patient (Figure 6.7). The patient was a 40 year-old woman with a new onset movement disorder. Repeat sample 3 months later was also positive (titres 1:200 both samples). She was not treated with immunotherapy, so we were unable to assess any response to therapy to support the pathogenicity and relevance of the antibodies found.

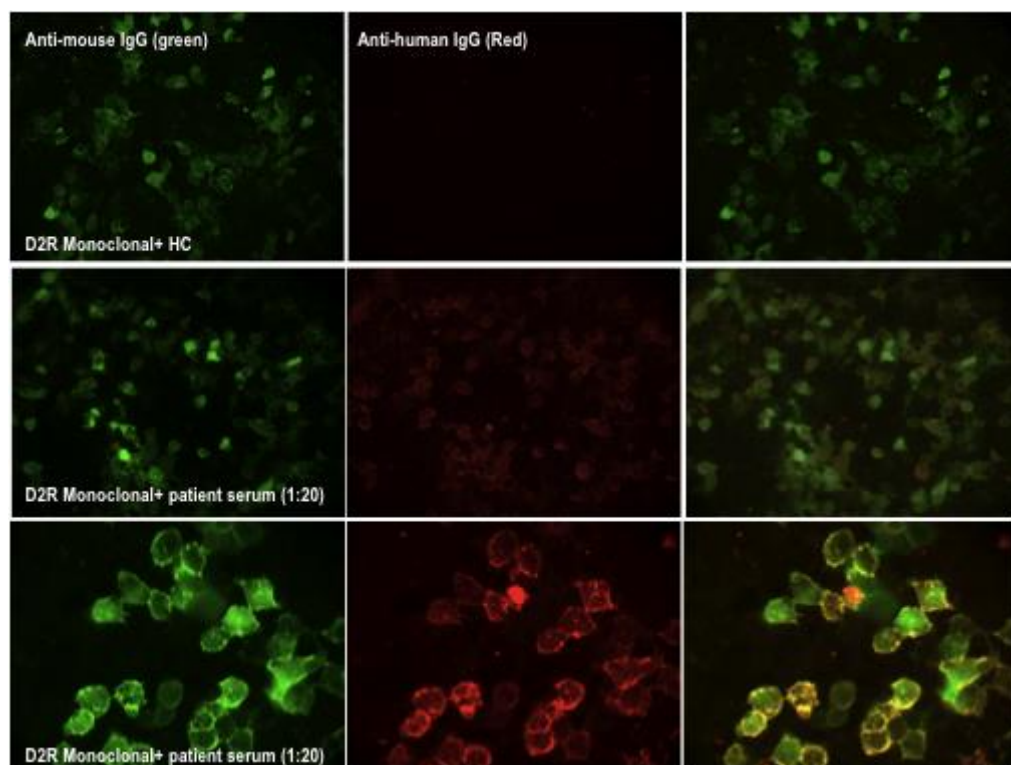


Figure 6.7 Cell based assay of D2R positive patient colocalised with monoclonal Anti-D2R commercial antibody.

Patient's serum (red) bound the extracellular surface of live D2R transfected HEK cells at 1:20 dilution and co-localize with commercial antibody (green)

6.6 Detection of antibodies to D2R using CBA FACS

The D2R (pIRES2-dsRed2 vector) expressed on cell-based assays and tested with flow cytometry did not identify D2R antibodies in any of the 25 patients with basal ganglia encephalitis or the 100 controls. The experiment was done initially at 4°C (Figure 6.8) and repeated at room temperature (Figure 6.9) with no difference in results (Figure 6.8-10). The adult patient who was positive on the D2R CBA was negative on FACS with a shift to the right of the non-transfected cells (Figure 6.8 and 9), which may suggest non-specific binding to HEK cells, although this had not been seen when testing for NMDAR-Ab.

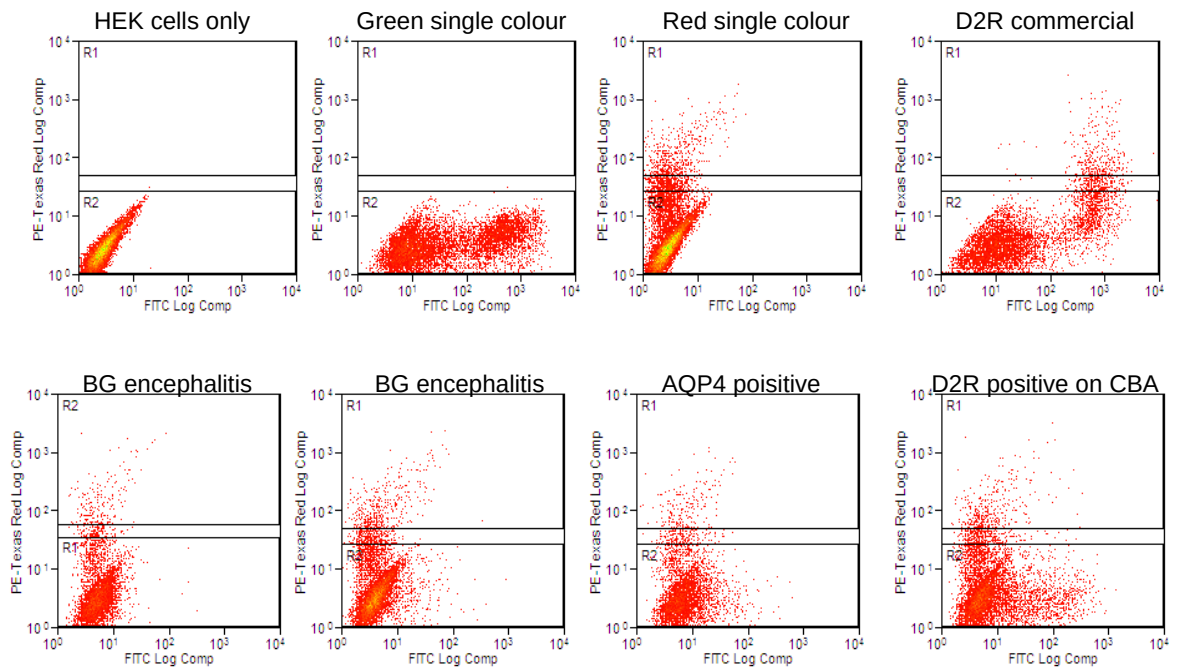


Figure 6.8 Flow cytometry using D2R pIRES2-dsRed2 vector at 4°C

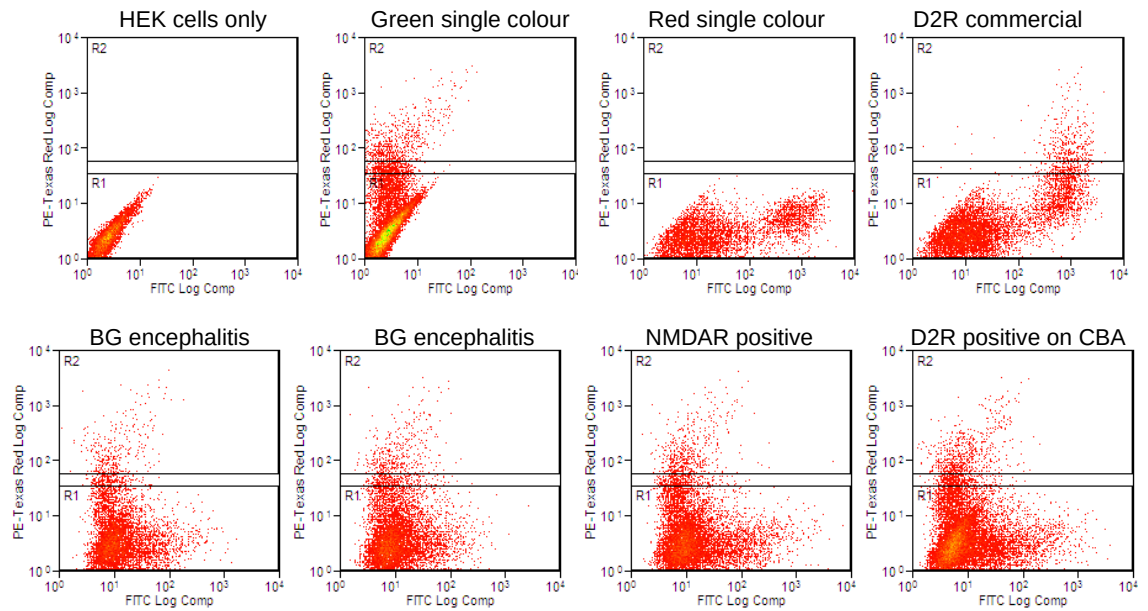


Figure 6.9 Flow cytometry using D2R pIRES2-dsRed2 vector at room temperature

Figure 6.8 and 6.9: Flow cytometry settings were established using, HEK cells only, EGFP transfected cells (green single colour), dsRed transfected cells (red single colour) and positive control (monoclonal antibody). Patients with BG encephalitis, and both disease and healthy controls were all negative. Positive patient on CBA (Figure 6-7) was negative on FACS analysis with shift to the right of both transfected and non-transfected cells. The experiment was repeated in both room temperature and at 4°C with no difference in results.

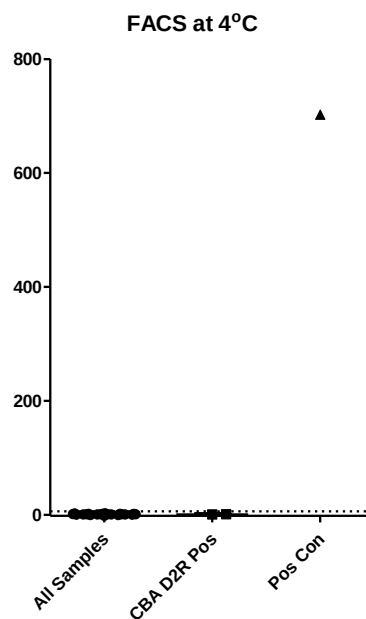


Figure 6.10 Δ MRFI scores (Y-axis) of serum tested on D2R FACS assay using pIRES2-dsRed2 construct

6.7 *Identification of novel antigens*

Given that the targeted approach to antigens localized to the basal ganglia did not reveal positive results, we then performed a more generalised approach was used. Serum samples were applied to rat brain sections and binding patterns evaluated. Serum was also applied to live hippocampal and mixed neuronal and astrocytic cultures in order to identify possible extracellular binding.

6.7.1 Immunohistochemistry and immunofluorescence on brain sections

Serum samples were tested using a range of dilutions (1:100 –1:3200) to identify specific staining patterns on rat brain sections (Chapter 2.6). Sera from three patients showed specific immunoreactivity: (i) Astrocytic staining (co-localising with GFAP) was evident in multiple brain regions (Figure 6.11) with the serum of a 5 year old girl, who presented with encephalopathy, prominent movement disorder and who responded to steroids. (ii) 'Neuropil' staining pattern was found on rat cortex using serum from a 12-year-old boy (Case 17) with steroid responsive encephalopathy, psychiatric symptoms and hypokinesia (Figure 6.12). Although he had a normal MRI, PET scan revealed reduced uptake in most of the frontal cerebral cortex (6.13). Neuronal staining of the granular layer of the dentate gyrus was found in a 2-year-old girl with acute onset encephalopathy, prolonged febrile seizures and dyskinetic movement disorder (Figure 6.14). All 25 patients with Tourette's syndrome and 25 healthy controls did not stain rat brain sections. By contrast, Figure 6.15 demonstrated characteristic 'neuropil' binding pattern of CSF from a patient with NMDAR-Ab encephalitis and co-localisation with the NRI commercial antibody. No patients CSF's samples were available for testing.

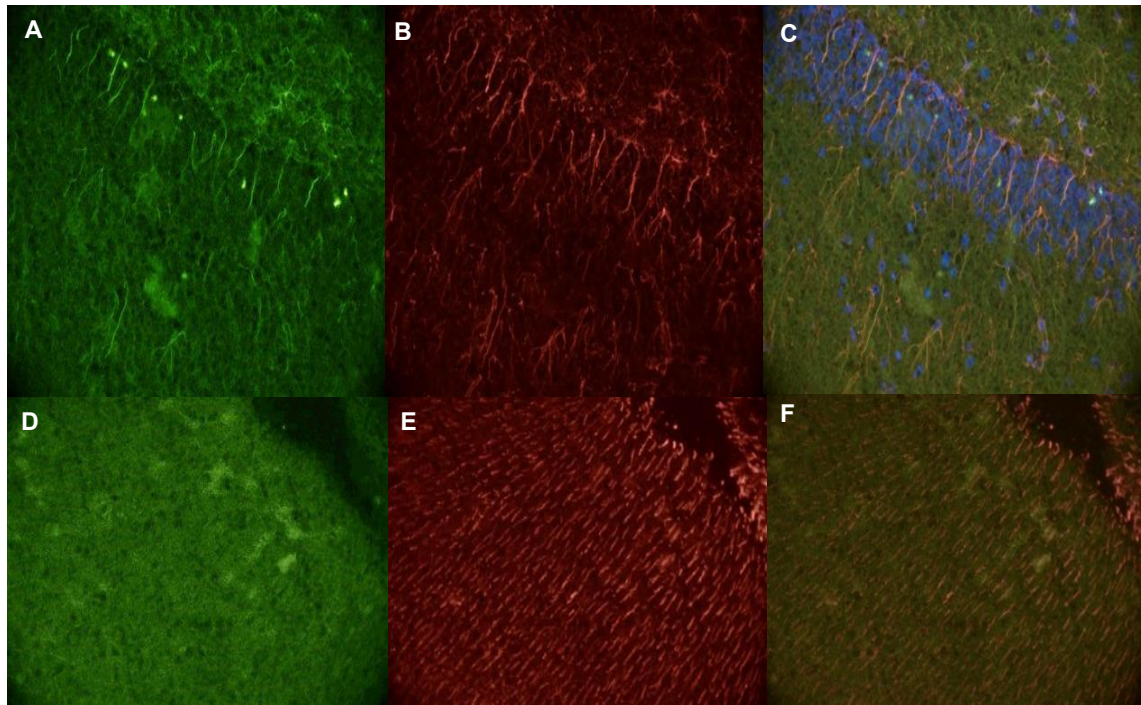


Figure 6.11 Immunohistochemistry using serum of a patient with basal ganglia encephalitis

Immunoreactivity of patient sera probed on a cross section of rat brain sections. A 5yr old girl, who presented with encephalopathy and prominent movement disorder and had a good response to steroid therapy. Patient sera immunoreactivity in the hippocampal region (A) has similarities to GFAP staining (B), with apparent co-localisation (C). Additional staining to the cerebellum (D) is distinctly different from GFAP immunoreactivity (E) and does not co-localise (F).

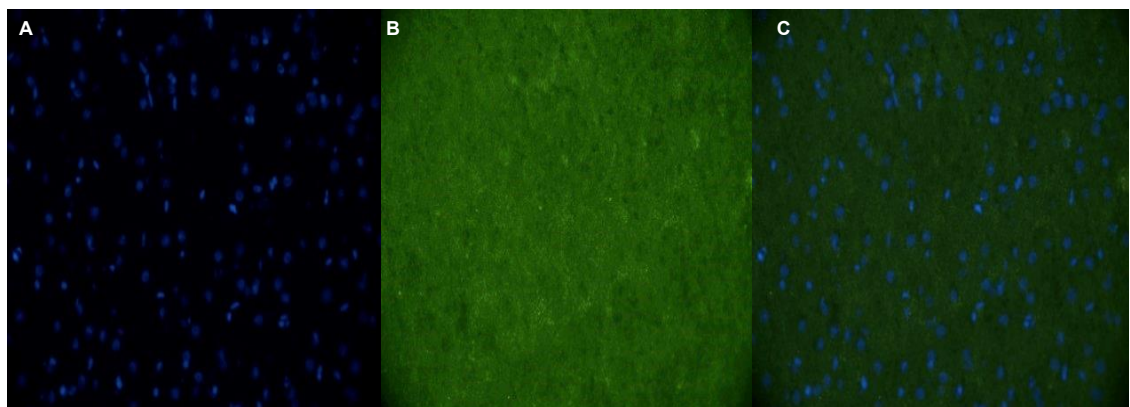


Figure 6.12 Neuropil staining in the cortex on IHC using serum of a patient with basal ganglia encephalitis.

Immunoreactivity of sera from a 12yr old boy with steroid responsive encephalopathy, psychiatric symptoms and hypokinesia (parkinsonism). On sagittal rat brain sections, 'neuropil' staining pattern was found in the cortex only (A-C).

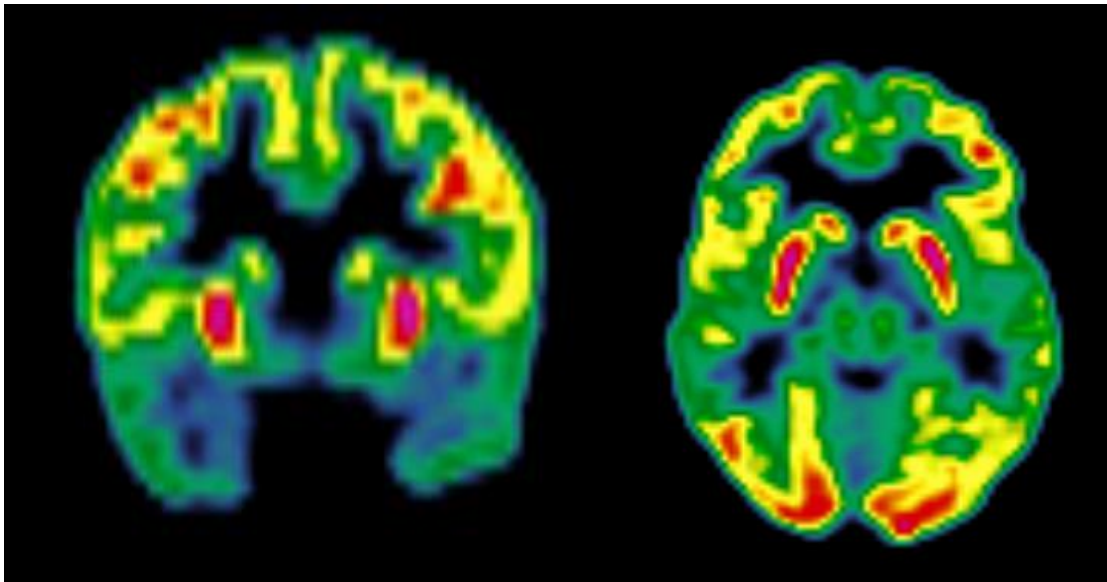


Figure 6.13 PET imaging of a patient with basal ganglia encephalitis.

PET demonstrates reduce uptake in the anterior cerebral cortex in both hemispheres suggestive of diffuse encephalitic process. The basal ganglia are normal. Interpretation of PET in children is challenging in view of the physiological changes seen with age, particularly when symmetrical (London and Howman-Giles, 2014) and these finding need to be replicated in a larger cohort with longitudinal analysis to show reversibility of changes observed.

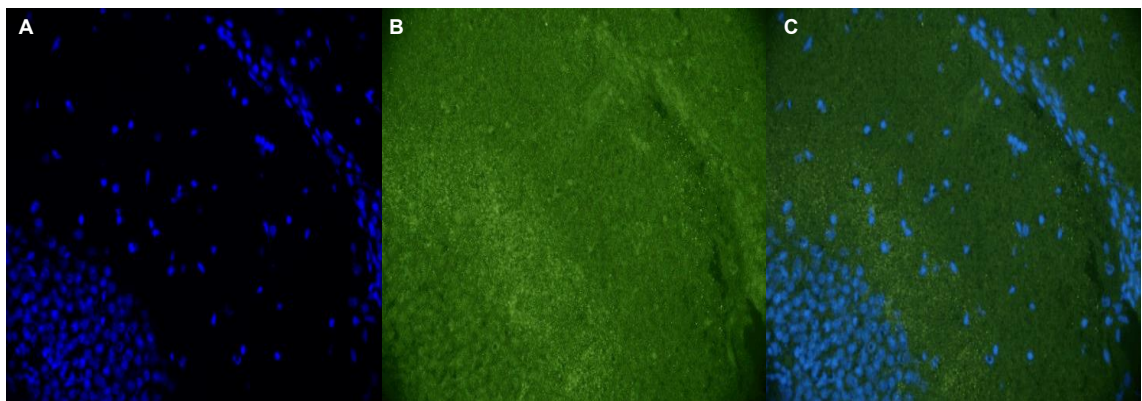


Figure 6.14 Neuronal staining granular layer dentate gyrus on IHC using serum of a patient with basal ganglia encephalitis.

Sera from a 2 year-old girl with acute onset encephalopathy, prolonged febrile seizure and dyskinetic movement disorder staining the granular layer dentate gyrus

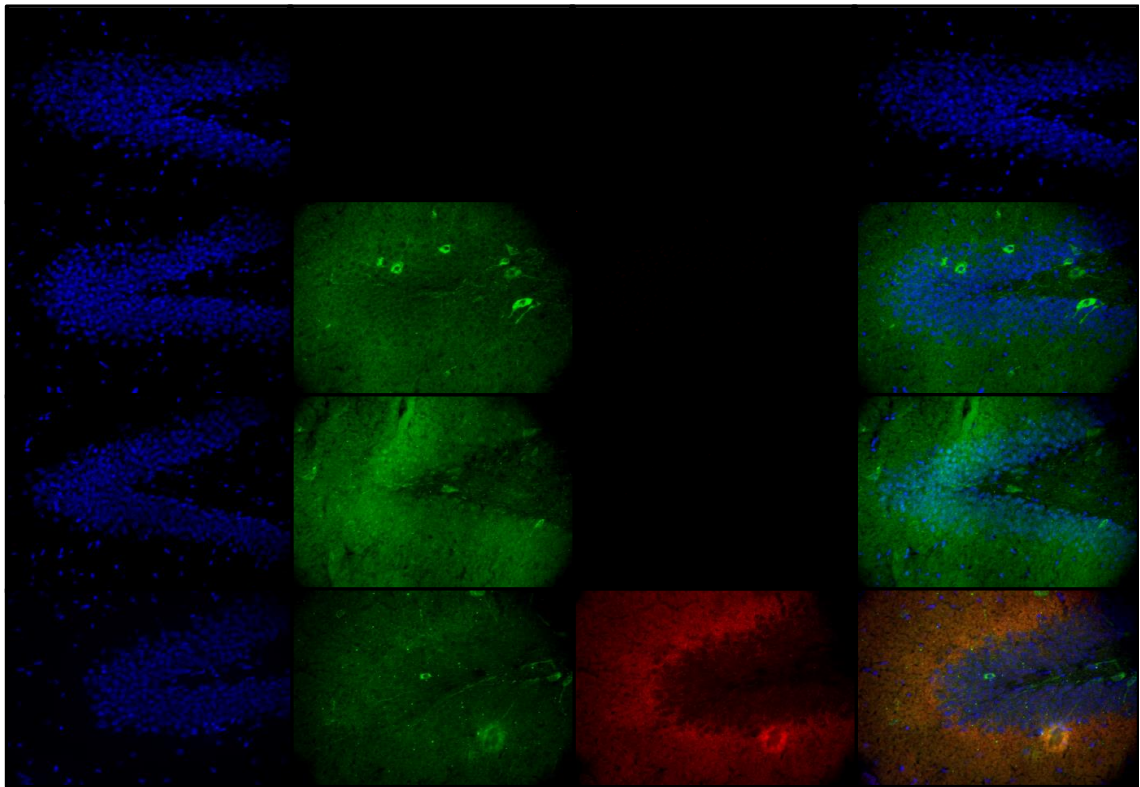


Figure 6.15 Immunohistochemistry on rat hippocampal tissue.

Tissue was stained in a serial method with no primary, primary monoclonal recognising mouse NR1 subunit of NMDARs (green), healthy(HC) and patient CSF (red), along with a DAPI counterstain (blue). Anti-NR1 primary recognised epitopes in all three conditions, while patient CSF resulted in a better co-localisation with the commercial antibody pictured in the dentate gyrus. The binding is consistent with autoantibodies that recognise the NR1 subunit of NMDARs in hippocampal tissue.

6.7.2 Primary hippocampal, mixed neurons, cerebellar and astrocytic cultures.

None of the patients' sera bound to live hippocampal neurons, mixed neuronal cultures or astrocytic cultures, including those who had brain specific staining by immunohistochemistry (Figures 6.11, 6.12 and 6.14). The NMDAR, CASPR2 and AQP4 antibody positive control sera bound to the different cell cultures, in the prototypic pattern, as previously observed. Figure 6.16 (mix neuronal cultures not shown).

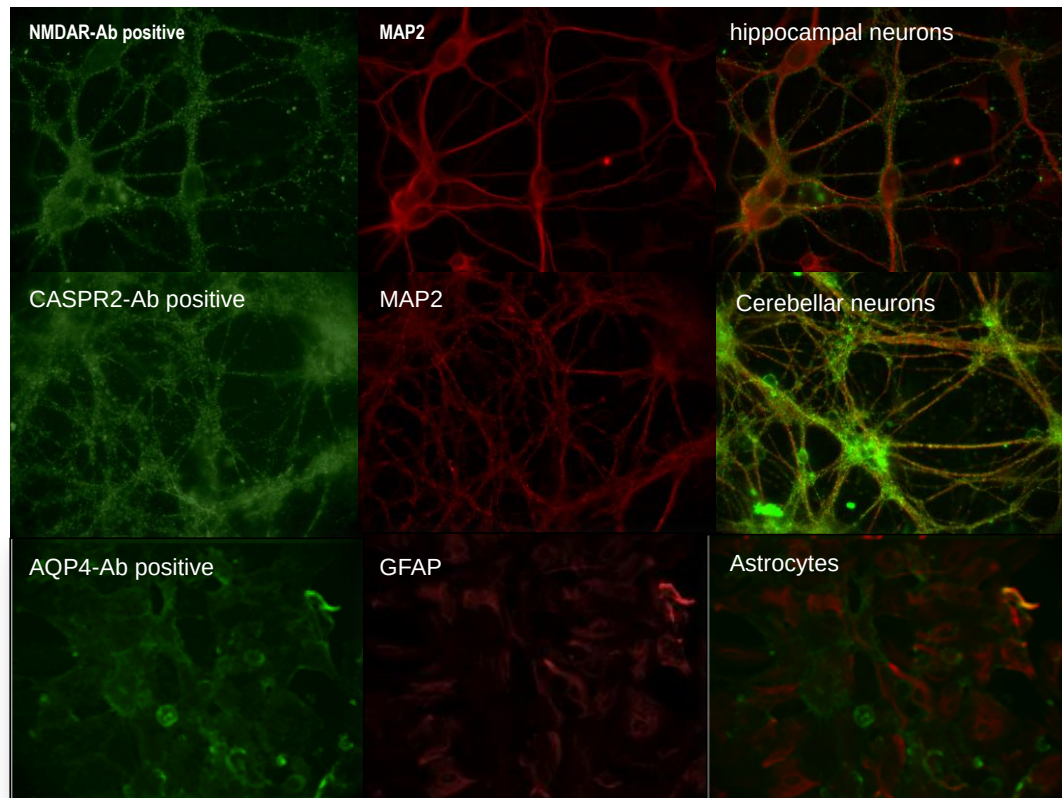


Figure 6.16 Positive binding to primary hippocampal neurons, cerebellar neurons and astrocyte cultures.

6.8 Discussion

The clinical phenotypes associated with paediatric autoimmune encephalitis are increasingly recognised but many patients are negative for the available autoantibody tests. The key observation in this chapter has been that antibodies to the dopamine receptors D1R and D2R were not identified in this cohort of 25 children with basal ganglia encephalitis, nor was there consistent evidence for an alternative antigen. The patients were defined by clinical and radiological (MRI pattern recognition) features. The lack of a disease-specific biomarker questions the autoimmune pathology proposed, and it is possible that there is a different pathobiological disease process. Alternatively, the negative results could reflect limited sensitivity of the current assays.

Selecting a cohort with distinct clinical phenotype of encephalitis with basal ganglia involvement was chosen in order to identify a unifying aetiology. Patients presenting with an

encephalitic processes affecting the basal ganglia have been reported with a number of clinical diagnoses including basal ganglia encephalitis, encephalitis lethargica, and bilateral striatal necrosis; although these phenotypes have been recognised clinically, despite rigorous testing for infections, autoimmune and inherited disorders, the primary aetiology often remains unknown (Beattie et al., 2013).

The incidence and demographics of patients presenting with basal ganglia encephalitis were similar to children presenting with NMDAR-Ab encephalitis (Table 6-1). Despite the clinical similarities MRI abnormalities were seen more frequently in patients with basal ganglia encephalitis whereas CSF OCB were less frequent. None of the children had a neoplasm. The lower frequency of immunotherapy in the basal ganglia encephalitis cohort reflects the confidence of clinicians in diagnosing autoimmune encephalitis in patients without known autoantibodies. Of note, patients who were found to have other (non disease specific) autoantibodies (VGKC-complex, GAD, TPO) were more likely to receive immunosuppression.

D2R antibodies were recently reported in 12/17 (71%) children with basal ganglia encephalitis and were also identified by the same group in patients with Sydenham's chorea and Tourette's syndrome (Dale et al., 2012) and two patients with neurological relapses following HSVE with or without NMDAR antibodies (Mohammad et al., 2014). D2R antibodies were not identified when tested by us (Hacohen et al., 2014c) and others (Armangue et al., 2014b) in patients who relapse following HSVE, adult patients presenting acutely with HSVE or more recently in 38 young females with a novel teratoma associated encephalopathy syndrome with prominent psychiatric features and myoclonus (Armangue et al., 2014c). In view of the clinical similarity of relapsing HSVE and Japanese encephalitis virus (JEV) patients who frequently develop a biphasic disease course or neurological relapses, with dyskinesias and abnormal movements (Pradhan et al., 2001), a cohort of 44 children with JEV was tested for neuronal antibodies. Of these 44 patients 4 had NMDAR-Ab but all were negative for D1R and D2R (unpublished data).

6.8.1 Lack of identification of a novel antigen in these patients may have resulted from multiple factors;

(3) The patients do not have an antibody mediated disease

Defining a group by the lack of a disease specific biomarker and where there is phenotypic overlap with infective, other inflammatory and genetic disorders is difficult. Identification of certain infectious agents at low levels, even in the CSF, for example HHV6 (Ward, 2014) and HHV7 (Schwartz et al., 2014) may not represent the primary aetiology. Furthermore, the co-occurrence of both infective and autoimmune aetiologies, and the clinical response for both entities to immunomodulation (steroids, IVIG), makes it difficult to identify the primary aetiology. In one study of patients with encephalitis and thalamic and basal ganglia abnormalities, 37% were positive for an infective organism, and 44% of the children were positive for a respiratory virus (Glaser et al., 2003). Although this association does not exclude a possible antibody-mediated disease, a primary infective aetiology cannot be ruled out.

Monogenetic disorders that predispose to recurrent and or familial disease such as autosomal dominant RANBP2 gene in acute necrotizing encephalopathy could have been the cause in some of these children. Children with acute necrotizing encephalopathy typically present following influenza infection with bithalamic and brainstem signal change on imaging. Importantly, a response to steroids and IVIG has also been reported in this group (Neilson, 2010). This strong interplay between infection and autoimmunity further complicates unravelling of primary autoimmune disorders that result from a specific immune response to an antigen and make it difficult to distinguish them from disorders propagated by immune responses but initiated by genetic or degenerative biology.

Finally, these patients may have an immune-mediated encephalopathy but not an antibody-mediated disease. The role of T-cells in encephalopathies was previously suggested in

some of the paraneoplastic conditions and in CNS vasculitis; both may present with a range of clinical and radiological phenotypes. Even in the prototypic autoimmune CNS disease, multiple sclerosis, the specific pathobiology is unknown.

(4) The patients have a known antibody but current test are not sensitive enough

The sensitivity and specificity of the different antibody detection methods are essential in evaluating patients with inflammatory CNS disorders. In a multicentre study comparing the different AQP4 antibody assays in patients with clinically defined neuromyelitis optica, sensitivities ranged from 48-78% (Waters et al., 2012). Striking differences in results may simply reflect different techniques used by different groups and explain the negative findings of D2R antibodies in our cohort. Nevertheless, the use of a similar method (cell-based assay) and the same construct for testing the cohorts makes this unlikely

Alternatively, further optimisation of the NMDAR-Ab assay may increase its sensitivity and identify some of the basal ganglia encephalitis patients as NMDAR-Ab encephalitis. A recent comparison of two different NMDAR-Ab cell-based assays showed that fixed cells yielded a higher sensitivity than did the more time consuming live-cell-based technique, with immunohistochemistry testing more often in agreement with CBA on fixed cells 77/108 (71%) than with CBA with live cells 63/108, 58%, ($p=0.0056$) (Gresa-Arribas et al., 2014).

Antibody clustering may increase the sensitivity of the NMDAR-Ab assays as previously shown for the detection of low-affinity antibodies to AChR. These antibodies were not detected in solution phase assays but were detected by live cell based assays, if the AChRs were clustered at the high density that is found at the neuromuscular junction. Using this technique has allowed the identification of 66% (25/38) of sera previously negative for binding to AChR in solution (Leite et al., 2008b). Clustering of CNS antigens could be one way of trying to increase the sensitivity of the assays.

Induction of autoantibodies is thought to occur outside of the CNS in the peripheral immune system and therefore autoantibodies should initially be detected in the blood (Vincent et al 2011). In the majority of these disorders, particularly NMDAR-Ab encephalitis, intrathecal antibody production has been reported. Absolute serum IgG levels are usually around 400 higher than those in CSF, with any lower ratios indicating that the specific antibody within the CSF is being produced intrathecally. For NMDAR antibodies the ratio is often much lower (median 1:15)(Irani et al., 2014). Although the serum levels remain higher than those in the CSF, CSF is claimed to be superior for testing NMDAR-Ab because it has much lower CSF concentrations and can be tested undiluted. In one study, 250 patients with NMDAR-Ab detected in CSF, serum antibodies were found in only 214 (serum sensitivity of 85.6%) (Gresa-Arribas et al., 2014).

The use of appropriate collection techniques and rigorous and shared standard operating procedures are essential. As the samples tested here were sent initially for a clinical test, the quality control for the sample processing is not available but they were sent in parallel to samples testing positive for NMDAR-Ab (in addition to a range of CNS and PNS known autoantibodies), and it is unlikely that the processing procedures should have affected the results.

NMDAR-Ab may still be present 15yr from the onset of symptoms even when the patients are asymptomatic (Hansen et al., 2013) and AQP4-Ab have been identified years before the clinical onset of NMO in patients with co-existent myasthenia gravis (Leite et al., 2012). By contrast, antibodies to GQ1b in patients with Bickerstaff's brainstem encephalitis (Shahrizaila and Yuki, 2013) are typically only positive acutely. Different clinical phenotypes in a single antibody-mediated disease may also present with different time courses as seen with MOG-Ab, that are typically only present acutely in monophasic ADS but persist in patients with a relapsing disease (Probstel et al., 2011, Rostasy et al., 2013).

It is also possible that new entities emerge over time, which although may not be the primary initiator, could still be relevant to on-going disease and can be used as disease biomarkers. Identification of NMDAR-Ab at the time of HSVE relapse (and not at the HSVE onset)

(Hacohen et al., 2014c) highlights that samples taken at the first clinical event may not be diagnostic for the current clinical course.

(5) Antigen discovery techniques need further optimization

Surprisingly, none of the 25 samples bound to the extracellular surface of live neurons, and only three showed specific (but different) staining patterns on rodent brain sections. The use of CSF (which was not available) rather than serum might have been superior, particularly for immunohistochemistry as mentioned above. Although using higher serum dilutions help to overcome this problem, specific immunostaining patterns could be masked at high concentrations by the high background and may disappear with continuous dilutions. Different antigens are expressed on different neuronal cultures at different time points and this also could have led to negative results. An important limitation of this study was that basal ganglia neurons were not used. Additionally, some of the antigens may have only been expressed on embryonic neurons, which were not tested here. The use of inappropriate conditioned medium was previously shown to cause false negative results for the identification of NMDAR on oligodendrocytes as the glutamate used in the conditioned medium was toxic to the NMDAR (Bakiri et al., 2008). Finally, if the antigen is only expressed in humans it would have been missed using the methods described above which were performed using rodent brain sections and cell-cultures.

6.9 Summary and conclusions

In this cohort of children with basal ganglia encephalitis no causative autoantibody was identified. In addition to the patients' demographics, clinical and paraclinical features that this group shares with children with NMDAR-Ab encephalitis, both groups were also diagnosed at a similar frequency. Importantly, these children responded to immunotherapy. Further studies exploring a range of other inflammatory, infective and even genetics aetiologies may establish the exact pathobiology of this interesting group of patients.

CHAPTER 7 - General discussion and future directions

The work presented in the thesis characterises children with a range of autoimmune encephalopathies with or without CNS antibodies. Studying cohorts of patients with CNS antibodies identified in diagnostic screening, resulted in the expansion of the disease spectrum associated with NMDAR autoantibodies (Chapter 4) with the identification of partial phenotypes, white matter disorders and post-HSV encephalitis relapses. On the other hand, in a different group of children with VGKC-complex antibodies (Chapter 5), the precise extracellular target was not identified in the majority, suggesting these antibodies may not be clinically relevant, but the antibodies appear to provide a biomarker for inflammatory disorders. Using strict clinical phenotyping, a cohort of children with basal ganglia encephalitis was studied to determine the existence of dopamine receptor or novel antibody targets, but despite the similarities to patients with NMDAR-Ab encephalitis, rigorous testing did not identify evidence of a pathogenic autoantibody. Overall, patients with clinically indistinguishable features may differ in the presence of serum or CSF antibodies and patients with similar antibody profiles may have clinically distinct disorders. This lack of absolute fidelity of clinical-antibody profiles challenges all clinicians. A topic of considerable debate relates to the decision of whether a diagnosis should be based on clinical or laboratory features- which should “win out” in the situation of a clinical-antibody paradox?

The sensitivity and specificity of the different assays requires strict clinical inclusion criteria in order to determine the applicability of the test in a clinical setting. It is important to distinguish technically false positivity from a biological valid seropositive result. The current “gold standard” remains the clinical presentation. Diagnosis of a specific disorder in antibody-positive patients with some, but not all features of a well-defined syndrome, rests on confidence in both the pathogenicity of the antibody and on the rigor of the test method. Antibody-positive patients with clinical features discordant for the disorder, a situation that may occur often with the use of

“antibody panels”, must be closely monitored to determine the positive predictive value of this antibody for clinical management.

Perhaps the strongest levels of evidence linking specific antibodies to the pathology of specific disorders are found in AQP-4 antibody in NMO and NMDAR antibody in NMDAR-Ab encephalitis. Identification of antibodies linked to specific disorders was initially enabled by collation of samples with similar clinical criteria for each disorder. Subsequently, detection of serum and CSF antibodies against specific antigens has led to an expansion of phenotypes of each disorder to include seropositive children with only some of the hallmark clinical features, or with features not initially considered part of the clinical phenotype.

By contrast, different antibodies can be associated with similar clinical phenotypes. Forms of limbic encephalitis in adults with antibodies to LGI1, GABA_BR or AMPAR, may not be clinically distinguishable. In children, the clinical expression may also be influenced by the age, both with respect to the ramifications of inflammation in the immature CNS, and by age-related antibody production.

It is becoming clear that patients may have multiple autoantibodies and some develop new antibodies over time, which may associate with a new neurological presentation. It remains to be defined whether patients with two distinct antibody- associated disorders have two distinct biologies, or a single immune defect that leads to poly-antigenic responses. There thus remains a significant complexity in achieving a specific diagnosis.

The clinical phenotypes of the different CNS autoantibodies are summarised in Table 7-1. Only the common antibodies reported in paediatric neuroimmune disorders will be discussed here, with emphasis on their clinical relevance and potential pathogenic role.

Antibody	Clinical Presentations	Specific Considerations	Disease course
NMDAR	- Encephalopathy - Psychosis - Movement disorder - Seizures - Dysautonomia - Preceding HSV encephalitis	- Mutism or language regression can occur - Lower risk of associated malignancies in children (i.e. teratoma) - High titres associated with more aggressive disease	- Prolonged disease course. 75% admitted to ICU. - Full recovery in 75%. - Relapse seen in 12%. - Immunoresponsive
	- Brainstem encephalitis - Optic neuritis - ADEM or ADEM plus ON - NMOSD	- More common association in paediatrics than adults - NMDAR antibodies are negative at time of HSV - PCR HSV negative at the time of NMDAR encephalopathy	- Response to immunotherapy similar to patients without prior HSV encephalitis - No reports of recurrence of HSV infection during treatment
		- May or may not have clinical NMDAR encephalopathy - Demyelinating presentations may follow or precede the NMDAR encephalopathy - Some patients have MOG Abs - The frequency of NMDAR antibodies in children with CNS demyelination is likely low	More frequent relapses of neurological deficits than is seen in typical NMDAR encephalitis
AQP4	- NMO - NMOSD	- ADEM-like attacks may occur, particularly in younger children - Oligoclonal bands found in fewer than 20% - Apparent worsening of disease, or at least failure to respond to MS-specific immunomodulatory therapies	- Typically relapsing disease although patients with a single episode of ON and TM are reported - Recovery from attacks may be poor - Relapse frequency reduced by immunosuppressive therapies
MOG	- ON - ON and TM - ADEM - ADEM plus recurrent ON	- Likely predictor of non-MS disease course - OCB negative	- Monophasic or relapsing course - Persistent of antibodies in relapsing demyelination and normalization in monophasic diseases
VGKC-complex	- Limbic encephalitis - Neuromyotonia - Morvan syndrome	Rare in children	Unknown due to lack of specificity of current test
LGI1	Limbic encephalitis	Not reported in children	- Often monophasic. - Clinical response to immunotherapy - Persistent memory deficits may be noted
CASPR2	Peripheral nerve hyperexcitability	Not reported in children	- Chronic disease - Symptomatic therapy
Contactin-2	- Limbic encephalitis - Neuromyotonia - Morvan syndrome	- Not reported in children - Reported in 6% of adult RRMS patients	Found in association with antibodies VGKC, LGI1 and CASPR2
D2R	- Basal ganglia encephalitis - Sydenham's chorea - Tourette's syndrome	Not reported in adults	- Full recovery in 42%. - Clinical improvement with immunotherapy in some
GlyR	- PERM - SPS	Rare	Immunotherapy responsive
GABA _B	Encephalitis with prominent seizures.	- Only 2 children reported - Associated with small cell lung cancer in adults	- One child died with refractory seizures - Some response to immunotherapy
GABA _A	Encephalopathy with refractory seizures and status epilepticus.	3/6 patients reported are <18yr	- Extensive cortical and subcortical MRI abnormalities - Positive TPO and GAD65 antibodies - Immunotherapy may be beneficial - Long-term outcome is poor
DPPX	- Cognitive dysfunction - psychiatric features - Resting tremor and myoclonus - PERM	- One adolescent patient reported - Associated with diarrheal illness	Immunotherapy responsive (aggressive).
mGluR1	- Cerebellar ataxia - Hodgkin's Lymphoma	Not reported in children	
mGluR5	Limbic encephalitis and Hodgkin's Lymphoma (Ophelia syndrome)	Rare	- Improvement with tumour resection - Immunotherapy responsive
AMPA	Limbic encephalitis.	Not reported in children	- Frequently relapse. - Response to immunotherapy
GAD	Stiff-person syndrome	Very rare in children but have been reported in patients with LE and refractory seizures	Usually chronic disorders

Table 7-1 Clinical phenotypes associated with CNS autoantibodies

7.1 *Antibodies in encephalitis*

NMDAR- Ab is the most common neuronal autoantibody identified in children (Armangue et al., 2012). Originally reported as a paraneoplastic syndrome in young adult females with ovarian teratoma (Dalmau et al., 2007), patients typically present with an encephalopathic syndrome with prominent psychiatric symptoms, a movement disorder and seizures. The symptoms are severe with 69% of the patients being admitted to intensive care (Titulaer et al., 2013). About 40% of patients are under the age of 18 years, and the association with malignancy is seen less frequency in the paediatric population as evident in our cohort with an ovarian teratoma being identified in only one of the 31 children with NMDAR-Ab encephalitis (Chapter 4). The recovery process can be slow up to 18 months and has been reported to be in reverse order of symptom presentation with cognitive (verbal fluency and executive function) and behavioural problems last to improve (Dalmau et al., 2011). Relapses occur in 12% of patients typically with a less severe presentation to the initial one (Tardieu and Deiva, 2013). Complete recovery is seen in 75% of the patients (Dalmau et al., 2011). Milder or incomplete phenotypes of NMDAR have been identified either as the initial acute manifestation (Dalmau et al., 2011), or at the time of relapsing symptoms

However, non-encephalopathic patients presenting with predominant movement disorders, milder cognitive features, with or without seizures, were found to have high titers of NMDAR antibodies in the serum and CSF (Rubio-Agusti et al., 2011, Hacohen et al., 2014d). These patients were immunotherapy responsive. Moreover, white matter changes indistinguishable from acquired demyelinating syndromes were found in patients with NMDAR-Ab antibodies with and without AQP4 and MOG antibodies in both adults (Titulaer et al., 2014) and children (Hacohen et al., 2014a), highlighting a unexpected overlap between the demyelinating and encephalitic groups.

Antibodies to the VGKC-complex were described in children presenting with a range of neurological conditions including encephalitis, global regression and focal and generalized

epilepsies (Hacohen et al., 2013) In adults, VGKC-complex antibodies are most frequently associated with limbic encephalitis characterized by seizures, amnesia, hyponatremia, movement disorders and MRI evidence of abnormal signal in the limbic region. Many of the antibodies bind to the VGKC-associated proteins LGI1. In contrast to adults the presence of antibodies to the VGKC-complex proteins (LGI1, CASPR2, contactin-2) are rarely detected in children (Armangue et al., 2012). Importantly, antibodies against the VGKC-complex can be detected in patients with non-specific neurological symptoms, and identification of these antibodies at high titres, in patients with an alternative aetiology such as prion disease (Jones et al., 2014) raises the question of pathogenicity in such individuals. The work present here, begins for the first time to investigate the target of these VGKC-complex antibodies, and showed that in a proportion of the patients the antibodies appear to be directed against intracellular epitopes of the Kv1 channels themselves, either with or without the intracellular scaffolding proteins PSD 93 and 95 (Chapter 5). Nevertheless, although not disease specific, VGKC-complex antibodies were more likely to be found in children with primary inflammatory disorders and hence can be used as a marker of inflammation. This suggests that these antibodies occur when the nervous system is damaged, perhaps because released epitopes of the VGKC-complex are able to induce autoantibodies.

7.2 *Autoantibodies in ADS*

Antibodies against the astrocyte water channel protein, aquaporin-4 (AQP4) were first described in 2004 in patients with NMO (Lennon et al., 2004). NMO preferentially affects the optic nerves and spinal cord. The discovery of AQP4-Ab has directly influenced the diagnosis and management of these patients. As AQP4-Ab have now been identified in patients with single or recurrent attacks of optic neuritis, myelitis (longitudinally-extensive), or brain/brainstem disease, their presence is included in patients diagnosed with NMO spectrum disorders (Sato et al., 2013). The value in identification of an antibody in NMO and NMOSD, led to use of B-cell targeting

therapies, such as rituximab, which clearly reduce relapse rates (Mealy et al., 2014). Additionally, several conventional multiple sclerosis (MS) therapies such as interferon beta (Palace et al., 2010) have been associated with disease worsening in patients with NMO. AQP4 seropositivity is more prevalent in children with relapsing NMO than in children with monophasic NMO (Rostasy and Reindl, 2013, Tillema and McKeon, 2012) suggesting this antibody may be used as a predictor for relapse in this childhood entity. Two studies looking at the prevalence of AQP4 antibodies in paediatric patients presenting with a first episode of demyelination have only identified these in 0.7% (2/279) (Banwell et al., 2011) to 4.5% (3/64) (Hacohen et al., 2014b) of children, which may reflect the rarity of NMO in certain populations. Nevertheless, the severity of NMO and the role for specific therapies prompts testing of children with ADS.

Antibodies to myelin oligodendrocyte glycoprotein (MOG) have been reported in 20-40% of children at first presentation of ADS (Reindl et al., 2013), and in a group described as AQP4-Ab negative NMO patients (Kitley et al., 2012). These antibodies have been reported in both monophasic and relapsing diseases (Reindl et al., 2013). Whether the presence of MOG antibodies should influence therapy remains uncertain.

7.3 *Measurement of antibodies*

The sensitivity of antibody detection is strongly influenced by the methods used and the experience of the individual interpreting these results. It is important to understand the methodological strengths and weaknesses of each respective technique as was highlighted by a comparative study of AQP4-Ab in NMO that identified significant differences in the sensitivity and specificity of the different assays (Waters et al., 2012). Timing of testing is also key. Importantly plasma exchange (PLEX) or intravenous immune globulin (IVIG) will impact the accuracy of antibody measurement particularly when tested in the serum. Antibody responses can fluctuate during the course of an illness, including during clinical remission. Patients with NMDAR-Ab have demonstrated markedly elevated antibody titers (both in the serum and CSF) at the time of

clinical symptom resurgence (Hansen et al., 2013). On the other hand, the presence of antibodies may pre-date the first clinical manifestation. For example, four patients evaluated for myasthenia gravis had AQP4-Ab, which while not associated with clinical features of demyelination at the time of testing, did predict a future clinical syndrome consistent with NMO many years later (Leite et al., 2012).

Furthermore, the detection of an autoantibody does not necessary implicate that the antibody contributes or mediates a clinical phenotype. Antibodies binding to extracellular antigens are now recognised as a likely cause of neurological disorders in both adults and children (Vincent et al., 2011). These cell-surface antigens are often essential to cellular function or neurotransmission and are generally expressed throughout the nervous system. The pathogenic mechanisms are different for different autoantibodies. The clinical presentation of NMDAR-Ab encephalitis is similar to the clinical symptoms seen in patients exposed to medications that are NMDAR antagonists. A loss of function of the NMDAR is further supported by data showing that the antibodies cause internalization of the receptor with selective, reversible decrease of NMDAR surface density, synaptic localization, and currents (Moscato et al., 2014). The lack of complement activation or activation of a further inflammatory cascade may explain the absence of immune cell infiltration or neuronal loss in the brain, as demonstrated in patients who have succumbed or who have undergone brain biopsy (Bien et al., 2012). By contrast, AQP4 antibodies mediate astrocyte damage by complement-dependent cytotoxicity, leukocyte infiltration, cytokine release and blood–brain barrier disruption, further leading to myelin loss, and oligodendrocyte and neuronal cell death (Papadopoulos and Verkman, 2013).

Antibodies against intracellular antigens, as seen in most paraneoplastic syndromes and in the case of GAD65, are generally considered unlikely initiators of disease, and are more likely the result from an immune recognition of tissue destruction. These antibodies, although not directly pathogenic, may still contribute to propagation of established disease (relapse, progression) by T-cell mediated mechanisms.

7.4 *The role of infections in precipitating or propagating neuroimmune disorders*

The inter-relationship between infection and neuroimmune disorders has been studied perhaps most stringently in MS. There exists strong epidemiological association between Epstein-Barr virus and multiple sclerosis in both adults and children. The possibility that individuals with MS may fail to regulate the normal symbiotic relationship between EBV and host immune cell homeostasis is supported by a recent study that demonstrated increased EBV oral shedding in children with MS compared to EBV-positive healthy age-matched paediatric controls, a finding that was not seen with other viruses (Yea et al., 2013). Some exposures, such as cytomegalovirus or helminthic infections may, in fact, lower MS susceptibility or result in milder disease (reviewed in Waldman et al., 2014). Similarly the identification of NMDAR antibodies in patients who relapse following herpes simplex virus encephalitis (HSVE) highlights the close relationship between viral infections and autoimmunity in children (Armangue et al., 2014b, Hacohen et al., 2014c, Mohammad et al., 2014). Neurological deterioration in children who have recovered from HSVE occurs in about 25% of reported cases in the paediatric literature, typically with a new onset encephalopathy and a movement disorder (Hacohen et al., 2014c). And although viral reactivation cannot be ruled out, serum and CSF studies do not support re-infection. The identification of NMDAR-Ab in these children, as well as the response to immunomodulatory therapies suggest that the CNS insult of HSVE leads to a secondary neuroimmune NMDAR encephalitis (Armangue et al., 2014b, Hacohen et al., 2014c).

7.5 *Genetics in neuroimmune diseases*

The pathogenesis of some genetically defined immune diseases has been eloquently delineated, leading to major advances in targeted therapies (Morato et al., 2014). While host genetic factors undoubtedly influence responses to CNS infection and to neuroimmune disorders, the processes appear heterogeneous and thus have not led to specific therapeutic strategies. The complex

interaction between genetics and neuroimmune disorders can be considered in several ways: (i) in the context of known genetic disorders that also appear to have neuroimmune disease associations, such as Leber hereditary optic neuropath (LHON mtDNA) mutations (Pfeffer et al., 2013); (ii) in the context of monogenetic, often familial, disorders that are characterised by widespread, non-specific inflammation, which may involve multiple systems, and is induced by deregulated activation of the innate immune system (Martinon and Aksentijevich, 2015), and (iii) by inborn errors of anti-viral interferon immunity with toll like receptor (TLR)3 mutations predisposing to HSVE (Sancho-Shimizu et al., 2011). While it is unlikely that most individuals with neuroimmune disorders have specific genetic mutations, the pathobiological pathways identified through the study of patients with these genetic mutations is likely to inform on CNS-directed immunological responses and may lead to new concepts in therapy.

7.6 *Creating Diagnostic Algorithms*

An approach to the investigation and diagnosis of neuro-immune disorders in children is provided in Figures 7.1 and 7.2 (this work was done in collaboration with Dr Brenda Banwell, The Children's Hospital of Philadelphia). In general, neuroimmune disorders manifest in previously healthy, typically developmentally and neurologically normal children and adolescents. Onset of neurological deficits is abrupt in most patients, although a prodrome of more subtle cognitive or behavioural change may precede encephalopathy and other features of patients with NMDAR-Ab encephalitis, and children with acute disseminated encephalomyelitis (ADEM) often experience fever, malaise or mild infectious symptoms before neurological impairment. Characterization of neuroimmune disorders is aided by dividing neurological features at presentation to those that localize to a single CNS site (clinically monofocal) or involve multiple CNS regions (polyfocal), and by further dividing patients with polyfocal/polysymptomatic deficits into those with encephalopathy and those with preserved alertness (Figures 7.1 and 7.2).

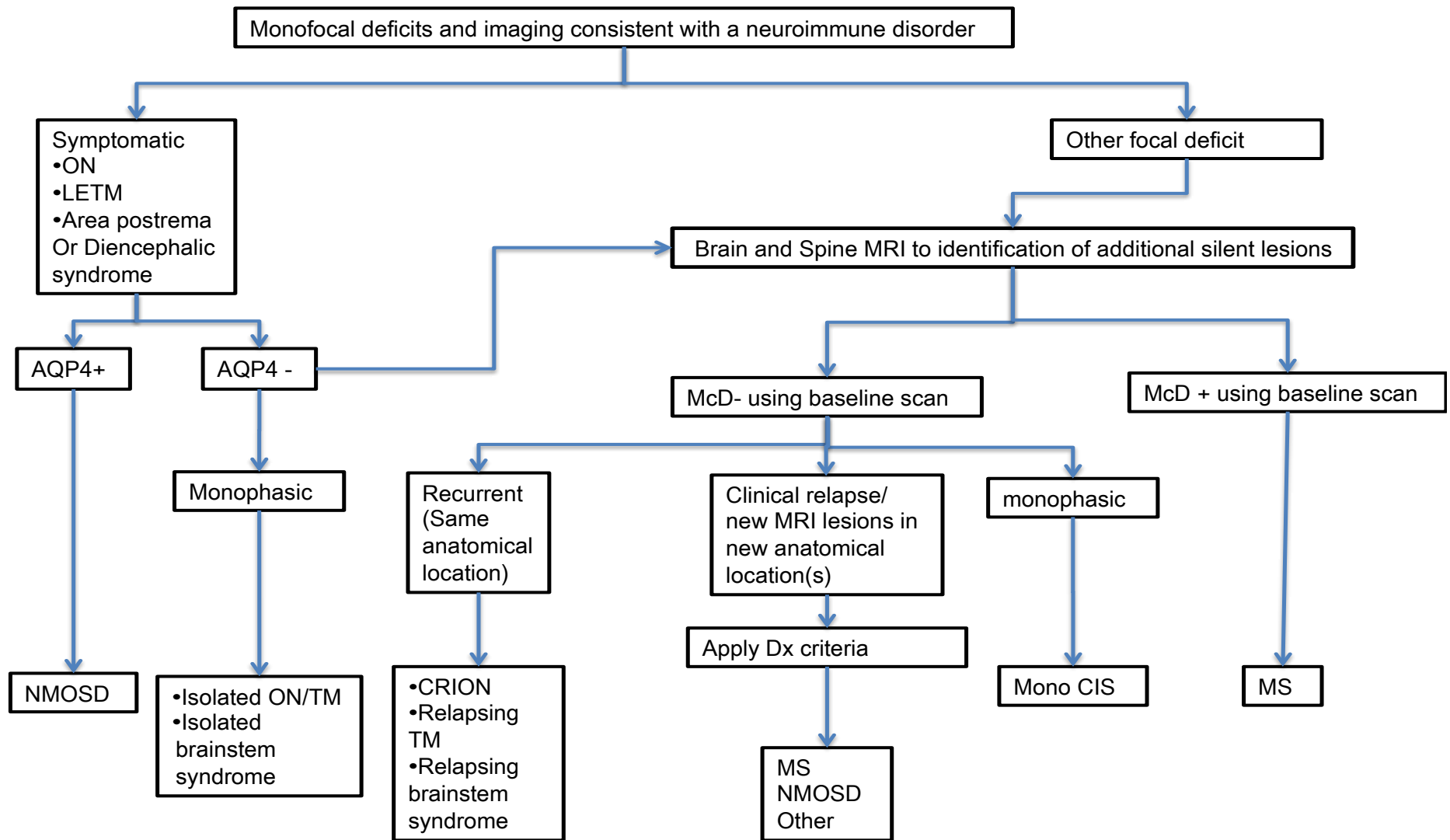


Figure 7.1 Diagnostic algorithm for patients presenting with a monosymptomatic deficit

Abbreviation: CIS clinically isolated syndrome; CRION Chronic relapsing inflammatory optic neuropathy; LETM longitudinally extensive transverse myelitis NMOSD neuromyelitis optica spectrum disorder ON optic neuritis McD McDonald criteria 2010; MS multiple sclerosis. MOG antibodies have not been added to this diagnostic algorithm and are likely to be a useful biomarker for a non-MS demyelinating phenotype in both monophasic and relapsing diseases (Hacohen et al., Neurol Neuroimmunol Neuroinflamm. In press)

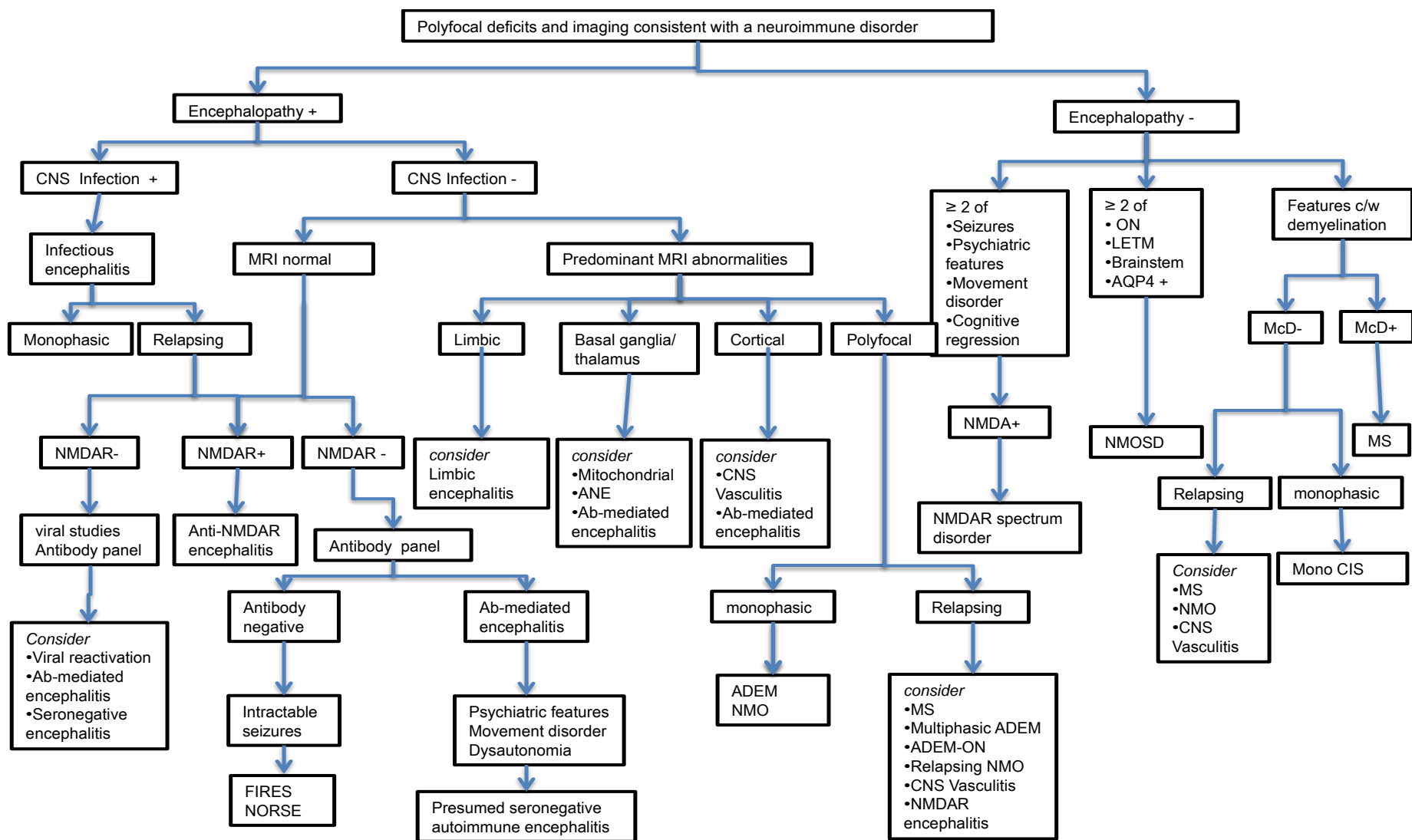


Figure 7.2 Diagnostic algorithm for patients presenting with polyfocal/polysymptomatic deficit

Abbreviation; ADEM Acute Disseminated Encephalomyelitis; ANE Acute necrotizing encephalopathy; FIRES Febrile infection-related epilepsy syndrome; LETM longitudinally extensive transverse myelitis; NORSE New Onset Refractory Status Epilepticus; NMOSD neuromyelitis optica spectrum disorder ON optic neuritis; McD McDonald criteria 2010; MS multiple sclerosis

7.7 *Treatment, outcome, and clinical trials*

The available biological data as well as clinical consensus strongly supports a favourable impact of anti-inflammatory and immunomodulatory therapies for children with neuroimmune disorders. Current therapeutic strategies are largely centre-specific; formal consensus guidelines are yet to be formulated, and no clinical trials have been performed. Meaningful advances in care require: 1) shared clinical data in order to fully appreciate the breadth of clinical severity and time course of different clinical disorders; (ii) shared and standardised care paradigms with consistent definitions of “adequate” and “insufficient” therapeutic responses: (iii) development of rigorous outcome metrics that are appropriate in the paediatric context; and (iv) delineation of biomarkers to predict prognosis and treatment response.

Strategies for treatment may include non-disease specific immunosuppression as well as target therapies. The current strategy for children with acute neuroimmune disorders is to commence treatment promptly with corticosteroids as first line. Although glucocorticoids may be anti-inflammatory, their use can be challenging in patients with CNS inflammation in whom the diagnosis of viral infection cannot be ruled out or in children with prior infection (such as those with HSV encephalitis followed by NMDAR encephalopathy). The interaction of corticosteroids and viruses is complex, and detailed analyses are limited. A recent *in vivo* study looking at viral replication in cell cultures transfected with HSV demonstrated that dexamethasone was associated with increased viral reactivation rate, but that this effect was abolished if dexamethasone was provided in conjunction with acyclovir (Turner et al., 2014). Evidence that corticosteroid administration and concurrent antibiotics reduces neurological disability in immunocompetent children with various forms of meningitis reduces concern regarding safety of corticosteroids in children with possible CNS infection. Other general strategies, such as intravenous immunoglobulins (Dalakas, 2014) and plasma exchange (Weinshenker et al., 1999) have clearly been of benefit in neuroimmune disorders.

Identification of specific antibody and immune cell profiles and appreciation of the pathogenic mechanisms in neuroimmune disorders may yield useful biomarkers for individual prognosis and treatment monitoring (Alvermann et al., 2014). As an example, Eculizumab, a therapeutic monoclonal IgG that neutralizes the complement protein C5, which was recently shown to reduce attack frequency, and stabilise or improve neurological disability measures in patients with aggressive neuromyelitis optica spectrum disorders (Pittock et al., 2013).

In summary, at this point, it remains unknown, why different neurobiological mechanisms can result in similar clinical phenotypes, and conversely why a single pathobiologic mechanism can produce a spectrum of disease manifestations. It has been shown in individual cases that a sequence of immune events or an underlying genetic predisposition may trigger immune activation in specific clinical syndromes. It can be hypothesized that further understanding of these disease mechanisms would allow us to potentially design therapies that specifically target these.

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