Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN
SCIENZE BIOMEDICHE E NEUROMOTORIE

Ciclo XXXI

Settore Concorsuale di afferenza: 06/D6

Settore Scientifico Disciplinare: MED/26

ANTIBODIES AGAINST NEURONAL SURFACE PROTEINS IN CENTRAL NERVOUS SYSTEM DISORDERS

Presentata da: Dott.ssa Maria Pia Giannoccaro

Coordinatore Dottorato **Supervisore**

Prof. Pietro Cortelli **Prof. Rocco Liguori**

Esame finale anno 2019

Abstract

Over the last two decades, the discovery of antibodies directed against neuronal surface antigens in patients with different forms of encephalitis has provided a basis for immunotherapies in previously undefined disorders. These important findings have raised questions regarding the role of neuronal antibodies in patients with other possibly immune-mediated diseases. Nevertheless, the pathogenicity of specific neuronal surface antibodies has not been adequately demonstrated in animal models.

The presence of neuronal surface antigens in other disorders was examined by three approaches in patients with narcolepsy type 1, in patients with chronic neurological conditions (temporal lobe epilepsy and patients with neurodegenerative disorders), and in healthy and other disease controls. To establish an animal model, antibodies against contactin-associated protein 2 (CASPR2) were injected intraperitoneally daily into mice that were given a single lipopolysaccharide injection to open the blood brain barrier. Behavioural performance was studied over five days, and the mouse brains carefully investigated for presence of bound antibodies and neuropathological changes.

Overall, patients with central nervous system disorders showed a higher frequency of antibodies compared to controls, but no antibodies specific to any one disorder were identified. Mice with high serum CASPR2 antibodies showed altered working memory and anxiety-like behaviours only in a social context. There were human immunoglobulins bound to the brain parenchyma along with a mild Purkinje cell loss and astrocytosis in the cerebellum, increased c-fos expression in the piriform-entorhinal cortex and hypothalamus, and microglial and astrocyte activation. These results not only support a pathogenic role for CASPR2 antibodies but provide the first demonstration in this field that a brief opening of the blood brain barrier is sufficient to allow access of antibodies into the brain with behavioural and neuropathic consequences. These findings are of relevance to other neuronal antibodies and stimulate further work in the field.

Acknowledgments

This thesis was made possible through the help, support, spiritual and material, and encouragement I received from many people I met during these years, to whom I will forever be indebted.

The work of this thesis has been entirely produced at the Nuffield Department of Clinical Neurosciences of the Oxford University, and I will always be grateful for the generosity and scientific support from the Department.

First and foremost, I would like to thank my supervisors, Prof Angela Vincent and Prof Rocco Liguori. Prof Liguori opened the possibility for me to go to Oxford and to join such a fantastic lab and wonderful city, where I developed my lab skills and my love for neuroimmunology, supporting me from the distance in any possible way and preparing the bridge for my future. However, none of this work would have been possible without Angela's unconditional and continuous support and inspirational figure. She gave me freedom to explore my own ideas, even when they were different from hers or the results were disappointing, but she never stopped her enthusiastic guidance. Working with her, seeing her passion for science, always accompanied by the care of the people around her, has been and will always be, truly inspirational.

I was very fortunate to have met and worked with Drs Ester Coutinho and David Menassa who both provided friendship and enthusiasm. Without our endless coffee breaks, many of the good ideas that ended up in this thesis would have not been possible. Ester was the first person to show me around the lab and the first to patiently teach me most of what I have learnt in the lab, without request or obligations. David taught me all the secrets of histology and confocal imaging.

I am extremely grateful for the help I received from all the other members of the neuroimmunology lab. I would like to acknowledge in particular Dr Leslie Jacobson who has always been extremely patient and helpful, both in the study of human cohorts and in the animal model experiments. Without his deliberate decision to support my experiments and to spend the Christmas and New Year holidays injecting mice, little of this work would have been possible. I want to thank also Prof Bethan Lang, under whose licence these experiments were done.

My acknowledgments go to all the other people who shared ideas, advice and experience. In particular, I want to thank Dr John Dawes for sharing his experience on the CASPR2 animal model. However, most of the patients' work would have never been possible without the clinicians. I particularly want to thank Prof Giuseppe Plazzi for sharing the narcolepsy samples and Dr Fabio Pizza who sent the cases and shared the clinical information, Dr Avoni for the epilepsy patients, Dr Sabina Capellari for the neurodegenerative patients and Dr Veria Vacchiano for collecting their clinical data and, obviously, Prof Rocco Liguori, who coordinated all this work. Apart from sharing their work with me, they have been a family, supporting me from the distance.

And last but not least, my gratitude goes to those in the backstage who made it all possible: my family and my friends, close and far.

Table of contents

List of figures

List of tables

Abbreviations

125I- αDTX Iodinated alpha-dendrotoxin
AA antibiotic-antimycotic
AARs Alternate arm returns
Abs Antibodies
ACh Acetylcholine
AChR Acetylcholine receptor
AD Alzheimer's disease
ADLD Autosomal dominant leukodystrophy
ADLTE Autosomal dominant lateral temporal lobe epilepsy
AE Autoimmune encephalitis
AEDs Antiepileptic drugs
ALS Amyotrophic lateral sclerosis
AMPAR Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANCOVA Analysis of covariance
ANOVA Analysis of variance
ASO Anti-streptolysin O
AQ4 Aquaporin 4
AR Accelerating rotarod
BBB Blood-brain barrier
BBMD: brief battery for mental deterioration
BSA Bovine serum albumin
CASPR2 Contactin-associated protein-like 2 (protein)
CBA Cell-based assay
CBD Corticobasal syndrome
CJD Creutzfeldt-Jakob disease
CMAP Compound muscle action potential
CNS Central nervous system
CNTNAP2 Contactin-associated protein-like 2 (gene)
CSA Continuous spontaneous alternation
CSF Cerebrospinal fluid
D2R Dopamine 2 receptor
DAPI 4',6-Diamidino-2-Phenylindole, Dihydrochloride
DLB Dementia with Lewy body
DMEM Dulbecco's modified eagle's medium
DNER δ/notch-like epidermal growth factor–related receptor
DPPX Dipeptidyl-peptidase-like protein-6
DRG Dorsal root ganglia
EAMG Experimental autoimmune myasthenia gravis
EDS Excessive daytime sleepiness
EEG Electroencephalogram
EGFP Enhanced green fluorescent protein
EHC Elderly healthy controls
ELISAs Enzyme-linked immunosorbent assays
EPHB2R Ephrin-B2 receptors
ESS Epworth sleepiness scale
FA Forced alternation test
FBDS Facio-brachial dystonic seizures
FCS Foetal calf serum

FTD Frontotemporal dementia
$GABA_AR$ Gamma-aminobutyric acid A receptor
$GABA_BR$ Gamma-aminobutyric acid B receptor
GAD-65 Glutamic acid decarboxylase 65
GAPDH Glyceraldehyde-3-phosphate dehydrogenase
GFAP Glial fibrillary acidic protein
GlyR Glycine receptor
HBSS Hanks's Buffered Saline Solution
HC Healthy control
HCRT1 and 2 Hypocretin 1 and 2
HCRTR1 and 2 Hypocretin receptor 1 and 2
HEK Human embryonic kidney
HEPES N-(2-hydroxyethyl)piperazine-N'-2-thanesulphonic acid
H&L Heavy and light chain
HL Hodgkin's lymphoma
HLA Human leukocyte antigen
HSV Herpes Simplex virus
ICV Intraventricular
Ig Immunoglobulin
IgG Immunoglobulin G
IgM Immunoglobulin M
IgLON5 Immunoglobulin-Like Domain-Containing Protein family member 5
IHC Immunohistochemistry
IP Intraperitoneally
IS Inverted screen test
IvIg Intravenous immunoglobulin
LDb Light-dark box test
LE Limbic encephalitis
LGI1 Leucine-rich glioma-inactivated 1
LPS Lipopolysaccharide
LRP4 Low-density lipoprotein receptor-related protein 4
LTP Long-term potentiation
MBT Marble burying test
MEM Minimum essential medium
MG Myasthenia gravis
mGluR1 Metabotropicpic glutamate receptor 1
mGluR5 Metabotropicpic glutamate receptor 5
mEPSC Miniature excitatory postsynaptic currents
MMSE Mini-mental state examination
MOG Myelin oligodendrocytes glycoprotein
MoS Morvan's syndrome
MRI Magnetic resonance imaging
MSA Multiple system atrophy
MTLE-HS Mesial temporal lobe epilepsy with hippocampal sclerosis
MTS Mesial temporal sclerosis
MuSK Muscle specific kinase
NB Narrow beams
NBS Neurobasal medium
NeuN Neuronal Nuclei

NGS Normal goat serum
NMDAR N-methyl-D-Aspartate receptor
NMJ Neuromuscular junction
NMT Neuromyotonia
NOR Novel object recognition
NSA Neuronal surface antigen
NSA-Abs Neuronal surface antigen-antibodies
NT1 and 2 Narcolepsy type 1 and type 2
NRX1 Neurexin 1α
OBs Oligoclonal bands
OCT Optimum citing temperature
OF Open field
OT Olfaction test
PBS Phosphate buffered saline
PBS-T PBS + Triton-X-100
PC Purkinje cells
PD Parkinson's disease
PDD Parkinson's disease-related dementia
PDDy PD with dysautonomia
PEI Polyethylenimine
PERM Progressive encephalopathy with rigidity and myoclonus
PFA Paraformaldehyde
PI Preference index
PLL Poly-L-lysine
PNS Peripheral nervous system
PPA primary progressive aphasia
PSP Progressive supranuclear palsy
RBD REM sleep behavior disorder
RIA Radioimmunoassay
RT Room temperature
SAP Spontaneous alternation performance
SARs Same arm returns
SCLC Small cell lung carcinoma
SD Standard deviation
SEM Standard error of mean
SNMG Seronegative myasthenia gravis
SPS Stiff person syndrome
TBS Tris-buffered saline
TPO Thyroid peroxidase
UD Undetermined dementia
UP undetermined parkinsonism
VGKC Voltage-gated potassium channel
WB Western blot
WBC white blood cells

Chapter 1: Introduction

"…the organism possesses certain contrivances by means of which the immune reaction, so easily produced by all kinds of cells, is prevented from acting against the organism's own elements and so giving rise to autotoxins…so that we might be justified in speaking of a "horror autotoxicus" of the organism. These contrivances are naturally of the highest importance for the individual."

With these words, in 1901, Paul Ehrlich proposed for the first time the idea that individuals *might* make antibodies that were toxic to themselves, posing the basis for the concept of autoimmunity (Ehrlich P, 1957). The mechanisms' "contrivances" were subsequently defined as "tolerance".

As Burnet theorised "The need and the capacity to distinguish between what is acceptable as self and what must be rejected as alien is the evolutionary basis of immunology" (Burnet M, 1969). Tolerance is achieved through central and peripheral mechanisms. Central tolerance is established in the thymus, where developing lymphocytes undergo positive selection in the cortex before maturing and entering the circulation; in the meantime, lymphocytes with potential reactivity against self-peptides are negatively selected and deleted in the thymic medulla. Once in the periphery, mature T cells are subjected to a secondary selection (peripheral tolerance) by which the majority of self-reactive T cells are deleted or rendered anergic. If immature B cells express surface IgM that recognises ubiquitous self-cell-surface antigens, they are eliminated by a process known as clonal deletion. Mature B cells are also under the control of peripheral tolerance.

However, despite the strict vigilance of central and peripheral tolerance, small numbers of potentially self-reacting lymphocytes can still 'escape' into the periphery, even in otherwise normal individuals. The existence of these potential self-reactive T and/or B lymphocytes, and the ability of the B cells to produce autoantibodies, does not necessarily lead to pathology (Salinas GF et al. 2013). Accordingly, autoimmunity can sometimes be classified as 'physiological' (Hang LM et al. 1997; Avrameas S and Selmi C, 2013). Physiological autoimmunity is usually transient, without evidence of clinical disease. This is exemplified by the presence of naturally occurring autoantibodies (Panda S and Ding JL, 2015), which help eliminate degraded self- and foreign antigens for maintenance of homeostasis. Therefore, to be defined as disease, the immune reaction needs to be associated with damage to the target tissue.

1.1 How to define autoimmunity: The Witebsky's criteria

Given the existence of naturally occurring auto-antibodies in the population, there is a need to apply specific criteria to establish the autoimmune nature of a disease. These criteria are based on the Koch/Witebsky postulates (reviewed in Rose NR and Bona C, 1993), which were later adapted to antibody-mediated diseases (Drachman DB, 1990), and are based on the following principles:

1. Presence of auto-antibody or self-reactive lymphocytes during the clinical manifestation in the sera or affected tissue;
2. Transfer of pathology by antibodies or lymphocytes from an affected individual to laboratory animals or to cells in culture;
3. Experimental disease must show immunopathological lesions that parallel those in the natural disease.

Three lines of evidence exist for the pathogenicity of autoantibodies:

- Circumstantial - these include clinical observations such as a) presence of genetic susceptibility (i.e recurrence in the same family and HLA association); b) association of antibodies with a specific clinical phenotype; c) response to immunotherapy.
- Indirect - reproduction of the autoimmune disease in experimental animals through active immunisation.
- Direct - from transfer of pathogenic antibody from a human subject to experimental animals.

1.2 Immunoglobulins structure and mechanisms of antibody-mediated autoimmunity

Immunoglobulins (Ig) are made of two heavy (H) and two light (L) chains, where the L chain can consist of either a κ or a λ chain. Each component chain contains one NH2-terminal "variable" (V) domain and one or more COOH-terminal "constant" (C) domains. In humans, Ig are of five different isotypes: M, G, D, A, E, which differ in structure, concentration and functional features. The choice of isotype is dependent upon the antigen itself and the signaling pathways that are activated, as well as the local microenvironment (Janeway CA Jr et al. 2001).

Immunoglobulin-antigen interactions take place between the paratope, the site on the Ig at which the antigen binds, and the epitope, which is the site on the antigen that is bound. *In vivo*, immunoglobulins tend to be produced against intact antigens in soluble form, and thus preferentially identify surface epitopes that can represent conformational structures.

IgGs are the main isotype found in the body and they have the longest serum half-life of all immunoglobulin isotypes. Papain digests IgG into two Fab fragments, each of which can bind antigen, and a single Fc fragment. Pepsin splits IgG into an Fc fragment and a single dimeric F(ab)2 that can cross-link as well as bind antigens.

Based on structural, antigenic and functional differences in the constant region of the heavy chain, IgG are divided into four subclasses, numbered in reference to the rank order (IgG1>IgG2>IgG3>IgG4) of the serum levels of these antibodies in the blood of healthy individuals. The IgG subclasses exhibit different functional activities. Activation of the complement cascade is an important means of clearance of opsonized pathogens. Affinity for C1q, which is the first component of the complement pathway, differs between members of the IgG subclasses, IgG3>IgG1>IgG2, while IgG4 antibodies fail to fix complement. Within the secondary antibody response, there is skewing in the predominant subclass that is induced. For example, IgG1 and IgG3 antibodies are generally induced in response to protein antigens whereas IgG2 and IgG4 are associated with polysaccharide antigens. The response to a given antigen can also result in a skewed IgG subclass response and this is frequently a source of investigation as it can correlate with protection from disease or for the design of vaccines. Specific subclasses can also be associated with individual disease processes. For example, antibodies against LGI1 or MuSK are mainly IgG4.

Fc receptors for immunoglobulin link the humoral immune compartment to the cellular immune compartment. The net result of binding of Ig to the Fc receptor is a function of the receptor, the cell on which it is expressed, and on ancillary signals. Fc receptors for IgG are termed FcγR. In humans, three classes of FcγR have been identified as FcγRI, II and III. IgG subclasses show different affinity for the three classes of FcγR (I, II and III). IgG1 and IgG3 bind to all three FcγR classes. IgG4 binds only FcγRII and III, albeit significantly weaker than the binding of IgG1, and IgG2 binds only to FcγRII.

1.3 Mechanism of antibody mediated damage

Antibodies can affect their targets through different mechanisms (reviewed in Ludwig RJ et al. 2017):

1. Complement activation. Complement is a component of the innate immune system. Antibodies activate complement through the classical pathway, where C1 binds with its C1q subunits to Fc fragments of IgG or IgM, which has formed a complex with the specific antigen.

C4b and C3b are also able to bind to the Fc domains of the antigen-associated IgG or IgM, to its Fc portion. Binding of C1q leads to the subsequent activation of serine protease C1r and C1s, propagating the cascade. Antibody-independent pathways are known as the alternative and lectin pathways. All three pathways converge at the assembly of C3 convertase, a proteolytic enzyme that cleaves C3 in to C3a and C3b molecules. The terminal stage of the complement cascade is the formation and insertion of the membrane attack complex (MAC, also known as C9) into the cell surface, which disrupts the integrity of the cell membrane, resulting in osmotic lysis and destruction of the targeted cell. In addition, cleaved peptide fragments C3a, C4a and C5a are anaphylatoxins and promote inflammation, and C3b fragments bind to the surface of antibody bound cells, and act to mark the cell for phagocytosis.

2. Antibody mediated cell-mediated cytotoxicity (ADCC). This is part of the adaptive immune response by which an effector cell actively lyses a target whose membrane-surface antigens have been bound by specific antibodies. ADCC requires an effector cell which classically is a natural killer (NK) cell; however, macrophages, neutrophils and eosinophils can also mediate ADCC.

3. Neutralization or alteration of function of targeted molecules. Antibodies to cellular receptors can activate intracellular signaling cascades thereby modulating the cellular function or triggering apoptotic pathways.

4. Binding to neurotransmitter receptors and mediating receptor internalization, with consecutive reduction of their surface expression. Antibodies to cell surface molecules can also block functions including cellular interactions with soluble molecules, other cells, or extracellular matrix components and can, thereby, change molecular or cellular function. These are the aspects that are most relevant to this Thesis.

5. Induction of inflammation at the site of autoantibody binding, which occur though recruitment of effector immune cells and relies of cytokines and chemokines.

1.4 The prototype of neurological antibody mediated disorders: myasthenia gravis

Myasthenia gravis (MG) is an autoimmune disorder characterised by muscle weakness and fatigability caused by antibodies directed against components of the postsynaptic muscle endplate localised at the neuromuscular junction (NMJ). Over two-thirds of all patients with MG begin with symptoms localised to their eye muscles (ocular MG) (Berrih-Aknin S and Le Panse R, 2014). The symptoms usually progress to other muscles during the first 2 years,

resulting in generalised MG. Approximately 15% of patients with MG have a thymoma, and 50% of thymoma patients develop MG (Romi F, 2011).

In 1960, Simpson suggested that MG was caused by autoantibodies to the acetylcholine receptor (AChR) (Simpson JA, 1960). Nowadays the pathogenicity of these antibodies is clearly established. Indeed about 90% of generalised MG is caused by pathogenic autoantibodies to muscle nicotinic AChRs. Plasmapheresis leads to a substantial, but temporary, improvement in muscle function (Pinching AJ et al. 1976). In animals, experimental MG (EAMG) can be induced by immunisation with AChR purified from the electric organ of Electrophorus electricus (Patrick J and Lindstrom J, 1973) or by injection of monoclonal antibodies (mAbs) to AChR (Tzartos S et al. 1987). Moreover, injection of patients' IgG or isolated AChR autoantibodies from affected individuals into laboratory animals passively transfers several features of MG (Toyka KV et al. 1977; Kordas G et al. 2014). The pathological autoantibodies are directed at conformation-dependent extracellular epitopes on AChRs, especially the AChR α1 subunits (Vincent A et al. 2001). The predominant isotypes of autoantibodies to AChR are IgG1 and IgG3. These autoantibodies impair neuromuscular transmission primarily by three mechanisms:

1) Focal complement-mediated lysis of the postsynaptic membrane that destroys the postsynaptic membrane that contains the AChRs and, therefore, disrupts synaptic morphology (Engel AG, 1984; Engel AG and Arahata K, 1987);

2) Cross-linking of AChRs by the autoantibodies on the surface of the postsynaptic membrane that accelerates endocytosis and lysosomal destruction of AChRs (Drachman DB et al. 1978);

3) Inhibition of AChR function by direct blockage of acetylcholine (ACh) binding sites (Bufler J et al. 1998) (Figure 1.1).

The loss of AChR numbers or function results in the recruitment of fewer muscle fibres and a decrement in the compound muscle action potential (CMAP).

AChR antibodies are present in around 80% of patients with generalised MG. Around half of those patients without AChR antibodies have antibodies, instead, to muscle-specific kinase (MuSK) (Hoch W et al. 2001). MuSK is an essential component for NMJ formation through its interaction with low-density lipoprotein receptor-related protein 4 (LRP4), a receptor for agrin (a protein secreted from the motor neuron terminal) (Kim N et al. 2008; Zhang B et al. 2008). MuSK mediates clustering and stabilisation of AChR in developing and mature muscle. Patients with MuSK-antibody mediated MG have more focal involvement and wasting of the involved muscles (Vincent A et al. 2003).

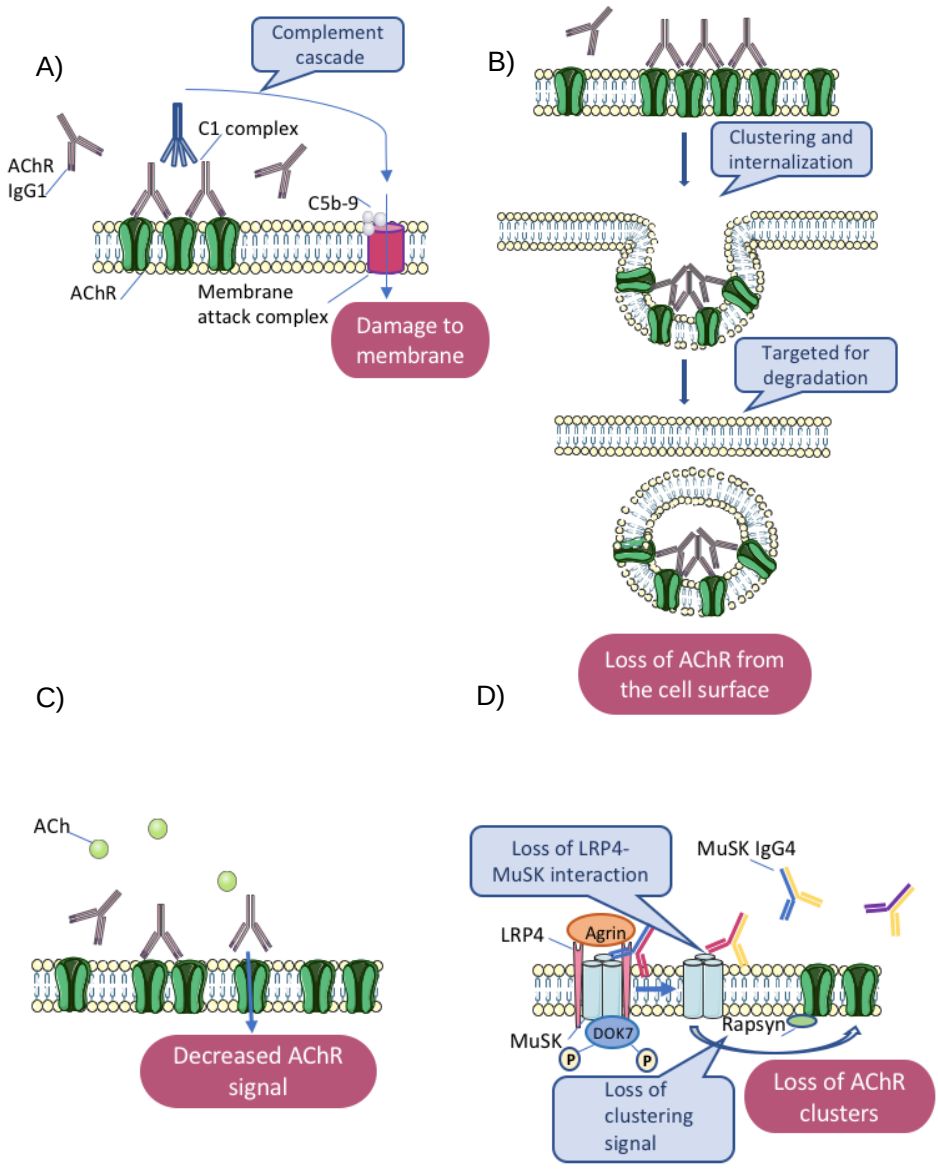

Figure 1. 1 Molecular mechanism of the antibodies associated with MG

A) Immunoglobulin G1 (IgG1) and IgG3 can activate the complement cascade via their Fc domains, which interact with complement proteins C1 and C1q. The complement cascade culminates in the formation of the membrane attack complex which disrupts the phospholipid bilayer, resulting in loss of the junction folds and acetylcholine receptors (AChRs). B) IgG1 and IgG3 can crosslink antigenic targets, leading to internalisation and degradation of the antigen in lysosomes. C) Some AChR autoantibodies can directly block AChR currents. D) Muscle skeletal receptor tyrosine-protein kinase (MuSK) autoantibodies are mainly of the IgG4 subclass. They inhibit agrin-induced binding of low-density lipoprotein receptor-related protein 4 (LRP4) to MuSK disrupting AChR clustering.

Autoantibodies to MuSK are mainly of the IgG4 isotype and the IgG4 fraction alone can transfer disease to mice (Klooster R et al. 2012). The binding of IgG4 autoantibodies to MuSK disrupts its interaction with agrin-bound LRP4 and directly suppress the postsynaptic tyrosine kinase pathway thus indirectly reducing the metabolic stability of endplate AChRs, but IgG1-3 also disrupt this pathway by a different mechanism (Koneczny I et al. 2013) (Figure 1.1).

The knowledge of the antibody mechanisms in MG have promoted the development of novel therapies, particularly therapies based on the inhibition of complement pathways (Soltys J et al. 2009). MG is an ideal disease for antigen-specific immunotherapy because of its clearly defined autoantigen. Theoretically, an antigen-specific immunotherapy would eliminate the pathogenic autoimmune response to autoantigen without affecting the other functions of the immune system, and thus avoiding severe adverse effects. The idea of specific immunosuppression of autoimmune response to AChR by administration of AChR peptides or fragments has been investigated in EAMG for many years. For instance, a vaccine using AChR cytoplasmic domains has been shown to be effective at specifically suppressing EAMG (Luo J and Lindstrom J, 2014), but none of the many published approaches have reached the clinic.

1.5 Antibody-mediated CNS autoimmune disorders

The brain has long been considered an immunologically privileged organ due to the presence of the blood brain barrier (BBB) which ideally prevents the access of antibodies and immune cells from the peripheral circulation. In the late ’60s there was the first description of an autoimmune limbic encephalitis (LE) in three patients with small cells lung carcinoma (SCLC) (Corsellis JA et al. 1968). Subsequently a few more cases were described and different onconeural antigens, such as Hu, Yo, CRMP5 and Ma2, were discovered (Dalmau J and Rosenfeld MR, 2008). The presence of antibodies against intracellular antigens (nuclear or cytoplasmic) have been described in patients with a range of central and peripheral neurological manifestations including LE, cerebellar ataxia and sensory neuropathy, and are commonly associated with an underlying malignancy; they are referred to as paraneoplastic neurological disorders. Despite the presence of an antibody, these disorders are not considered to be antibody-mediated due to the following lines of evidence (reviewed in Dalmau J and Rosenfeld MR, 2008):

1) Intracellular antigens are inaccessible to circulating antibodies;

2) Attempts to transfer human disease by passive transfer of patient IgG or active immunisation of the specific antigen have failed to reproduce neurological features reminiscent

of disease *in vivo*, despite inducing antibody responses (Graus F et al. 1991; Greenlee JE et al. 1995);

3) Patients generally do not improve clinically with immunotherapies aimed to reduce antibody levels.

Indeed, these diseases are considered to be T-cell mediated as supported by neuropathological findings showing extensive cytotoxic T-cell infiltrates and neuronal death in patients' post mortem brain tissue (Dalmau J et al. 1991; Giometto B et al. 1997). These paraneoplastic antibodies are likely to be bystanders of a T-cell immune response; however, their detection is important for the identification of an underlying malignancy. Since these antibodies are not pathogenetically relevant, they will not be discussed further.

Neuromyotonia (NMT), also known as Isaacs' syndrome, is a rare disorder characterised by spontaneous and continuous muscle activity at rest, which manifests clinically as visible muscle twitching (90%), cramps (70%), myopathy, stiffness and impaired muscle relaxation (reviewed in Maddison P, 2006). Indirect evidence for an autoimmune aetiology came from the demonstration that plasma exchange significantly reduced the number of neuromyotonic discharges, and injection of the antibodies into mice (passive transfer) produced a raised threshold to d-tubocurarine in the diaphragm muscle (Sinha S et al. 1991; Newsom-Davis J and Mills Kr, 1993). In 1995, Shillito P et al. developed a radioimmunoprecipitation assay (RIA) using mammalian brain extract as a concentrated source of Kv1 type voltage-gated potassium channels (VGKCs). VGKCs were labelled with iodinated alpha-dendrotoxin (125I-αDTX), a neurotoxin derived from the venom of the Dendroaspis angusticeps (Green mamba snake) which binds specifically to the Kv1.1, 1.2 and 1.6 subtypes of the Shaker channel family (Scott VE et al. 2002). The authors found that three of six patients studied precipitated 125I-αDTX counts that were significantly higher than controls levels (Shillito P et al. 1995), indicating that their antibodies had bound to the VGKC or related proteins.

In 2001, Liguori et al. reported a 76-year-old man with Morvan's syndrome (MoS) and antibodies against VGKCs (Liguori R et al. 2001). The striking response to plasmapheresis, the presence of oligoclonal bands in the cerebrospinal fluid (CSF), and the absence of morphological alterations but suggestion of diffusion of IgG into the thalamus and striatum at postmortem, when the patient died subsequently, all supported an antibody-mediated basis for the condition. This began a shift from the paradigm that only peripheral antibodies could cause disease, to recognition that antibodies could also affect the central nervous system. This was supported by the finding of VGKC-antibodies in patients with limbic encephalitis, mainly

without tumours or onconeural antibodies, first in two patients (Buckley C et al. 2001) and then in two small case series (Vincent A et al. 2004; Thieben MJ et al.2004).

In 2005, the field began to extend to other antibody-mediated CNS diseases. Seven patients with subacute limbic encephalitis were described, six with antibodies that bound the neuropil of hippocampus or cerebellum on rat brain sections and one to an intracellular antigen (Ances BM et al. 2005). Only in one case was the pattern of reactivity compatible with VGKC-antibodies whereas in the other cases the antigens appeared diverse, but all expressed on the neuronal cell membrane and dendrites. All patients, except the one with antibodies to intracellular antigens, had dramatic clinical and neuroimaging responses to immunotherapy or tumour resection. On the basis of these features the authors suggested a classification of antibody-associated encephalitis with implications for prognosis and treatment. One group comprises patients with antibodies to intracellular antigens including most of the previously characterised paraneoplastic antibodies, whereas the other comprised patients with antibodies that react with the brain neuropil and includes VGKC antibodies and the collectively termed 'novel neuropil antibodies'.

Soon after, a new disorder, presenting with prominent psychiatric symptoms, seizures, memory deficits and decreased level of consciousness in young women, in association with ovarian teratomas, was described (Vitaliani R et al. 2005). This disorder was considered to represent a new category of severe but treatment-responsive paraneoplastic encephalitis and further studies led to the identification of the antigen as the GluN1 subunit of the N-methyl-D-aspartic acid receptor (NMDAR) (Dalmau J et al. 2007).

In 2010, it was found that the VGKC antibodies were actually directed against two proteins that co-precipitate with the Kv1 subunits, contacting-associated protein 2 (CASPR2) and leucine rich glioma inactivated protein 1 (LGI1) (Irani SR et al. 2010), rather than the VGKC itself. In the following years, in the effort of elucidating the cause of previously considered idiopathic encephalitis, several new neuronal surface antigen-antibodies (NSA-Abs) involved in autoimmune encephalitis were identified, each one associated with partly characteristic clinical features, as summarised in Table 1.1. In the meantime, more and more studies focused on demonstrating the pathogenic role of the antibodies and on elucidating the underlying pathogenetic mechanisms (summarised in Table 1.2). In the following paragraphs the methods of detection, the triggering factors and the main antibodies against neuronal surface antigens (NSAs) will be described (Figure 1.2).

Table 1. 1 Neuronal surface antibody-mediated autoimmune encephalitis

Antigen	Main clinical syndromes	Other syndromes	CSF features	Associations	HLA	Equivalent genetic syndrome
	Antibodies against synaptic receptors					
N-methyl-D-aspartate receptor (NMDAR)	NMDAR encephalitis: psychiatric syndrome, seizures, amnesia, movement disorders catatonia, autonomic instability	Few cases with purely psychotic features; few in cryptogenic epilepsy syndromes	Lymphocytosis in early stages (70%) and OBs after (>50%); Abs usually present	Ovarian teratoma in about 50%; post-HSV encephalitis	Unknown	*GRIN1* mutations (encoding for GluN1) associated with severe intellectual disability, seizures, movement disorders and dysmorphic features
Glycine Receptor (GlyR)	PERM, SPS	LE, brainstem encephalitis; cryptogenic epilepsy	Pleocytosis in half of the cases, OBs (20%)	Thymoma (<10%)	Unknown	*GLRA1* (encoding α1 subunit) or *GLRB* (encoding β subunit) associated with hereditary hyperekplexia
α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor (AMPAR)	LE	Psychosis	Lymphocytosis; OBs; abs usually present	Tumor in 50% cases (lung, thymoma, breast)	Unknown	*GRIA2* (subunit 2) and *GRIA3* (subunit 3) mutations associated with intellectual disability and autism
Gamma-aminobutyric acid A receptor ($GABA_AR$)	LE with prominent seizures	Psychiatric syndromes; various presentation including SPS, opsoclonus, ataxia	OBs; abs can be absent in the CSF	Tumor in 70% cases (thymoma, lung, and breast)	Unknown	*GABRA1*, *GABRB3*, *GABRG2* and *GABRD* associated with different idiopathic epilepsy syndromes
Gamma-aminobutyric acid B receptor ($GABA_BR$)	LE	Ataxia, opsoclonus, status epilepticus	Common pleocytosis; rare OBs	Tumour in 60 % (mainly lung)	Unknown	No
Dopamine receptor 2 (D2R)	Basal ganglia encephalitis with movement disorders and psychosis	Sydenham chorea and Tourette syndrome	Pleocytosis	Streptococcus infections	Unknown	Mutations associated with myoclonus dystonia or schizophrenia
Metabotropic glutamate receptor 1 (mGluR1)	Cerebellar ataxia		Common pleocytosis; rare OBs	Usually associated with tumors (HL and T cells lymphoma)	Unknown	*GRM1* mutations associate with psychiatric disorders and epilepsy
Metabotropic glutamate receptor 5 (mGluR5)	Ophelia syndrome (limbic encephalopathy in patients with HL)	Schizophrenia (0.1% cases)	Lymphocytosis; abs presence unknown	Hodgkin lymphoma (2/2 patients)	Unknown	No

Continuation table 1.1

	Antibodies against synaptic proteins or other cell surface proteins					
Leucine-rich glioma inactivated 1 (LGI1)	LE with or without FBDS and or hyponatremia	Cryptogenic epilepsy	Usually normal, rare OBs; Abs can be absent	Tumor in 10% cases	DRB1*07:01 DQB1*02:02 haplotype (in HLA class II genes, as well as with B*44:03 and C*07:06 in the HLA class I region	Mutations associated with autosomal dominant lateral temporal lobe epilepsy (ADLTE) with prominent auditory seizures
Contactin-associated protein like 2 (CASPR2)	LE, MoS, NMT	Cerebellar ataxia, movement disorders, cryptogenic epilepsies, Guillain-Barre–like syndrome	Usually normal; rare OBs; abs can be absent	Tumor in 30% cases (mainly thymoma)	Unknown	Mutations associated with autism, epilepsy and intellectual disability
Dipeptidyl-peptidase-like protein-6 (DPPX)	Cognitive impairment, brainstem symptoms and diarrhea	Cerebellar ataxia, PERM	Pleocytosis OBs; Abs usually present	B cells tumour (<30% cases)	Unknown	Mutations associated with susceptibility to ALS and with autism or familial ventricular fibrillation
Ig-Like Domain-Containing Protein family member 5 (IgLON5)	NREM sleep disorder, abnormal movement and behaviours obstructive sleep apnoea and stridor, occasional gait instability and brainstem symptoms	Dementia, movement disorders; isolated dysphagia	Pleocytosis Abs usually present	Tauopathy	HLA-DRB1*1001 and HLA-DQB1*0501 (all patients investigated)	No
Neurexin3α	Prodromal fever, headache or GI symptoms, followed by confusion, seizures, and decreased level of consciousness		Pleocytosis	No	Unknown	Genetic variation at this locus has been associated with a range of behavioral phenotypes

Abbreviations: Abs: antibodies; ALS: amyotrophic lateral sclerosis; CSF: cerebrospinal fluid; HL: Hodgkin's lymphoma; HLA: human leukocyte antigen; HSV: Herpes Simplex virus; FBDS: facio-brachial dystonic seizures; GI: gastrointestinal; LE: limbic encephalitis; MoS: Morvan's syndrome; NMT: neuromyotonia; OBs: oligoclonal bands; PERM: progressive encephalopathy with rigidity and myoclonus; SPS: stiff person syndrome.

Table 1. 2 Molecular and pathophysiological mechanisms of antibodies to NSA

Target	Main epitope	Other epitopes	IgG subclasses and other Ig classes	Mechanism of Abs	Functional consequences	Passive transfer
NMDAR	GluN1	GluN2a-2b	IgG1; IgA, IgM	Cross-linking and internalisation with reversible reduction in cluster density; surface receptors laterally displaced out of synapse	Reduced NMDAR currents, reduced LTP and hyper-glutamatergic state; reduction in strength of interaction between NMDAR and ephrin-B2 receptors	Yes
GlyR	α1 subunit		IgG1	Cross-linking and internalisation in HEK cells	Unknown	No
AMPAR	GluA1, GluA2		N.A.	Internalisation and degradation with reduction of surface synaptic AMPAR	Decreased AMPAR-mediated currents; changes in the pattern of action potential firing in neurons	No
$GABA_AR$	α1 subunit	β3/γ2	IgG	Cross-linking and internalisation with selective reduction of $GABA_A$ receptor clusters at synapses	Unknown	No
$GABA_BR$	R1 subunit		mainly IgG1	Unknown	Unknown	No
Kv4.2	DPPX		IgG; no	Unknown but reduced expression of DPPX and Kv4.2 in hippocampal neurons	Hyper-excitability of enteric neurons	No
D2R	D2		IgG	Unknown	Unknown	No
IgLON5	Ig-like domain 2		IgG4 but also IgG1	Internalisation with reduction of IgLon5 expression	Unknown	No
mGluR1	mGluR1		IgG	Unknown	Reduced mGluR-mediated current in cerebellar slices	Yes

Continuation of table 1.2

mGluR5	mGluR5		IgG; unknown	Unknown	Unknown	No
LGI1	LRR and EPTP repeat domains		IgG4, rarely IgG1	Unknown but disruption of the interaction with ADAM22 and reduction of AMPAR; possible complement activation	Increased spontaneous depolarisations in hippocampal CA3; enhanced hippocampal mossy fibre to CA3 pyramidal cell transmission	No but reported cats with epilepsy and LGI1 abs
CASPR2	Discoidin domain	Lam1, Lam2, Egf1 domains	IgG4 but also IgG1	Alteration of the interaction with contactin2; possible complement activation	Reduction of hippocampal synaptic gephyrin clusters/ disruption of inhibitory synaptic contacts of GABAergic neurons; reduction of Kv1.1 expression on DRG neurons with hyperexcitability	PNS symptoms peripheral administration model; model of maternal-fetal transfer
Neurexin3α	Unknown		IgG	Reduced expression and reduced synaptic number	Unknown	No

DRG: dorsal root ganglia; HEK: human embryonic kidney cells; LTP: long-term potentiation; PNS: peripheral nervous system.

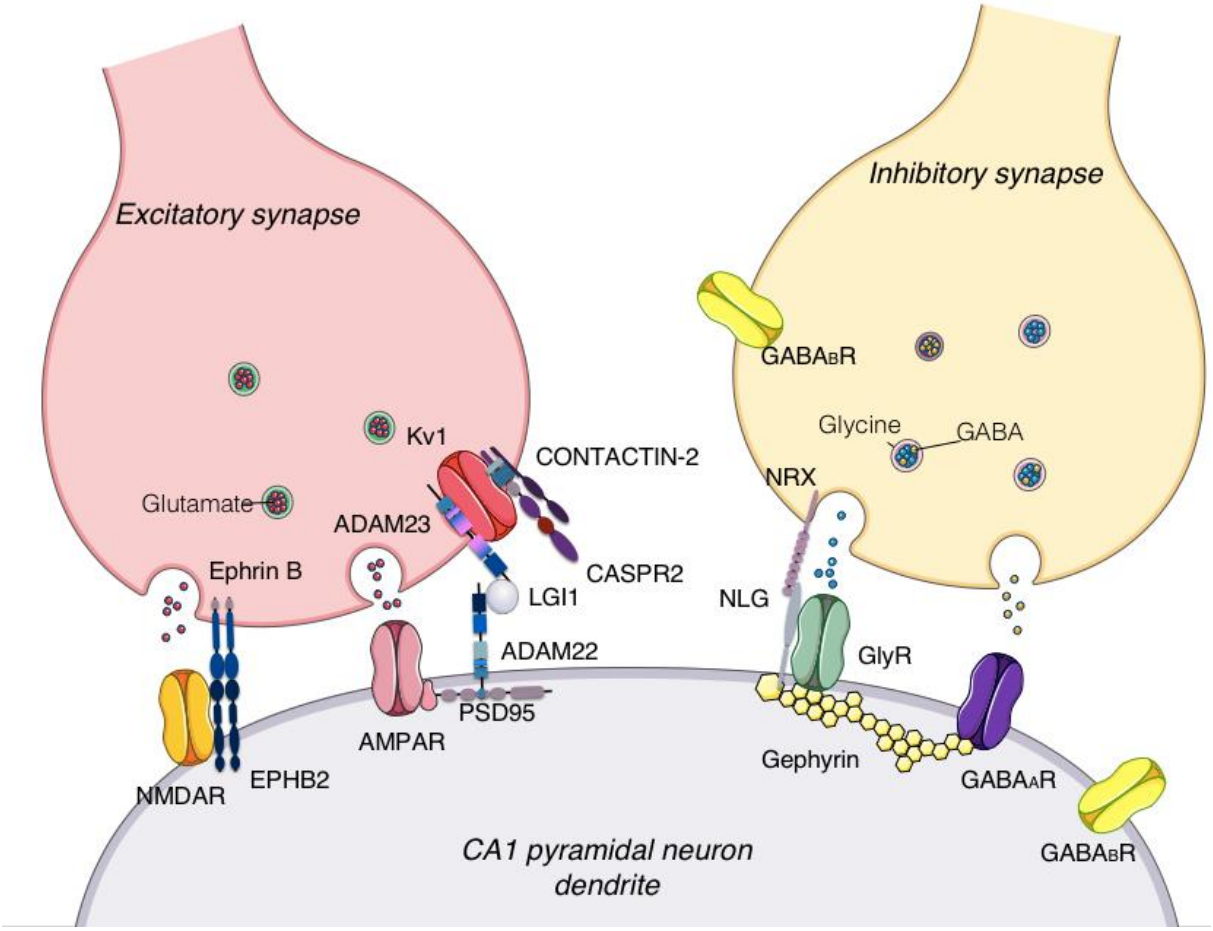

Figure 1. 2 Schematic representation of central excitatory and inhibitory synapses and main antibodies targets

The proteins targeted by antibodies associated with autoimmune encephalitis are proteins and channels expressed on the neuronal surface, often at both pre- and post-synaptic levels on inhibitory (gabaergic) and/or excitatory (glutamatergic) neurons in the CNS (adapted from Giannoccaro MP et al. 2018).

1.5.1. Methods of detection of NSA-Abs

Since the antibodies directed against NSA-Ab are directed against extracellular portions of the protein and its conformational epitopes, it is important that the method of detection of these antibodies preserves the native structure of the protein. Cell-based assays (CBAs), which measure the binding visually (Figure 1.3) or by flow cytometry, using live cells that preserve the structure of membrane proteins, meet these requirements. CBAs have been shown to be superior for the detection of NSA-Ab compared to other methods such as western blots (WB) or peptide enzyme-linked immunosorbent assays (ELISAs), in which the proteins are not necessarily conformational (Waters PJ et al. 2012).

CBAs and flow cytometry use live cells transiently transfected with the protein of interest and then incubated with patient's serum, and antibody binding is detected by a secondary immunofluorescent anti-human antibody. This method is, of course, only valid for known antigens.

In order to identify new antigenic targets two commonly used methods are:

1) Immunohistochemistry on rat brain sections: antibodies to neuronal antigens usually bind the neuropil, each with a specific pattern;

2) Immunofluorescence on hippocampal rat neurons in culture: the majority of NSA-Ab recognise protein expressed on hippocampal neurons.

If binding is found with these screening methods, the following steps are the identification of the target by mass spectroscopy and the establishment of a new specific CBA.

1.5.2. Triggering causes of autoimmune encephalitis

Tumours

NSA-Ab can be associated with cancer and the frequency and type of tumour varies accordingly to the type of disease and the autoantibody. Some autoimmune encephalitis rarely associates with tumours, as in the case of LGI1 encephalitis (Irani SR et al. 2010; Lai M et al. 2010), whereas others show strong association with cancer such as small cell carcinoma with $GABA_B$ receptor antibodies, or teratoma with NMDAR encephalitis (Hoftberger R et al. 2013; Jeffery OJ et al. 2013; Dalmau J et al. 2007). In the cases with tumours, the neoplastic cells express the neural surface antigen against which the antibodies are directed. Therefore, it is thought that the ectopic expression of neuronal proteins by the tumour breaks immune tolerance for these proteins contributing to the development of the immune response (DeLuca I et al. 2009).

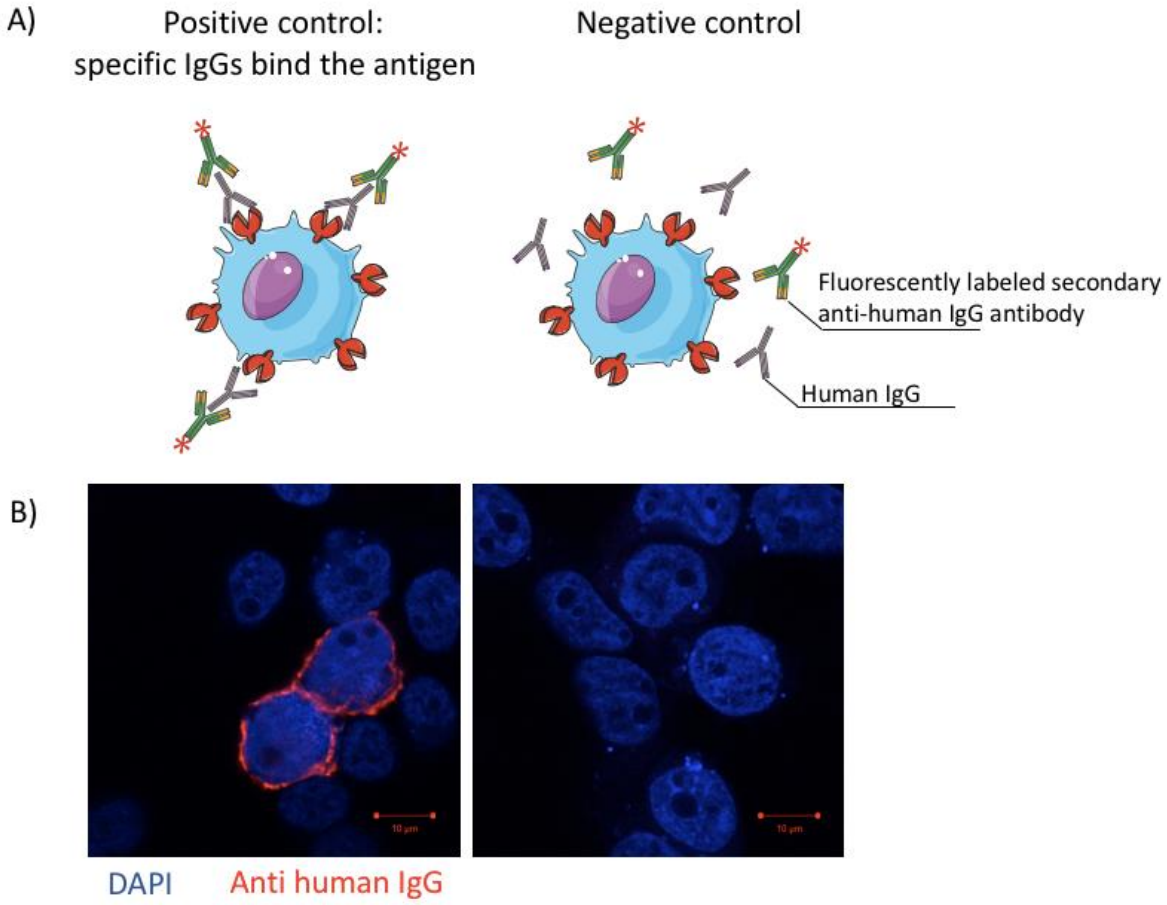

Figure 1. 3 Schematic representation of a cell-based assay (CBA)

A) Schematic representation of human embryonic kidney (HEK) cells expressing a protein of interest after transfection. On the left: an example of positive serum containing specific IgG directed against the target protein; bound IgG antibodies on the cell surface are detected by a secondary anti-human IgG antibody. On the right: a negative serum does not contain antibodies specific for that antigen. B) Microphotographs of HEK cell from a positive (left) and negative (right) control.

In the case of ovarian teratoma, the tumour itself contains mature or immature neural tissue (Dalmau J et al. 2007) which express NMDARs that likely contribute in triggering the autoimmune response. In line with this hypothesis, the tumours of these patients contain larger amounts of inflammatory infiltrates compared with the teratomas from patients who do not develop anti-NMDA receptor encephalitis (Tuzun E et al. 2009).

Herpes Simplex and other viruses

Several recent reports describe the occurrence of NSA-Abs within weeks of herpes simplex virus (HSV) infection of the CNS (Armangue T el al. 2015, Armangue T et al. 2013; Hacohen Y et al. 2014). Most cases show new synthesis of antibodies against NMDAR and less frequently against other receptors or NSA ($GABA_A$ receptors, dopamine receptors, uncharacterised cell surface proteins) (Armangue T el al. 2015; Hacohen Y et al. 2014; Mohammad SS et al. 2014). In these cases, treatment with immunotherapy rather than viral therapy associates with neurological improvement. This observation suggests that autoantibodies may be generated in the context of inflammation and neural damage caused by a primary viral infection. Indeed, a possible link between other infections and anti-NMDA receptor encephalitis has been suggested (varicella zoster virus, mycoplasma), but the number of cases is too small to confirm this association (Gable S et al. 2009; Schabitz WR et al. 2014).

In a substantial number of patients with autoimmune encephalitis (AE), the trigger of the autoimmune response is unknown; in these cases, screening studies for an occult tumour or recent viral infections are negative. The young age and lack of tumour risk factors in some patients, as well as long clinical follow-up during which patients remain tumour free, support the existence of other unknown immunological triggers. The fact that some patients have other autoantibodies with a frequency higher than expected in the normal population suggest an underlying predisposition to autoimmunity (Florance NR et al. 2009; Lai M et al. 2009; Tuzun E et al. 2011).

Genetic susceptibility to develop AE has only been investigated in a few of these disorders. Recent studies show specific human leukocyte antigen (HLA) associations in patients with LGI1 and IgLON5 antibodies (Gelpi E et al. 2016; Van Sonderen ARD et al. 2017). LGI1-antibody associated encephalitis with HLA-DR7 and HLA-DRB4 was identified. Interestingly, this haplotype association did not appear to apply to patients who developed the disorder in the context of a systemic tumour, suggesting that the absence of those haplotypes could raise suspicion for an underlying tumour or paraneoplastic mechanism (Van Sonderen ARD et al. 2017; Binks S et al. 2018).

1.5.3. Anti-NMDAR encephalitis

Anti-NMDAR encephalitis, the classical syndrome associated with NMDAR antibodies, is the most commonly recognised AE. Up to 70% of patients have prodromal symptoms consisting of headache, fever, nausea, vomiting, diarrhoea, or upper respiratory-tract symptoms. Psychiatric symptoms, such as memory problems, confusion, abnormal behavior, paranoia and hallucinations may also be present, in addition to seizures, dyskinesia, autonomic instability, catatonia, hypoventilation, lethargy and language deficits. Hypoventilation and memory problems appear more frequently in adults (Dalmau J et al. 2011). Although initially only recognised as a neurological syndrome in the context of ovarian teratoma, it is now known that the majority of patients have no neoplasia (around 60-70%) and that the disease can affect men and women of all ages (Irani SR, Bera K et al. 2010, Titulaer MJ et al. 2013).

Despite the severity of the clinical picture, brain MRI is normal in approximately half of patients, with hyperintensity visible in the other half (Dalmau J et al. 2011; Dalmau J et al. 2008; Irani SR, Bera K et al. 2010). However, EEG often show diffuse slow and disorganized activity, and some epileptic discharges, and CSF analysis reveals lymphocytosis early in the disease, sometimes associated with raised protein levels (Dalmau J et al. 2008; Dalmau J et al. 2011; Irani SR, Bera K et al. 2010). As the disease progresses, CSF-specific oligoclonal bands and evidence of intrathecal NMDAR autoantibody synthesis can be detected (Irani SR, Bera K et al. 2010). The prognosis of NMDAR-encephalitis is largely time-dependent, including time to diagnosis, time to tumour removal (when present), and time to initiation of immunosuppressive therapy.

Overall about 75% of patients experience only mild long-term deficits or recover completely, but the remaining quarter have severe sequelae, and mortality due to intensive care complications can be up to 7% (Dalmau J et al. 2011; Wandinger KP et al. 2011; Titulaer MJ et al. 2013).

In paraneoplastic cases, response to tumour removal and first-line therapy is approximately 80%, whereas patients without tumours have an initial response rate of 48%. Second-line therapy, most commonly rituximab and/or cyclophosphamide, is often needed in patients without tumours and in those with a late diagnosis. Approximately 65% of patients show substantial improvements with second-line immunotherapy (Dalmau J et al. 2011; Titulaer MJ et al. 2013). Patients can relapse, especially those without tumours, although relapse symptoms are typically less severe than the initial presentation (Titulaer MJ et al. 2013).

Pathogenic mechanism of NMDAR antibodies

The NMDAR is a subtype of ionotropic glutamate receptor composed of two GluN1 (also called NR1) and two GluN2/3 (also called NR2 and NR3) subunits that form a central ion channel. Patients' antibodies were shown to react against the NR1 subunit and to significantly decrease NR1, NR2A, NR2B and NMDAR surface and total cluster densities in a titre-dependent fashion with consequent reduction of synaptic NMDAR currents but without disruption of the neuronal structure. The reduction of NMDAR expression is mediated by the antibodies that cross-link NMDARs, resulting in their internalisation, and appeared to be independent from complement deposition (Hughes EG et al. 2010). In parallel, the antibodies alter receptor trafficking. At the synaptic level they induce dispersal of GluN2A-NMDAR, preventing their dynamic synaptic retention through the blockade of the interaction between the extracellular domains of GluN1/2 subunits and ephrin-B2 receptors (EPHB2R) whereas at the extrasynaptic level they reduce the GluN2B-NMDAR dynamics through endocytosis (Mikasova L et al. 2012) (Figure 1.4). All together these changes affect long term potentiation, which is the cellular substrate of learning and memory, explaining some, but not all, the symptoms observed in patients.

The pathogenicity of NMDAR-Abs has been partially demonstrated in animal models. In mice, a single intraventricular (ICV) injection of immunoglobulin G (IgG) from individuals with anti-NMDAR encephalitis induced, in association with the chemoconvulsant pentylenetetrazol, more frequent and severe seizures than a single injection with IgG from control individuals (Wright S et al. 2015). Continuous intraventricular infusions of CSFs pooled from individuals with anti-NMDAR encephalitis to mice over 14 days reproduced some of the clinical features observed in patients such as memory deficits, anhedonia and depression-like behaviours but no other core features such as motor disorders or seizures. IgG deposition and a decrease in NMDAR clusters was observed in the hippocampi of NMDAR-Abs injected mice, which resolved within days after discontinuing the infusion (Planagumà J et al. 2015). These two animal models support the proposed mechanisms of cross-linking, internalisation and altered NMDAR trafficking, and together with the absence of complement activation, explain why patients often respond promptly to immunotherapy. Indeed, a good clinical outcome correlates with a significant decrease of NMDAR-Abs in patients' CSF (Dalmau J et al. 2008).

However, recent findings demonstrated that patients may have long term cognitive deficit and structural hippocampal damage as shown by brain MRI, in which severity correlated with disease duration and delayed and inefficient treatment (Finke C et al. 2016). This suggests that, while the acute effects of NMDAR-Abs are functional and reversible, their persistence can

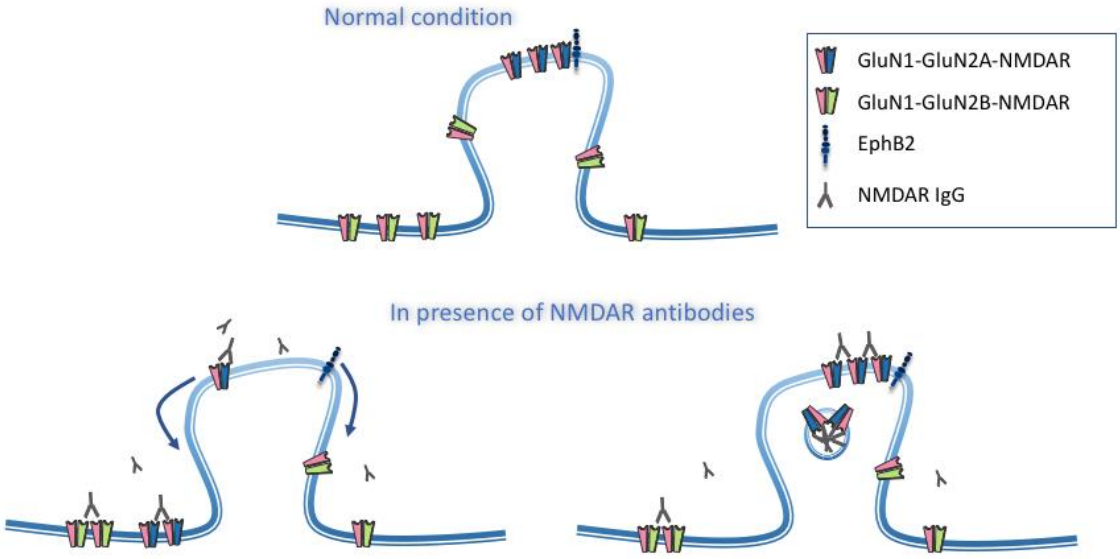

Figure 1. 4 Schematic representation of the proposed functional effects of NDMAR

In normal conditions GluN2A receptors are anchored at the synaptic levels through the interaction with the ephrin receptor, EPHB2R, whereas GluN2B are more extrasynaptic. Patients' IgGs against NMDAR prevent the interaction between NMDAR and EPHB2R in the synaptic area causing their lateral displacement. Moreover, patients' IgGs reduce surface NMDAR expression, likely by causing their internalisation and degradation (adapted from Mikasova L et al. 2012).

impact on neuronal function in a way that is not yet understood. Nevertheless, it is possible that the long-term damage is related to the accumulation of excitatory mediators such as glutamate at synaptic levels as occurs when NMDAR-antagonists are administered to normal animals. Indeed, patients' CSFs and purified IgGs resulted in an acute increase of both glutamate levels and the excitability of the motor cortex in a rat model (Manto M et al. 2010). It is worth noting that in experimental animal studies, NMDAR-antagonists are able to produce reversible or irreversible effects depending on the dosage and duration of the administration (Newcomer JW et al. 2000).

1.5.4 LGI1 antibody related syndromes

After the discovery that VGKC antibodies were associated with some cases of NMT, their presence was also identified in patients with CNS diseases (Buckley C et al. 2001; Vincent A. et al. 2004). It was subsequently found that the 'VGKC' autoantibodies actually bound to contactin-associated protein 2 (CASPR2) or LGI1 (Irani SR et al. 2010; Lai M et al. 2010); both of these proteins co-precipitate with the Kv1 subunits, which explains why the autoantibodies were able to precipitate the dendrotoxin-labelled VGKCs.

Autoantibodies to LGI1 (LGI1-Abs) are the most common autoantibody in patients with limbic encephalitis (LE), a clinical syndrome characterised by the acute development of mood changes, anxiety, short term memory deficit and seizures due to an inflammatory process involving the limbic system that includes the medial temporal lobes, hippocampus, amygdala, and fronto-basal and cingulate cortices (Graus F et al. 2016). LGI1-Ab encephalitis preferentially affects elderly patients (median age 60 years) with a male predominance (Irani SR et al. 2010; Lai M et al. 2010). Most patients do not have cancer (5% have thymoma).

The onset of an overt limbic dysfunction can be preceded by episodes of facio-brachial or crural seizures that last a few seconds and occur many times during the day; these episodes have been described as facio-brachial dystonic seizures (FBDS) (Irani SR et al. 2011; Irani SR et al. 2013). MRI studies show basal ganglia hyperintensity in 42% of patients with this type of seizure, suggesting a basal ganglia contribution (Flanagan EP et al. 2015). About 70% of the patients show substantial neurological improvement after immunotherapy, but only 35% are able to return to their baseline cognitive function. Clinical relapses occur in 24–35% of the patients (Ariño H et al. 2016; Van Sonderen ARD et al. 2016).

Pathogenic mechanism of LGI1 antibodies

LGI1 mutations have been associated with an autosomal dominant lateral temporal lobe epilepsy manifesting often with auditory features (Kalachikov S et al. 2002), and epilepsy is also one of the features of the autoimmune encephalopathy associated with LGI1-Abs. However, the autoimmune form of epilepsy in this case is characterised by contractions of homolateral face, arm and sometimes leg, known as FBDS, whereas auditory seizures are rare (Irani SR et al. 2011). LGI1 antibodies are predominantly IgG4. Since these antibodies do not fix complement and are less effective than IgG1 in crosslinking and internalising the target antigen, they are thought to affect their targets by interference with protein-protein interactions.

LGI1 is a protein secreted by the presynaptic terminals of neurons. This protein binds to ADAM22 and ADAM33, two proteins involved in cell-cell adhesion and located post-synaptically and pre-synaptically respectively. LGI1 forms a bridge between these two proteins establishing a physical contact between the pre- and the post-synapse which is believed to play an important role in synaptic maturation (Fukata Y et al. 2006; Owuor K et al. 2009). Binding to ADAM22, LGI1 regulates AMPA receptor-mediated synaptic currents in the hippocampus. Binding to ADAM23, LGI1 selectively prevents inactivation of the presynaptic voltage-gated potassium channel Kv1.1 mediated by a cytoplasmic regulatory protein, Kvβ (Schulte U et al. 2006).

LGI1 antibodies disrupt the ligand-receptor interaction of LGI1 with ADAM22, resulting in reversible reduction in synaptic AMPARs in cultured hippocampal neurons (Ohkawa T et al. 2013) (Figure 1.5). This observation, along with the presence of the antibodies in the CSF and the good response to immunotherapy support a pathogenic role of these antibodies. Indeed, their pathogenicity has been recently confirmed by a passive transfer animal model which showed severe memory impairment, but not seizures, in LGI1-Ab injected mice (Petit-Pedrol M et al. 2018). Moreover, LGI1-Abs have been detected in the sera of cats with a spontaneous form of autoimmune encephalitis with complex partial seizures with orofacial involvement. Postmortem analysis in these cases, showed complement deposition, a feature also shared by post-mortem examination of the small number of available brains from patients with LGI1-related encephalitis (Klang A et al. 2014). The finding of complement deposition suggests that LGI1-Abs are able to induce neuronal death through complement activation which may explain why, despite a good response to immunotherapy, only 35% of patients return to their baseline cognitive function (Ariño H et al 2016). This appears to be inconsistent with the observation that LGI1-Abs belong to the IgG4 subclass, which is classically considered unable to activate complement. However, the post-mortem findings, which are infrequent, may reflect

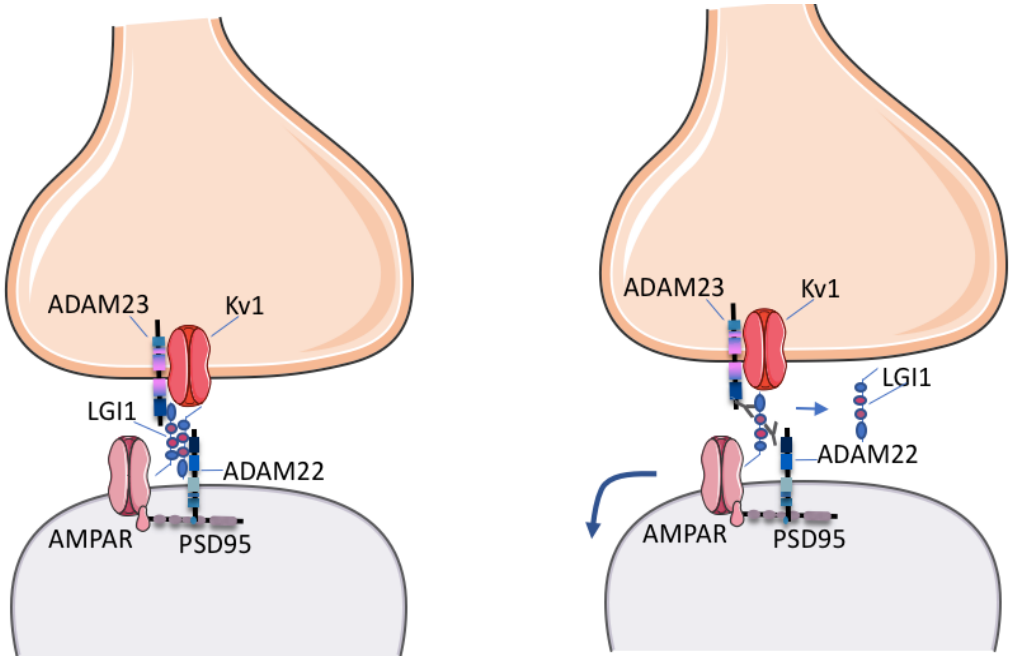

Figure 1.5 Schematic representation of the proposed functional effects of LGI1 antibodies

LGI1 interacts with presynaptic ADAM23 and postsynaptic ADAM22 organizing a trans-synaptic protein complex that includes presynaptic Kv1.1 potassium channels and postsynaptic AMPA receptors. LGI1-Abs interfere with the normal interactions of LGI1 probably decreasing the levels of the postsynaptic AMPA receptors and altering the function of the presynaptic Kv1 channels, leading to increased neuronal excitability (adapted from Van Sonderen A, Petit-Pedrol M et al. 2017).

the relatively small, but more pathogenic, IgG1 antibodies that are present in patients with limbic encephalitis (Irani SR et al. 2010; Thompson J et al. 2018).

1.5.5 CASPR2 antibody related syndromes

CASPR2 is one of the targets within the complex of proteins that includes the voltage-gated potassium channels (VGKCs). CASPR2 antibodies (CASPR2-Abs) have been associated with CNS symptoms including cognitive impairment, memory loss, hallucinations, delusions, cerebellar symptoms and epilepsy as well with peripheral nerve involvement with pain, neuropathy and hyperexcitability causing neuromyotonia (NMT). Some patients present with Morvan Syndrome (MoS), characterised by the combination of neuromyotonia, neuropathic pain, encephalopathy with hallucinations, and a characteristic sleep disorder described as agrypnia excitata (Liguori R et al. 2001; Provini F et al. 2011). This term (agrypnia - loss of sleep; excitata - increased motor activity and restlessness) was developed to describe the sleep disturbances that occur in several pathogenically unrelated diseases such as fatal familial insomnia (a prion disease) or delirium tremens (Montagna P and Lugaresi E, 2002). Patients with agrypnia excitata have severe insomnia, dream-like stupor (hallucinations and enacted dreams), sympathetic hyperactivity (hyperthermia, perspiration, tachypnea, tachycardia, and hypertension), and motor agitation. Key neurophysiological features include the loss of slow-wave sleep, which represents the transitional process of falling asleep, and the presence of abnormal REM sleep without atonia in the antigravity muscles (Montagna P and Lugaresi E, 2002).

Approximately 20% of patients with CASPR2-Ab associated symptoms have an underlying thymoma (Vincent A and Irani SR, 2010; Irani SR et al. 2010; Irani SR et al. 2012; Van Sonderen A, Arino H et al. 2016). Immunotherapy and treatment of the tumour (when appropriate) resulted in improvement in 93% of the patients, but 25% had relapses (Van Sonderen A, Arino H et al. 2016).

Pathogenic mechanisms of CASPR2-Abs

CASPR2 is a neurexin-related cell adhesion molecule expressed in the central and peripheral nervous system and CASPR2-Abs react with both the brain and peripheral nerve tissues (Irani SR et al. 2010). CASPR2 is essential for clustering Kv1.1 and Kv1.2 at the juxtaparanodes of myelinated axons (Figure 1.6). These channels are important for repolarisation of the nerve axon, avoiding repetitive firing and helping to maintain the internodal resting potential.

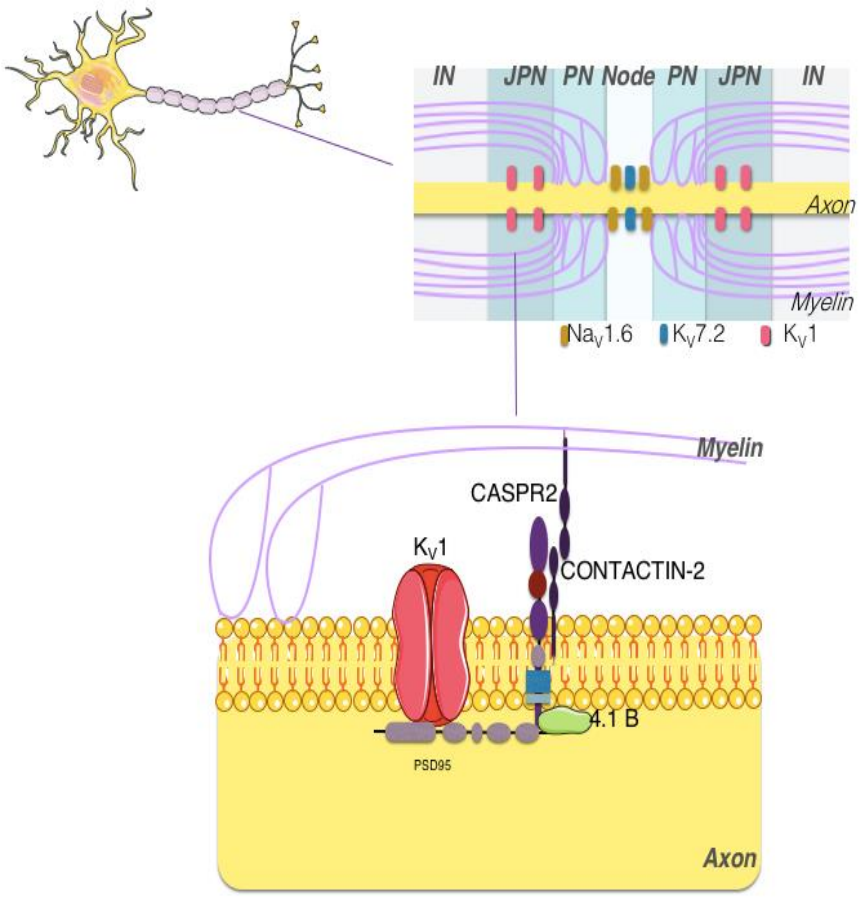

Figure 1.6 Schematic representation of CASPR2 protein

CASPR2 localises at the juxtaparanode of myelinated axons. CASPR2 binds to contactin-2/TAG-1 via its extracellular domain and links to PDZ-binding proteins, and to the cytoskeleton via protein 4.1B, stabilizing Kv1 channels (from Giannoccaro MP et al. 2018).

For CASPR2-Abs the mechanisms of action are still largely unclear. CASPR2-Abs are mainly IgG4, and therefore the most likely common mechanism of their action is a disruption of the interaction of CASPR2 with its associated molecules rather than internalisation and complement activation which are typical of IgG1, 2 and 3 antibodies. Accordingly, a recent study showed that CASPR2-Abs do not reduce CASPR2 expression on the surface of cultured hippocampal neurons, but they appeared to act by altering CASPR2 interaction with contactin-2 (Patterson KR et al. 2018). However, these findings contrasted with the pathology in a case showing reduced CASPR2 expression in the brain (Sundal C et al. 2017) and with two pathological cases associated with the presence of complement deposition (Liguori R et al. 2001; Kortvelyessy P et al. 2015). These discrepancies might be related to several factors; in particular, IgG1 CASPR2 antibodies are present in many of the patients, and may be responsible for complement mediated neuronal loss, and different antibody levels may explain some of the limitations of the *in vitro* studies, which only partially reproduce the variety of mechanisms involved *in vivo*.

How CASPR2-Abs produce the CNS symptoms is still largely unknown. CASPR2 is expressed throughout the brain as well as at the juxtaparanodes of motor and sensory axons (Figure 1.6). CASPR2-Abs have been described in association with several different disorders ranging from cerebellar ataxia to a wide range of movement disorder. Such a broad spectrum of presentations could be related to difference in epitope specificity, antibody titres and or the site of antibody production. A recent study showed that CASPR2-Abs can bind to different epitopes, although the majority reacted against the first extracellular discoidin domain. No correlation of antibody specificity with the phenotype was made (Olsen Al et al. 2015). One study found CASPR2-Abs in the CSF and serum in patients with autoimmune encephalitis, whereas they were detected only in the serum in patients with NMT or MoS (Joubert B et al. 2016). The highest antibody titres appear to be specific for LE, MoS and ataxia (Bien CG et al. 2017), and CASPR2 antibodies are usually low in patients with neuromyotonia, explaining why they might not have been detected in the CSF.

A study, using cultured hippocampal neurons, showed that serum IgGs targeted inhibitory interneurons where they reduced the number of synaptic gephyrin clusters (Pinatel D et al. 2015) which anchors $GABA_A$ receptors. More recently, however, treatment of cultured dorsal root ganglia (DRG) neurons with CASPR2-Abs caused a reduction in Kv1 channel surface expression and consequent neuronal hyperexcitability (Dawes JM et al. 2018). The same study produced the first passive transfer model of CASPR2-Abs by intraperitoneal (IP)

injections showing that these antibodies are able to cause mechanical-pain hypersensitivity in the exposed mice. However, the effects on the CNS were not investigated.

Interestingly, mutations in the CNTNAP2 gene, encoding for CASPR2, are associated with focal epilepsy, schizophrenia and autism spectrum disorder (Friedman JI et al. 2008). Two recent studies using a maternal-fetal transfer model showed that the offspring of dams injected with CASPR2-Abs demonstrated behavioural disorders and neuropathological features (Brimberg L et al. 2016; Coutinho E et al. 2017) raising interesting questions about the role of these antibodies in neurodevelopment disorders.

1.5.6 $GABA_A$ receptor antibody related syndromes

Autoantibodies to the $GABA_A$ receptor ($GABA_AR$) were first reported in some patients with AE characterised by encephalitis, prominent seizures or status epilepticus, and multifocal MRI abnormalities (Ohkawa T et al. 2014; Petit-Pedrol M et al. 2014). They were then found in patients with a wider spectrum of disorders (Pettingill P et al. 2015; Spatola M et al. 2017) such as focal neurological signs, hemiparesis, dyskinesias, aphasia or oculomotor disturbances. CSF findings are variable and abnormal in most cases and include pleocytosis, elevated protein concentration, or oligoclonal bands (Petit-Pedrol M et al. 2014). Coexistence of other autoantibodies occurs in some patients including thyroid peroxidase (TPO), GAD65, and $GABA_B$ receptor antibodies (Ohkawa T et al. 2014; Petit-Pedrol M et al. 2014). Approximately 75% of the patients develop multifocal FLAIR and T2 hyperintense MRI abnormalities involving various cortical and subcortical brain regions (Spatola M et al. 2017). This finding provides a clue towards the identity of the disorder. Approximately 30% of the patients have an underlying tumour, mainly a thymoma; older patients are more likely to have a tumour than younger patients (Spatola M et al. 2017). Treatment with anticonvulsants is frequently ineffective, and pharmacologically induced coma can be necessary to control seizure activity. In a recent review of all reported cases, 86% of the patients improved with immunotherapy and the other 14% died of status epilepticus or secondary medical complications (Spatola M et al. 2017).

Pathogenic mechanisms of $GABA_A$ -Abs

The $GABA_A$ receptor is a ligand-gated ion channel that mediates the majority of fast inhibitory transmission in the brain (Macdonald RL and Olsen RW, 1994). $GABA_ARs$ are heteropentamers consisting of five homologous subunits forming the channel pore. In patients

with autoantibodies against the $GABA_AR$, the predominant targets are α1 and β3, and less frequently the subunit $\gamma 2$ (Petit-Pedrol M et al. 2014; Pettingill P et al. 2015; Spatola M et al. 2017). In cultured primary neurons, application of patients' antibodies led to a reduction of synaptic and extrasynaptic density of $GABA_AR$ receptors (Ohkawa T et al. 2014; Petit-Pedrol M et al. 2014; Pettengill P et al. 2015). Animal models have not yet been reported.

1.5.7 $GABA_B$ receptor antibody related syndromes

Antibodies against the $GABA_B$ receptor ($GABA_BR$) associate with LE accompanied by prominent seizures or status epilepticus, with 50% of patients having an underlying SCLC (Hoftberger R et al. 2013; Lancaster E et al. 2010; Jain A et al. 2015). Most patients have complete or substantial neurological improvement after immunotherapy and tumour therapy when needed. The neurological outcome is similar in patients with or without tumour, but the long-term prognosis is dictated by the presence of SCLC and cancer recurrence.

Pathogenic mechanism of $GABA_BR$-Abs

The $GABA_BR$ is a G protein-coupled receptor for the inhibitory neurotransmitter GABA. The receptors are heterodimers comprised of two subunits, GABA-B1 and GABA-B2, which are both necessary for receptor function (Bettler B et al. 2004). GABA-B1 binds GABA with its extracellular domain, and GABA-B2 activates G proteins intracellularly.

$GABA_BR$ autoantibodies bind the extracellular domain of the GABA-B1 subunit (Lancaster E et al. 2010). The potential pathogenic effects of patients' autoantibodies were recently examined using cultured rat hippocampal neurons. In culture, neurons develop numerous synapses and spontaneously produce synaptic currents and actions potentials. This electrical activity is attenuated by the application of the $GABA_BR$ agonist baclofen. Application of patients' autoantibodies did not modify the levels of cell surface or synaptic receptors but abrogated the effects of baclofen on culture excitability, suggesting that $GABA_BR$-Abs may directly block the function of the receptor (Jain A et al. 2015). Although the exact mechanism whereby antibodies block $GABA_BR$ function is unknown, the findings provide a plausible explanation for the extremely common seizures and life-threatening status epilepticus seen in patients with LE related to these autoantibodies.

1.5.8 AMPA receptor antibody related syndromes

Most patients with AMPAR-Abs develop a typical LE, 40% of them showing additional symptoms beyond the limbic system, and only a few patients presenting with a different syndrome, such as rapidly progressive dementia or psychosis (Hoftberger R et al. 2015; Lai M et al. 2009). About 70% of the patients have an underlying tumour including SCLC, thymoma, ovarian or breast cancer, or teratoma. Approximately 70% of the patients respond to immunotherapy or treatment of the tumour, most showing a partial neurological response. Patients who do not receive aggressive immunotherapy are more likely to have clinical relapses. The presence of concurrent paraneoplastic antibodies was found associated with additional symptoms and a poor prognosis (Hoftberger R, et al. 2015).

Pathogenic mechanisms of AMPAR-Abs

The AMPA receptor is an ionotropic glutamate receptor that mediates most of the fast-excitatory transmission in the brain (Shepherd JD and Huganir RL, 2007). The majority of AMPA receptors are tetramers composed of GluA1, 2, 3, or 4 subunits that combine in a brain region-dependent manner (Palmer CL et al. 2005). The highest levels of GluA1/2 and GluA2/3 receptors are found in the hippocampus, subiculum, cerebellum, caudate-putamen, and cerebral cortex (Sprengel R, 2006). The AMPAR-Abs are directed against extracellular epitopes of the GluA1 or GluA2 subunits (Lai M et al. 2009). Preincubation of cultured rodent neurons with patients' IgG to either GluA1 or GluA2 led to a decrease of synaptic clusters of AMPA receptor subunits and to a reduction of fluorescence intensity of the remaining AMPA receptor clusters (Lai M et al. 2009; Peng X et al. 2015). As a result, frequency and peak amplitude of AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSC) were reduced in primary neurons following incubation with antibodies to the GluA1 and GluA2 receptor subunit (Lai M et al. 2009; Peng X et al. 2015). These preliminary findings point toward a direct pathogenic effect of patients' AMPA receptor autoantibodies on synaptic function, but the evidence is sparse and animal models have not been developed.

1.5.9 GlyR antibody related syndromes

Antibodies against the glycine receptor (GlyR-Abs) have been described in patients with stiff-person syndrome (SPS) and with progressive encephalitis with rigidity and myoclonus (PERM). SPS is characterised by muscle stiffness, rigidity, and painful spams predominantly involving the paraspinal, abdominal, and lower extremity muscles. The spasms can be

spontaneous or triggered by movement or sensory (tactile, auditory) and emotional stimuli. Patients with SPS have a normal brain and spinal MRI, and the CSF is often normal except for the presence of oligoclonal bands in 35% of the patients (Saiz A et al. 2008). PERM has a more aggressive clinical course compared with SPS. In addition to encephalomyelitis with rigidity and myoclonus, other symptoms include sensory problems (pruritus and neuropathic pain), brainstem dysfunction (nystagmus, ophthalmoparesis, dysphagia, trismus), and dysautonomia (profuse sweating, dry mouth, bladder dysfunction). Brain and spinal MRI are usually normal, but the CSF frequently shows pleocytosis (Carvajal-González A et al. 2014).

Pathogenic mechanisms of GlyR-Abs

Glycine receptors (GlyRs) are pentameric proteins belonging to the superfamily of ligand gated ion channels. These receptors can exist as α1-4 subunits homers or as heteromers consisting of α and β subunits. The different α subunits are differentially expressed in the CNS and the binding of autoantibodies to these different subunits could explain the range of spinal cord and brainstem symptoms observed in individuals with GlyR autoantibodies (GlyR-Abs). However, a recent study showed that the majority of sera from GlyR-Ab positive patients bind to α1β heteromers, and no correlation between different subunit binding affinities and clinical phenotype was found (Carvajal-González A et al. 2014).

Under physiological condition, glycine-mediated activation of the GlyR leads to an influx of Cl^- into the neurons and results in hyperpolarisation of the membrane potential with consequent reduced excitation (Legendre P, 2001). Indeed, incubation of HEK293 cells expressing GlyRs with IgG derived from individuals with GlyR-Abs resulted in internalisation and targeting of the receptors to lysosomes. Therefore, if a similar response occurs *in vivo*, the expected reduced glycinergic neurotransmission may explain the symptoms seen in patients with PERM.

1.5.10 GAD antibody syndromes

Highly raised antibodies against glutamic acid decarboxylase 65 (GAD-65) have been detected in 60-100% of patients with SPS (Costa M et al. 2002; Chang T, Alexopoulos H, McMenamin M et al. 2013) and at much lower titres in 80% of newly diagnosed type 1 diabetes mellitus (Baekkeskov S et al. 1990). GAD antibodies have also been detected in some patients with subacute cerebellar ataxia (Honnorat J et al. 1995; Saiz A et al. 1997; Abele M et al. 1999) and limbic encephalitis with prominent temporal lobe epilepsy (Malter MP et al. 2012).

Pathogenic mechanisms of GAD-Abs

GAD65 is a rate-limiting enzyme of GABA synthesis. Despite being an intracellular antigen, it is still unclear if these antibodies paly a pathogenic role. A loss of GAD activity may lead to decreased GABA synthesis and a reduction in inhibitory interneuron activity, resulting in hyperexcitability and epileptiform activity. However, it is not clear how the GAD antibodies can exert this effect on an intracellular enzyme. Specific and distinct staining of hippocampal neurons by sera from GAD-positive patients with drug-resistant epilepsy, ataxia and SPS has been demonstrated (Vianello M et al. 2006) and implies that the sera may contain additional specificities. *In vitro* studies have suggested that IgG from GAD-positive patients can be pathogenic, selectively suppressing GABA-mediated transmission on cerebellar Purkinje cells (Mitoma H et al. 2000), and a few patients have responded to IvIg treatment (Abele M et al. 1999). It is unclear whether the pathogenicity lies in the GAD antibodies or in an additional unidentified antibody. The immunisation of experimental animals with GAD65 resulted in the production of antibodies that immunoprecipitated GAD and bound to GAD intracellularly but also to the surface of cerebellar neurons in culture. Immunised animals did not show any behavioral abnormality, but they showed immunoglobulin diffusion into the brainstem, and a partial loss of GAD-EGFP expressing GABAergic neurons in the brainstem, suggesting the possible existence of a humoral response to antigen expressed on the surface of these neurons (Chang T et al. 2013).

1.5.11 Proposed criteria for the diagnosis of autoimmune encephalitis

Until a few years ago, the most common cause of acute encephalitis was considered to be infectious. However, with the discovery of so many new antibody targets, it has become clear that autoimmune encephalitis is more common than previously thought. Given the importance of an early treatment in determining patients' outcome however, it is clear that a prompt diagnosis cannot rely solely on the presence of antibodies. Moreover, despite the increasingly large number of targets, some CSF and sera are negative for known antibodies ("seronegative" autoimmune encephalitis). Recently a Consensus paper established guidelines to help the clinician in the early identification and treatment of patients with suspected autoimmune etiology (Graus F et al. 2016).

A patient with new onset encephalitis should be considered as having a possible autoimmune encephalitis if all three of the criteria shown in Table 1.3 are met. Of course, this diagnosis is only possible if a series of conditions, listed in Table 1.4, are excluded. According

Table 1. 3 Diagnostic criteria of possible autoimmune encephalitis

The diagnosis of POSSIBLE autoimmune encephalitis can be made if all the following criteria are met:
1) Subacute onset (rapid progression of less than 3 months) of working memory deficits (short term memory loss), altered mental status (defined as decreased or altered level of consciousness, lethargy, or personality change), or psychiatric symptoms;
2) At least one of the following:
· New focal CNS findings
· Seizures not explained by a previously known seizure disorder
· CSF pleocytosis (white cell count of more than five cells per mm3)
· MRI features suggestive of encephalitis (hyperintense signal in T2-weighted fluid attenuated inversion recovery sequences highly restricted to one or both medial temporal lobes, or in multifocal areas involving grey matter, white matter or both compatible with demyelination or inflammation)
3) Reasonable exclusion of other causes.

Table 1. 4 Differential diagnosis of autoimmune encephalitis

DISORDER
CNS infections
Septic encephalopathy
Matabolic encephalopathy
Drug toxicity (including use of illicit drugs, direct neurotoxic effects of prescribed drugs or through induction of seizures, posterior reversible encephalopathy, idiosyncratic reaction, drug interaction or withdrawal)
Cerebrovascular disease
Neoplastic disorders
Creutzfeldt-Jakob disease
Epileptic disorders
Rheumatologic disorders (i.e. lupus, sarcoidosis…)
Kleine-Levin syndrome
Reye syndrome
Mithocondrial diseases
Inborn errors of metabolism (children)

to these guidelines, the diagnosis of definite autoimmune limbic encephalitis can be made when all four criteria listed in Table 1.5 are met. If one of the first three criteria is not met, a diagnosis of definite LE can be made with the detection of antibodies against cell-surface, synaptic, or onconeural proteins. Finally, if antibodies are negative, the diagnosis of autoantibody negative but probable autoimmune encephalitis can be made when all four of the criteria listed in Table 1.6 have been met.

1.6 NSA-Abs in "discrete" neurological disorders

After the discovery of the NSA-Abs and their report in the classical syndromes described above, an increasing number of studies investigated the presence of these or other antibodies in neurological and psychiatric disorders, either because their presentation might involve some of the symptoms observed in autoimmune encephalopathies, or because their subacute onset and clinical course suggested an autoimmune etiology. The significance of these findings is still often unclear, but it has been suggested that these cases may represent a less severe form of limbic encephalitis.

1.6.1 NSA-Abs in epilepsy

Seizures are frequently observed in patients with NSA-Abs either in the course of the disease either at onset, and sometimes they represent the main clinical feature. Although the mechanisms of seizures in AE are unclear, the pathogenic role of the antibodies is likely.

More recently, however, these antibodies have been reported also in unselected patients with recent onset and chronic forms of isolated epilepsies. McKnight et al. compared 139 epilepsy patients, composed of 26 patients with definite concomitant autoimmune disorders (systemic lupus erythematosus, Hashimoto's encephalopathy, antiphospholipid syndrome, etc.), 46 patients with suspected concomitant autoimmune disorders, and 67 patients with solely drug resistant epilepsy, to a control group of 150 patients. They reported VGKC complex antibodies in 11.5 % of the patients with epilepsy, spread across all 3 patient populations, vs 0.5 % of the control subjects, as well as GAD65 antibodies in 3.6 % of the epilepsy patients and none of the controls. Among the group with VGKC-complex antibodies, 6 patients presented with a short duration of seizures and five of them were treated with immunotherapy and had a good response. By contrast, GAD65-positive patients presented with a long history of drug resistant epilepsy and did not respond to immunotherapy (McKnight K et al. 2005).

Table 1. 5 Diagnostic criteria for defined limbic encephalitis

The diagnosis of DEFINED limbic encephalitis can be made if all the following criteria are met:
1) Subacute onset (rapid progression of less than 3 months) of working memory deficits, seizures, or psychiatric symptoms suggesting involvement of the limbic system;
2) Bilateral brain abnormalities on T2-weighted fluid-attenuated inversion recovery MRI highly restricted to the medial temporal lobes (18Fluorodeoxyglucose (18F-FDG) PET can be used to fulfil this criterion);
3) At least one of the following:
• CSF pleocytosis;
• EEG with epileptic or slow-wave activity involving the temporal lobes;
4) Reasonable exclusion of other causes (see table 1.4).

Table 1. 6 Diagnostic criteria for possible autoimmune encephalitis

The diagnosis of PROBABLE autoimmune encephalitis can be made if all the following criteria are met:
1) Rapid progression (less than 3 months) of working memory deficits (short-term memory loss), altered mental status, psychiatric symptoms;
2) Exclusion of well-defined syndromes od autoimmune encephalitis (e.g., typical limbic encephalitis, Bickerstaff's brainstem encephalitis, acute disseminated encephalomyelitis)
3) Absence of well characterised autoantibodies in serum and CSF, and at least two of the following criteria:
• MRI abnormalities suggestive of autoimmune encephalitis;
• CSF pleocytosis, CSF specific oligoclonal bands or elevated CSF IgG index, or both;
• Brain biopsy showing inflammatory infiltrates and excluding other disorders (i.e. Tumour).
4) Reasonable exclusion of other causes (see table 1.4).

Another study of 106 female patients with chronic epilepsy found VGKC-complex antibodies in 6.5% of the subjects. Only one patient was positive for voltage gated calcium channel (VGCC) and none for GAD65. Seizures were mainly generalised tonic-clonic (GTCS) and MRIs were normal in patients with VGKC-complex antibodies (Majoie HJ et al. 2006).

Quek et al. retrospectively investigated 32 patients with refractory epilepsy and a suspected autoimmune aetiology, and found 18 with VGKC-complex antibodies, 7 with GAD65-antibodies, and one patient with NMDAR-antibodies. In this study, 81% of patients improved after immunotherapy, with 67% achieving seizure freedom within a period of 10 months (Quek AM et al. 2012). Overall, patients with autoantibodies often had frequent, antiepileptic drug (AED)-refractory seizures with neuropsychiatric comorbidities.

Another study, found 5 NMDAR antibody positive patients and 3 GAD65 antibody positive patients in a group of 19 female patients with new onset unexplained epilepsy (Niehusmann P et al. 2009). Brenner et al. reported the presence of neuronal antibodies in 46 of 416 adult patients with epilepsy (11%). Among those patients, 21 (5%) had VGKC-complex Abs, 12 (3%) had GlyR-Abs, 7 (1.7%) had GAD65-Abs, and 7 (1.7%) had NMDAR-Abs (Brenner T et al. 2013). The presence of GlyR-Abs is of interest as these antibodies are usually associated with a completely different phenotype.

Many of these studies have two particular limitations: 1) they are retrospective and 2) they include patients with suspected autoimmune etiology. To determine the prevalence of NSA-Abs associated with immune-mediated epilepsy, Dubey et al (2017) enrolled 112 consecutive patients with epilepsy of unknown etiology. Serum Abs suggesting a potential autoimmune etiology were detected in 39 (34.8%) cases; 15 (13.4%) had thyroid peroxidase (TPO)-Ab, 14 (12.5%) had GAD65-Ab, 12 (10.7%) had VGKC-complex Ab (4 of whom were positive for LGI1-Ab), and 4 (3.6%) had NMDAR-Ab. The presence of GlyR-Abs was not investigated. More than one Ab was detected in 7 patients (6.3%): 3 (2.7%) had TPO-Ab and VGKC-complex Abs, 2 (1.8%) had GAD65-Ab and VGKC-complex Ab, 1 had TPO-Ab and GAD65-Ab, and 1 had Hu Ab and GAD65-Ab. Thirty-two patients (28.6%) had a single Ab marker. Even after excluding TPO-Ab and low-titre GAD65-Ab, Abs strongly suggesting an autoimmune cause of epilepsy were seen in 23 patients (20.5%). Certain clinical features, such as autonomic dysfunction, neuropsychiatric changes, viral prodrome, facio-brachial dystonic spells or facial dyskinesias, and mesial temporal sclerosis abnormality on magnetic resonance imaging, correlated with seropositivity (Dubey D et al. 2017). In patients who were seropositive, reduction in seizure frequency was associated with use of immunomodulatory therapy.

In another consecutive series, including 111 patients with mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE-HS), 30 healthy volunteers and 50 patients with relapsing remitting multiple sclerosis, antibodies against CASPR2 were found in 11 patients, uncharacterised VGKC-complex antibodies in four patients, GlyR-Abs in 5 patients, NMDAR-Abs in 4 patients and $GABA_AR$ in 1 patient; these antibodies were not found in controls. The history of status epilepticus, diagnosis of psychosis and positron emission tomography or single-photon emission computer tomography findings in temporal plus extratemporal regions were found significantly more frequently in the seropositive group (Vanli-Yavuz EN et al. 2016).

A few studies investigated the presence of NSA-Abs in children with various forms of epilepsy, with a frequency of positivity around 10% (Dhamija R et al. 2011; Suleiman J et al. 2011; Suleiman J, Brilot F et al. 2013; Suleiman J et al. 2013; Wright S et al. 2016). Interestingly, Wright S et al. (2016) found neuronal antibodies at low levels in 9.5% of 178 patients with new-onset pediatric epilepsy. However, these antibodies did not necessarily persist over time, and the development of antibodies *de novo* in later samples suggests they could be due to a secondary response to neuronal damage or inflammation.

Overall, these data suggest that these antibodies are present in increased numbers in the general epilepsy population, even when these patients do not present as typical limbic encephalitis. In those patients with a more abrupt onset of seizures, multiple studies provide consistent observational evidence to suggest a preferential response to immunotherapy over AEDs (Quek AM et al. 2012; Toledano M et al. 2014; Dubey D et al. 2017). However, the results of these studies are not conclusive and there are still unanswered questions such as the pathogenic role of the antibodies, the optimal methods used to identify patients with epilepsy and autoantibodies, and the rationale for immunotherapy.

1.6.2 NSA-Ab in degenerative disorders

Many NSA-Ab related syndromes associate with cognitive impairment and sometimes this is the first and prevalent manifestation. Case series have demonstrated that progressive dementia without delirium may represent an autoimmune neurological disorder (Geschwind MD et al. 2008; McKeon A et al. 2007). Given the fact that these are potentially treatable disorders, several studies tried to identify the main features associated with an “autoimmune dementia”. These have been classically defined as: 1) subacute onset with a rapidly progressive course; 2) coexisting organ-specific autoimmunity; 3) inflammatory CSF; 4) presence of MRI changes

suggestive of an inflammatory process (McKeon A et al. 2010; Castillo P et al. 2006; Geschwind MD et al. 2008; McKeon A et al. 2007).

In a setting when an autoimmune etiology for the dementia is strongly suspected, Flanagan et al. reported the presence of autoantibodies targeting the VGKC complex in 15% of cases. In the same cohort, 64% of cases responded to immunotherapy. A shorter delay from symptom onset to initiation of therapy for autoimmune dementia increased the likelihood of response (Flanagan EP et al. 2010). However, 41% of immunotherapy-responsive dementia patients had normal brain MRIs, and many patients showed normal CSF and EEG; almost 9% of the disorders had been initially diagnosed as Creutzfeldt-Jakob disease (CJD) (Flanagan EP et al. 2010).

Despite the clear existence of cases of immunotherapy-responsive dementia associated with NSA-Ab, the presence of several neuronal antibodies has been reported in patients with ascertained prion disease (Mackay G et al. 2012; Fujita K et al. 2012; Angus-Leppan H et al. 2013; Fujita K, Yuasa T, Takahashi Y et al. 2012; Rossi M et al. 2015), challenging the significance of this finding and further complicating this diagnostic dilemma in cases with more chronic presentations. In another study, 6.4% of patients with suspected CJD had autopsy findings of potentially treatable diseases, with immune-mediated disorders being the most frequent (Chitravas N et al. 2011). In a large cohort of 346 patients with suspected CJD 1.7% showed the presence of NSA-Ab in CSF versus none of 49 patients with definite CJD. The target antigens included CASPR2, LGI1, NMDAR, aquaporin 4 (AQ4), Tr (DNER [δ/notch-like epidermal growth factor–related receptor]), and an unknown protein. All patients improved or stabilized after appropriate treatment (Grau-Rivera O et al. 2014). Indeed, most patients with well diagnosed CJD do not have these antibodies (Rossi M et al. 2015).

However, not all patients with antibody-mediated dementia present with a rapidly evolving clinical picture. Autoimmune-mediated cognitive decline can progress slowly over many months, and therefore may be mistaken for a primary neurodegenerative disorder such as Alzheimer's disease (AD) or frontotemporal dementia (FTD) (Castillo P et al. 2006; Geschwind MD et al. 2008; McKeon A et al. 2007). To explore the prevalence of NSA-Ab in patients with defined primary dementias, Coban A et al. (2004) investigated 50 patients finding NMDAR antibodies in one case presenting with dementia with Lewy body (DLB) phenotype. Despite the fact that this patient had some features suggestive of an autoimmune etiology, the presence of atypical features in the remaining cases failed to predict the presence of an NSA-Ab, highlighting the necessity to identify other markers predictive of an autoimmune dementia.

The presence of NMDAR antibodies of the IgA isotype was initially described in a small cohort of patients with atypical dementia. A subgroup of positive patients partially responded to immunotherapy (Pruss H et al. 2012). Purified IgA containing NMDAR IgA antibodies caused substantial loss of NMDARs and further synaptic proteins in primary hippocampal cultures, resulting in marked changes of NMDAR-mediated currents (Pruss H et al. 2012). These results were further explored in a large series of 660 cases including different neurological disorders and controls. Serum NMDAR antibodies of IgM, IgA, or IgG subtypes were detected in 16.1% of 286 dementia patients and in 2.8% of 217 cognitively healthy controls. Higher prevalence of serum antibodies was detected in patients with "unclassified dementia" followed by progressive supranuclear palsy (PSP), corticobasal syndrome (CBD), Parkinson's disease-related dementia (PDD), and primary progressive aphasia (PPA). Among the unclassified dementia group, 60% of 20 patients had NMDAR antibodies, accompanied by higher frequency of CSF abnormalities, and subacute or fluctuating disease progression. Immunotherapy in selected prospective cases resulted in clinical stabilisation, loss of antibodies, and improvement of functional imaging parameters. Epitope mapping showed varied determinants in patients with NMDAR IgA-associated cognitive decline. However, antibodies were rarely found in CSF (Doss S et al. 2014), and therefore their role in the clinical manifestations is unclear.

As emerged from this and other reports, not only dementias but also other neurodegenerative disease, mainly presenting with movement disorders, can be associated with the presence of NSA-Ab. Patients with LGI1, IgLON5, DPPX and GAD65 antibodies have been misdiagnosed with Parkinson's disease, PSP or multisystem atrophy (MSA) (Pittock S et al. 2006; Tobin W et al. 2014; Kurtis MM et al. 2015; Sabater L et al. 2014; Kannoth S et al. 2015). However, the prevalence of NSA-Ab in a population of patients with a diagnosis of parkinsonism has not yet been reported. REM sleep behavior disorder (RBD) is associated with neurodegeneration and can anticipate by decades the onset of a neurodegenerative disease, in particular MSA and PD. RBD has been reported in patients with LGI1-antibody-associated limbic encephalitis (Iranzo A et al. 2006; Irani SR et al. 2010) and with IgLON5-antibody linked neurodegeneration (Sabater L et al. 2014). Finally, status dissociatus, a complete breakdown of the boundaries of the different states of being, which are wakefulness, REM sleep, and non-REM sleep, with motor hyperactivity, which may be observed in the final phase of several different neurodegenerative disorders, has also been reported in patients with CASPR2-Abs, and less commonly, LGI1-Abs or NMDAR-Abs (Stamelou M et al. 2012;

Abgrall G et al. 2015), widening the overlap between neurodegenerative and autoimmune conditions.

Finally, the question of the relation between antibodies and neurodegeneration is made more complex by the description of IgLON5 antibodies. These were firstly described in a small group of patients with prominent sleep-related movement disorders, mainly characterised by a non-REM sleep parasomnia with simple or finalistic movements, resembling daytime activities such as eating, drinking or manipulating objects. Other sleep abnormalities included RBD and periodic limb movements of sleep. Breathing disorders, including obstructive sleep apnea (OSAS) and stridor, both in sleep and wakefulness, are almost a constant feature and are often associated with respiratory failure. Other clinical manifestations include ataxia, bulbar signs, abnormal eyes movements and dysautonomia (Sabater L et al. 2014). Over time other clinical manifestations were reported including dementia and chorea (Simabukuro MM et al. 2015; Brüggemann N et al. 2016; Haitao R et al. 2016). In the first case series, all eight patients reported received immunotherapy, but six of them died during the follow-up period (Sabater L et al. 2014). To date more than 60 patients have been reported and response to immunotherapy seems to occur in certain cases and to some extent (Bonello M et al. 2017; Simabukuro MM et al. 2015; Brüggemann N et al. 2016; Haitao R et al. 2016; Honorat JA et al. 2017; Montagna M et al. 2108). However, mortality remains high (Gaig C et al. 2017; Honorat JA et al. 2017). Neuropathology, intriguingly show the presence of hyperphosphorylated three- and four-repeat tau aggregates in neurons, and neuronal loss predominantly in the hypothalamus and the brainstem tegmentum, and absence of inflammatory infiltrates (Sabater L et al. 2014; Gelpi E et al. 2016). These findings suggest neurodegeneration as the primary disease mechanism, which would fit with the observed absence of a significant response to immunotherapy. However, all genotyped patients had HLA-DQB1*0501 and HLA-DRB1*1001 alleles, suggesting a genetic susceptibility for autoimmunity (Sabater L et al. 2014, 2016).

The IgLON5 antibodies target the extracellular domain of the protein and are predominantly of the IgG4 subtype, and to a lesser extent IgG1. The latter are responsible for the internalisation of IgLON5 in neuronal cultures (Sabater L et al. 2016), whereas this effect was not seen with IgG4 antibodies, which are likely to act in a different way, and which may lead to secondary neurodegenerative changes. However, it is also possible that their presence is secondary to a primary tauopathy. Future studies have to address the link between autoantibodies and neurodegeneration.

1.6.3 NSA-Ab in Narcolepsy with cataplexy

Narcolepsy is a chronic sleep disorder, characterised by excessive daytime sleepiness (EDS) and cataplexy, often in association with hallucinations at sleep onset (hypnagogic) or/and at awakening (hypnopompic) and sleep paralysis (Yoss RR and Daly DD, 1957); the presence or absence of cataplexy defines two groups of patients; narcolepsy with cataplexy (narcolepsy type 1, NT1) and narcolepsy without cataplexy (narcolepsy type 2, NT2) (AASM, 2005). The disease, and in particularly NT1, has been linked to the loss of a small group of neurons in the posterior lateral hypothalamus producing hypocretin 1 and 2 (HCRT-1 and 2) (also known as orexin A and B) (Scammell TE, 2003; Peyron C et al. 2000; Thannickal TC et al. 2000). Patients with narcolepsy and cataplexy have low concentrations of hypocretin in the CSF, typically below 100 ng/L and most often undetectable with conventional radioimmunoassays (Nishino S et al. 2000; Nishino S et al. 2001). Although it is clear that the loss of orexin neurons is responsible for many of the symptoms observed in NT1 patients, the cause of this loss in unknown. The specific and highly selective depletion of hypocretin-secreting neurons and the strong association with the HLA DRB1*06:02 (Mignot E et al. 1997) led to the hypothesis that narcolepsy is an autoimmune driven process within the hypothalamus. However, to date, many observations supporting this hypothesis are circumstantial and clear evidence of an ongoing autoimmune process are missing, although, recently, autoreactive T cells clones directed against hypocretin were found in narcolepsy patients (Latorre D et al. 2018), supporting an autoimmune T cell-mediated disorder. It remains to be established if these T cells are primary or if they are secondary to the destruction of hypocretin neurons produced by other mechanisms.

Immunohistochemical studies screening for hypothalamic antibody binding (Knudsen S et al. 2007; Martinez-Rodriguez JE et al. 2007; Overeem S et al. 2006), as well as antibody studies screening for specific antigens such as the hypocretin precursor-peptide (Black JL III et al. 2005), HCRT1 and 2, hypocretin receptors 1 and 2 (HCRTR1 and 2) (Tanaka S et al. 2006) and other neuronal and non-neuronal autoantigens in narcoleptic patients (Black JL III et al. 2002) gave negative or inconclusive results. A possible explanation for these inconclusive findings is the fact that in many cases the studies were performed on patients with symptoms of an unspecified or lengthy duration, which could result in a negative study if the antibodies are only transiently present. The relevance of timing in the autoimmune process related to narcolepsy is suggested by the observation that a better, though insufficient, control of symptoms could be obtained by human IvIg administered within a few months from disease onset (Dauvilliers Y et al. 2004), and from the anecdotical report of symptom disappearance

and normalization of HCRT-1 CSF levels when the IvIg was administered within the first 15 days from onset (Dauvilliers Y et al. 2009).

Sleep disturbances are common in patients with autoimmune encephalitis (Vincent A et al. 2011), who often present with low levels of orexin A in the CSF (Mignot E et al. 2002; Küçükali Cİ et al. 2014). LGI1 antibodies, associated with limbic encephalitis, appear to co-localise with orexin neurons (among other neurons; Irani SR et al. 2012). Also, NMDAR antibodies were recently found in three orexin-deficient NT1 patients who presented with psychiatric disturbances (Tsutsui K et al. 2012). However, although 4/13 patents stained on rat brain sections, screening for antibodies associated with encephalitis (i.e. CASPR2, LGI1, NMDAR) was negative in post vaccine (Pandemrix®) narcolepsy cases (Thebault S et al. 2015). Moreover, the association between narcolepsy, psychosis and presence of NMDAR-Ab was not confirmed by a small subsequent study (Dauvilliers Y et al. 2016). Finally, new interesting clues about antibody-mediated pathogenic mechanisms in narcolepsy came from Ahmed and colleagues, who found antibodies against the HCRTR2 in 85% of post-Pandemrix® narcolepsy cases as well as in 35% of controls (Ahmed SS et al. 2015). The significance of this finding is unknown, and HCRTR2-antibodies were only rarely found in patients with idiopathic narcolepsy (Giannoccaro MP et al. 2017).

1.7 Open questions

Despite their relative rarity, antibody-mediated CNS diseases have been one of the most intriguing and research-driving fields in neurology over the last 15 years. However, several questions remain unanswered. Among these, is the site of production and distribution of the antibodies. To cause symptoms the antibodies must access the brain. Are they produced in the periphery and gain access to the brain when or where the BBB is less efficient, or are they produced locally in the brain? These two scenarios are not mutually exclusive, and their importance may change during the course of the disease. In NMDAR encephalitis the autoantibodies are present in patients' serum and CSF, the latter usually showing intrathecal synthesis and high antibody concentrations (Dalmau J et al. 2008). B-cells are able to cross the BBB where they are believed to undergo antigen driven re-stimulation, clonal expansion and differentiation into antibody producing plasma cells (Hauser SL et al. 2008). Indeed, NMDAR encephalitis responds to cyclophosphamide and rituximab therapies, both able to deplete B cell populations. However, the contribution of intrathecal versus systemic production is not clear.

In some diseases, it may be essential for CNS symptoms, as implied in cases of CASPR2-Abs (Joubert B et al. 2016), or the antibodies in the CSF may just reflect higher serum levels.
Very little is known about the origin of these antibodies. The role of tumours in paraneoplastic syndromes is well accepted, and the role of infections in precipitating the disease is also common, with clear relationship between HSV and NMDAR-Ab encephalitis. However, the role of triggering infections in other forms of autoimmune encephalitis or of other environmental factors have not been explored and epidemiological studies are missing. Genetic factors may also contribute to the pathogenesis of these disorders. Indeed, distinctive HLA associations are linked to clinical features and antibody subtypes in myasthenia (reviewed in Nacu A et al. 2015) and a recent study showed a strong association between LGI1 antibodies and HLA II DRB1*07:01–DQB1*02:02 haplotype (Kim TJ et al. 2016). The strong association of LG1 antibodies to HLA-DRB1*07:01 was confirmed in another study, which also found an association between CASPR2 antibodies and the HLA-DRB1*11:01 (Binks S et al. 2018).

Similarly, autoimmune encephalopathy with the IgLON5 autoantibody appears to be associated with specific HLA genotypes (Sabater L et al. 2014). However, the presence of a specific HLA locus association in other NSA-Abs syndromes is still unknown. In the future, more studies will be oriented in exploring the influence of genetic and environmental factors.

Another major question relates to how these antibodies can produce specific syndromes when the antigens are often expressed throughout the CNS. Epitope differences may play a role as well as the site of access of the antibodies into the CNS. Also, the relevance of different Ig classes and whether they cause disease requires further study. High frequency of IgM and IgA have been found in patients with dementia or different psychiatric disorders (Doss S et al. 2014; reviewed by Pollak TA et al. 2016). A recent study showed that, independently from the Ig class and the associated clinical condition, all NMDAR-Abs directed against the GluN1 subunit are able to provoke NMDAR1 internalisation in human-induced pluripotent stem cell-derived neurons and reduction of glutamate-evoked currents in NR1-1b/NR2A-expressing Xenopus oocytes; these results showed that all antibodies had pathogenic potential (Castillo-Gómez E et al. 2016). If this is confirmed and expanded to other antibodies, the diagnostic significance of this finding needs to be revised.

To be considered as pathogenetic some line of evidence needs to be satisfied. For antibodies the most compelling line of evidence derives from passive transfer studies of the human antibody into an animal model. This has been accomplished for some antibodies, i.e. NMDAR given intraventricularly (Planagumà J et al. 2015; Wright S et al. 2015), but for many others such evidence in still lacking. Indeed, although many of the NSA-Abs have been

associated with specific and recognisable clinical syndromes, there can be problems about their significance. This occurs when they are found in cases where the clinical expression of disease and a particular autoantibody have not been previously described, or in cases with a very restricted clinical phenotype such as epileptic syndromes or neurodegenerative disorders. Moreover, there is insufficient data regarding the prevalence of these antibodies in the general population. From a recent revision of the literature (Lang K and Pruss H, 2017) it emerged that NSA-Ab are overall rarely found in the healthy controls (HC) (mean 0.23%), however this result was variable for different antibodies and HC population size. Indeed, in a cohort of 1,703 HC samples analyzed for antibodies against AMPAR1/2, AQP4, DNER, DPPX, $GABA_BR$, GlyR, mGluR5, myelin oligodendrocytes glycoprotein (MOG), NMDAR/NR1, CASPR2, and LGI1, no positive samples were found for AQP4, DNER, $GABA_BR$, and mGluR5. The most frequent antibody was that against the NMDAR/NR1 in 20 cases (1.2%) (Dahm L et al. 2014). On the other hand, the same study revealed that NSA-Ab were present in 1.5% of disease controls, with frequencies varying among different subgroups from 0.25% to 3.46% (Lang K and Pruss H, 2017). These findings, together with the possibility of antibodies in epilepsy, narcolepsy or neurodegenerative cases raises several questions on the utility of antibody screening in cases without an obvious autoimmune suspicion, and on the clinical significance of positive findings. One could speculate that at least some of these positives in patients as well as controls could be "false positive".

The other possibility is that these antibodies are often present in the sera but do not cause disease because they are unable to reach their target. Therefore, in these cases, a "second hit", like disruption of the BBB, could be necessary to allow the manifestation of the disease and access of B cells into the cerebral compartment. Indeed, in many diseases and healthy controls, the positive finding of serum antibody has not been accompanied by CSF testing.

Another possible scenario is that the antibodies in healthy individuals are pathogenic but do not cause an obvious clinical phenotype because the changes induced go undetected – that is they are not sufficient to cause neurological symptoms. It is also possible that functional differences (i.e. affinity) in the antibodies play a role in their pathogenic potential. Finally, a positive neuronal antibody in a patient not initially suspected to have an antibody-mediated disease can still be "true-positive" and represent the further expanding phenotypic spectrum seen in most antibody-specific syndromes. Investigations into these restricted clinical phenotypes are relevant due to the implications for patients' treatment and prognosis.

1.8 Aims of this PhD

The aim of this PhD was to investigate the presence and pathogenic role of antibodies directed against neuronal surface antigens in different neurological conditions. Methods are described in Chapter 2. At first, the presence and frequency of these antibodies were explored in three different cohorts of patients. These included those (1) for which an autoimmune aetiology is strongly suspected (narcolepsy type 1), (2) patients with chronic neurological conditions with unknown or not primarily an immunologically-related aetiology (patients with temporal lobe epilepsy and patients with neurodegenerative disorders), and (3) healthy and disease (seronegative myasthenia gravis patients) controls. The presence of the antibodies was related to patients' clinical features (results are presented in Chapter 3). Finally, the pathogenic effects of antibodies against neuronal surface antigens *in vivo* were examined by a passive transfer animal model, using lipopolysaccharide (LPS) to open the blood-brain barrier. For this experiment, CASPR2 antibodies were chosen for several reasons: (1) they were one of the most common antibodies found in our cohorts; (2) they are some of the most commonly observed antibodies in patients with autoimmune encephalitis; (3) there are no previous studies attempting to model their CNS effects; (4) most importantly, plasma derived from the plasmapheresis of a patient with CASPR2 related encephalitis was available for the study. In this animal model, the behavioural and neuropathological effects of the antibodies were evaluated (results in Chapters 4 and 5).

Chapter 2: Materials and Methods

2.1 Antibody screening in human cohorts

The CNS disorders cohorts were provided from the UOC Clinica Neurologica of the IRCCS Istituto delle Scienze Neurologiche di Bologna. Population controls from elderly individuals (age over 60 years) and serum samples from patients with seronegative myasthenia gravis (SNMG), which served as disease controls, were kindly provided by the Nuffield Department of Clinical Neurosciences. It is important to note that all this work was retrospective, and no patients or controls were prospectically included in the study. The initial work included the search for antibodies in different cohorts in order to assess their frequency in specific neurological disorders and their association with specific phenotypes. In a second step the frequency of antibodies among patients with different neurological disorders and healthy controls were compared.

2.1.1 Narcolepsy type 1 cohort

Narcolepsy type 1 (NT1) patients' sera were collected at the Sleep Centre of the IRCCS Istituto delle Scienze Neurologiche di Bologna. Inclusion criteria were diagnosis of narcolepsy with cataplexy on unknown etiology or related to infections and vaccination (i.e. Pandemrix); patients were excluded if brain imaging documented the presence of brain lesions potentially responsible for secondary narcolepsy. Both children and adults could participate to the study. According to these criteria, sera from 59 consecutive patients (22 F, 37 M) diagnosed with NT1 accordingly to the ICSD, 2nd edition (AASM, 2005) were included. All patients underwent MSLT the day after a complete polysomnography and protracted EEG monitoring. CSF HCRT-1 levels and HLA status at locus DQB1*0602 were available in all patients.

2.1.2 Epilepsy cohort

Patients were collected by different adult and pediatric epilepsy centers in the Bologna area. Inclusion criteria was an ascertained diagnosis of temporal lobe epilepsy, with or without family history, and with or without mesial temporal sclerosis. Exclusion criteria were history and/or imaging evidence of stroke, tumor, trauma, vascular malformation, abscess or infectious lesion. No age limits were established. On the basis of these criteria, this cohort included 73 patients (34 F, 39 M).

2.1.3 Neurodegenerative cohort

Patients consecutively admitted for a neurodegenerative disorder to our clinic were included in this study if they satisfied the following inclusion criteria: parkinsonism or cognitive impairment/dementia of suspected neurodegenerative aetiology in absence of other causes (i.e. vascular dementia, drugs, syphilis). Patients with family history or mutations in genes associated to parkinsonism and/or dementia could be included. Patients with abnormal complete blood count, blood biochemistry analyses, thyroid function tests, sedimentation rate, vasculitic/rheumatological antibody screening, and cranial MRI were included only if these alterations were not considered responsible for the clinical picture. Sixty-one patients (21 F, 40 M), diagnosed with different neurodegenerative conditions, on the basis of current clinical criteria, were included.

2.1.4 Control cohorts

These included 50 population controls aged over 60 years (from a study started in the 1990s and used in Vincent A et al. 2004) and 159 patients with SNMG collected from 2000-2003. As specific ethics were not available for these samples to be used as controls, they were anonymized and unfortunately age and gender are not available.

2.2 Methods

2.2.1 Cell-based assays (CBAs)

Proteins that are expressed on the neuronal surface and relevant for neurological disorders were chosen for the screening of the cohorts using human embryonic kidney (HEK) 293 T cells after transfection with the plasmid of interest.

HEK 293 cells were cultured in Dulbecco's modified eagle's medium (DMEM) media supplemented with 10% foetal calf serum (FCS) (Biowest®) and 1% each of penicillin G and streptomycin 1 in 175 cm 3 plastic flasks at 37°C 5% CO2. After 3-4 days, cells were collected after 1-minute incubation with trypsin (GIBCO®, 0.5% diluted in PBS) and centrifuged (1100 RCF, 5 min) at room temperature (RT). Pellets were resuspended in 10 ml of HEK culture medium and counted on a hemocytometer using Tryptan blue to stain dead cells to exclude in the counting. $4.6x10^5$ HEK cells were seeded in 6 well plates containing 4 borosilicate glass

coverslips (13 mm, VWR) and coated with poly-L-lysine (PLL). 24 hours after seeding, cells were transiently transacted with the plasmid encoding for the protein of interest associated in some cases with the enhanced green fluorescent protein (EGFP) encoding plasmid. A total of 3 or 3.2 µg of DNA respectively was diluted in 50 µL DMEM, 0.82 µL of 20% glucose and 1.5 µL of polyethylenimine (9 mM, PEI) per well. After 12-16 hours of incubation (37°C) the media was replaced, and cells cultured for a further 24 hours (hr). EGFP was used as a marker of the successful transfection.

Indirect immunofluorescence

Sera antibodies were diluted in DMEM supplemented with N-(2-hydroxyethyl)piperazine-N'-2-thanesulphonic acid (HEPES) buffer (4.6 mg/ml) and 1% bovine serum albumin (BSA) and incubated with coverslips transferred to individual well on a 24 wells plate (1 hr, RT). For NMDAR, LGI1, AMPAR, IgLON5 and HCRTR2 constructs, sera were screened at 1:20 dilution; $GABA_AR$ antibodies were screened at 1:50 dilution; GlyR, $GABA_BR$ and CASPR2 were screened at 1:100. These dilutions were those in use by the routine laboratory.

After incubation, coverslips were washed 3 times in DMEM/HEPES and fixed in paraformaldehyde (PFA) (4%, TAAB®). After a further three washes, secondary antibodies (usually Alexa Fluor™ 568 anti-human IgG H&L chain rises in goat) were incubated in the dark (1 hr, RT). Finally, cells were washed in DMEM/HEPES (x3) and phosphate buffered saline (PBS) (x3) and the coverslips were mounted onto glass microscope slides (VWR®) using mounting medium (Dako®) containing a 1:1000 dilution of 4',6'-diaminidino-2-phenlindoledichloride (DAPI) in order to stain cells nuclei. This protocol was subjected to minor changes due to the use of different secondary antibodies, as described in the appropriate sections.

Antibody binding to the expressed antigen was observed using a fluorescence microscope (Leica DM 2500™). Successful transfection and antibody binding were usually confirmed by green (EGFP) and red (secondary antibody against human IgG) fluorescence respectively. A subjective visual scoring system was adopted to assess the presence and the intensity of the antibody binding to the cells of each coverslip (adapted from Leite I et al. 2008). In all experiments, positive and negative controls were included. The scoring system was the following: 0= no labelling; 1= weak, considered as a low positive; 2= moderate; 3= moderate-strong; 4= strong. Also, intermediate scores were possible and therefore 0.5 point could be added at each number when the observed staining was in between two scores. For instance, a score of 1.5 was given when observed binding was stronger than the low positive control but

did not satisfy criteria for score 2. Any result with observed reactivity (score ≥0.5) was reassessed by repeat assay. As anti H&L chain secondaries can bind IgG as well as IgM, to further confirm the positivity and the relevance of the observed staining, secondary antibodies against the human IgG Fc fragment were used to confirm that the antibodies were IgG. In positive cases, serum dilutions were performed, when possible, to assess the antibody titre. All samples were tested blind to the serum identify and retested on HEK cells transfected with a different construct to exclude non-specific binding to the cells.

Pictures were taken using an Imaging Rolera XR™ camera or with Leica Confocal microscope under the same conditions. No specific features within any image was enhanced, obscured, removed or introduced unless explicitly stated.

2.2.2 Screening approaches

2.2.2.1 Immunostaining on rat and mouse brain sections

Rat brain collection and preparation

Sprague Dawley rats were decapitated, and their brains removed by careful dissection, cut sagittally and immediately immersed for 1 hour in 4% PFA at RT. After fixation, the half brains were transferred in a sucrose solution at 40% and stored at 4 °C. The passage in sucrose solution ensure cryoprotection of the tissue. Brains were left in the solution for 24-48 hours and then embedded in optimum citing temperature (OCT) compound (Tissue-Tek®) and snap-frozen in dry-ice cooled isopentane. Frozen brains were wrapped in foil and stored at -80°C. Frozen tissue was mounted with OCT compound and cut on a cryostat (10 µm) (ThermoScientific Cryotome® FSE) sagittally. Cut section were transferred onto SuperFrost® Plus slides, dried overnight and stored at -20°C wrapped in aluminum foil.

Immunohistochemistry (IHC)

Slides were allowed to dry at RT and hydrophobic wells were created around each section using a Dako® delimiting pen. All procedures were performed at RT if not otherwise specified. After a rinse in cold tris-buffered saline (TBS) solution, endogenous peroxidase blocking was achieved by submersion in a solution of TBS-0.3% H2O2 for 15 min RT. Slides were than washed with TBS (3 x 5 min) and unspecific binding was blocked in normal goat serum (NGS) (10% in TBS for 1 hr). Sections were rinsed in TBS and then incubated in primary antibodies (1:100 dilution in 5% NGS-TBS) overnight at -4 °C in a humidity chamber. The day after,

slides were washed with TBS (3 x 5 min) and incubated with secondary antibodies (Biotinylated Goat Anti-Human IgG Antibody, Vector lab,1:1000 in TBS) for 2 hours. After washing with TBS (3 x5 min), in order to amplify the signal of the secondary antibody, a further incubation was performed with the avidin-biotin complex (ABC, Vectastain Elite ABC Kit Standard) (1:100 in TBS, 1 hr). Sections were further washed with TBS (3 x 5 min) and the reaction developed using brown 3,3'-diaminobenzidine DAB (ImmPACT DAB Peroxidase (HRP) Substrate, Vector lab) prepared as per manufactures instructions. Slides were left to dry in the fume hood before proceeding with dehydration procedure by immersion in progressive concentration of ETOOH solutions followed by p-Xylene. Slides were mounted with DPX mountant for histology (Sigma-Aldrich®) and viewed using a light Microscope (Nikon Eclipse E400). Section location was identified by comparison with a rat brain atlas (Paxinos G and Watson C, 2007).

Screening of patient sera binding to sagittal sections was assessed in the hippocampus, cortex, thalamus and cerebellum. Binding to these areas was scored using a subjective scoring system (0= no binding, 0.5= weak/questionable binding, 1=weak definitive binding, 2= moderate, 3= strong) in each brain area and appearance of binding. All patients showing a score ≥ 0.5 in at least 1 brain area was repeated and the final score was the mean value.

2.2.2.2 Immunostaining on primary hippocampal neuronal cultures

Neuronal cultures

The protocol for neuronal cultures was adapted from Kaech S and Banker G (2006). Following sacrifice of the pregnant dam, E17-19 fetuses were removed from the uterus and placed on ice. Brains were removed and immersed in ice cold antibiotic-antimycotic (AA) supplemented Hanks's Buffered Saline Solution (HBSS) (Gibco®). After midline sagittal bisection of the brain and involution of the cortex, the hippocampus was identified and dissected from each side. When all of the hippocampi had been removed, they were gently transferred with a Pasteur pipette to a Falcon tube containing 5 ml of a solution of HBSS-0.5% trypsin and incubated for 20 min at 37°C. After removal of the trypsin solution, the tissue was resuspended in complete minimum essential medium (MEM) (1X MEM alpha, Gibco®, supplemented with 10% FCS, 1% AA solution), triturated by repeatedly pipetting up and down and then centrifuged (1000 RCF for 10 minutes) before being re-suspended in complete MEM. Dissociated cells were plated-out in complete Neurobasal medium (NBS) (Neurobasal media 1 X, Gibco®, supplemented with 1% AA solution, 0.5 mM L-glutamate, Sigma, and 1% B27 supplement) in

6 well-plates containing 5-9 borosilicate glass coverslips (12 or 10 mm, VWR) previously coated with PLL. One third of media was replaced with fresh NBS medium every 5 days and maintained in culture for 12 days. Three days after plating, cytosine arabinoside was added at a concentration of 5 µM to curb glial proliferation.

Indirect immunofluorescence

After 12 days in culture, antibody binding studies to neurons were performed. Coverslips were transferred to individual wells on a 24 well plate and incubated with serum (1:100) diluted in NBS medium supplemented with HEPES buffer (4.6 mg/ml) and 1% BSA. Coverslips were then washed 3 times (NBS-HEPES) and fixed with PFA 3% in PBS (10 min, RT). After 3 additional washes with PBS, neurons were incubated with Alexa Fluor® Goat anti-human IgG H&L 488 secondary antibody (1:1000 in complete NBS-1%BSA) (45 min, RT). After further washing with PBS (3x), cells were permeabilised with PBS-T 0.3% (15 min, RT) prior to incubation with anti-microtubule associated protein 2 (MAP2) commercial antibodies (1:1000 in PBS-T 0.1%-5% NGS) (1 h, RT). Following PBS washes, appropriate secondary antibodies were added (Alexa Fluor® Goat anti Mouse 568) (45 min, RT). After final PBS washes, coverslips were mounted with DAPI and visualised using a fluorescence microscope (Leica DM 2500™) or a confocal microscope (Zeiss® LSM 710 TM).

Binding of patients' IgG to neurons was considered positive only if MAP2 staining was present, showing the neurons to have been healthy. The binding was scored subjectively on a scale of 0-3 (0= no binding, 1=weak definitive binding, 2= moderate, 3= strong). In the case of repeated experiments, the final score was the mean of the previous scores.

2.3 Passive transfer animal model

2.3.1 IgG purification

The plasma from the CASPR2-Ab positive patient and the serum form the healthy donor were centrifuged, diluted 1:1 with Hartmann's solution and incubated with Protein G Sepharose column beads (Sigma-Aldrich, Inc.) overnight at 4°C on a roller. The Sepharose-IgG mixture was then percolated trough a chromatography column before elution of IgG with 0.1M glycine solution (pH 2.3); the eluate was immediately neutralised with 100 µl of 1M Tris pH 8. The protein concentration of the elution was measured using a Coomassie Plus assay kit (Pierce,

USA). The eluted fractions were pooled, dialysed against 2 liters of Hartmann's physiological solution two times over 24 hours at 4°C, concentrated by dialysis against polyethylene glycol and filter-sterilized. The concentration was determined using NanoDrop 3300 (ThermoScientific, UK), and IgG was stored at 4°C. The specificity of the binding and the antibody titre of the CASPR2-IgG in the original plasma were already reported (Coutinho E et al. 2017). Absence of other antibodies in the plasma of the patient with CASPR2-Ab related encephalitis was demonstrated by showing the absence of binding to CASPR2-null neurons and brain sections (Coutinho E et al. 2017).

2.3.2 Animals

Nineteen C57Bl6 male mice aged 6 weeks (18-22 g) were purchased from a licensed breeding establishment (Charles River). The animals were housed in group of four or five under standard laboratory conditions (ad libitum access to food and water; 12:12 light:dark cycle, with lights on at 7:00) and monitored daily during the experimental period. Mice were tagged and randomly assigned to each experimental group. All *in vivo* experiments were performed in the Biomedical Services Unit at the John Radcliffe Hospital in accordance with the United Kingdom Home Office Animals in Scientific Procedures Act (1986) and in accordance with institutional guidelines.

2.3.3 Intraperitoneal injections

Two cohorts of mice were injected intraperitoneally (IP) with purified IgG from either a patient with CASPR2-antibodies (CASPR2-IgG, n=10 mice) related encephalitis or a healthy control (HC-IgG, n= 9). Animals were injected daily for 8 days. At day 3 all mice were injected IP with lipopolysaccharide (LPS, 1 mg/Kg). Further details are provided in results.

2.3.4 Behavioural testing

Behavioural testing was done during the light phase. The animals were brought to the experimental room approximately 15 minutes before testing. A rest period of at least 2 hours was allowed between a test and the following one. Between mice the walls and the floor of the different apparatus were cleaned with 70% ethanol and water and dried with a dry tissue. All tests were performed at baseline and after starting of the IgG injections, from day 5, in order to

allow animals to recover after the LPS injection, which could have affected behaviour. The olfaction test and the reciprocal social interaction test were performed only after the IgG administration.

2.3.4.1 Accelerating rotarod (AR)

Accelerating rotarod was used to assess motor coordination (Deacon R, 2013). The mice were placed place on the rotating rod, facing away from the direction of rotation. The rotarod was initially set with a speed of 4 rpm for the initial 10 seconds after which an acceleration of 20 rpm/minute was applied. Time to fall was recorded. If the mouse had fallen off during the initial 10 seconds it would have another try, to a maximum of 3 trials (Figure 2.1).

2.3.4.2 Kondziela's inverted screen test (IS) or grip test

This test was used to assess muscle strength. The inverted screen consisted of a 43 caesurae of wire mesh consisting of 12 mm squares of 1 mm diameter wire, surrounded by a 4 cm deep wooden beading to prevent the mice from climbing to the other side. At the beginning of the test the mouse was placed in the centre of the apparatus on the deeper side and the screen was quickly inverted while the timer was started. The inverted screen was held 50 cm above a padded surface for a maximum of 5 minutes. The time to fall was recorded. The average of three trials was taken as a final result (Figure 2.1).

2.3.4.3 Narrow beams (NB)

Narrow beams or static rods were used as another test of coordination (Deacon R, 2013). For this test three wooden rods of varying thickness (35 (rod 1), 22 (rod 2) and 9 (rod 3) mm diameter) each 60 cm long were fixed to a laboratory shelf such that the rods horizontally protrude into space at a height of about 60 cm from a padded floor. The end of the rod near the bench has a mark 10 cm from the end, to denote the finishing line. The mouse was placed at the far end of the widest rod and the timer was started. The orientation time (time taken to orientate 180° from the starting position towards the shelf) and transit time (the time taken to travel to the shelf end) were recorded for a maximum of 5 minutes. If the mouse had not fallen and had arrived at the end of the rod, was transferred on the next smaller rod. If the mouse had not reached the end by this time the test was ended, and the mouse transferred to the next rod (Figure 2.1).

2.3.4.4 Marble burying test (MBT)

This test was used to assess the presence of repetitive, compulsive-like behaviors (Angoa-Pérez M et al. 2013). Standard polycarbonate mouse cages with fitted filter-top covers were fitted with fresh mouse bedding to a depth of 5 cm and its surface leveled by inserting another cage of the same size onto the surface of the bedding. Ten standard glass toy marbles (assorted styles and colors, 14 mm diameter) were placed on the surface of the bedding in 2 rows of 5 marbles. The mouse was placed into a corner of the cage containing the marbles and left undisturbed for 30 min. Food and water access was allowed during the test. At the end of the test the mouse was returned to its home cage and the marbles buried were counted and the number expressed as percentage. A marble was scored as buried if two-thirds of its surface area was covered by bedding (Figure 2.1).

2.3.4.5 Open field (OF)

The open field was used to assess motricity but also anxiety. The apparatus consisted of a dark closed arena of 50 x 50 cm divided into 10 cm squares illuminated with a 60 W lamp posed 45 cm above the centre of the floor of the box. The mouse was placed in a corner square facing the wall and its movement recorded on camera for 5 minutes. The latency to move, the number of peripheral and central square entered (four paws), the number of rears (both back paws on the ground but not part of grooming), the number of grooming and time spent grooming, the time spent in the peripheral and in the central squares, the time spent moving and the time spent freezing were recorded. The number of faecal boli and the presence/absence of urine were also recorded (Figure 2.1).

2.3.4.6 Light-dark box (LDb)

The LD box test was used to assess anxiety. The apparatus consisted of an open white compartment 30 x 20 x 20 cm joined by a 3 x 3 cm opening to a dark box 15 x 20 x 20 cm. The white compartment was illuminated by a 60 W lamp placed 45 cm above the centre of the floor. One side of the white compliment of the box was transparent allowing detection of the mouse movement. The mouse was placed in the middle of the light side facing away from the opening and a count-down timer for 5 min started. The latency to cross with all four feet to the dark side, the latency to cross with all four feet to go back for the first time to the light side, the total time spent on the light side and on the dark side and the number of transitions through the opening were registered for the test. The number of faecal boli and the presence/absence of urine were also recorded (Figure 2.1).

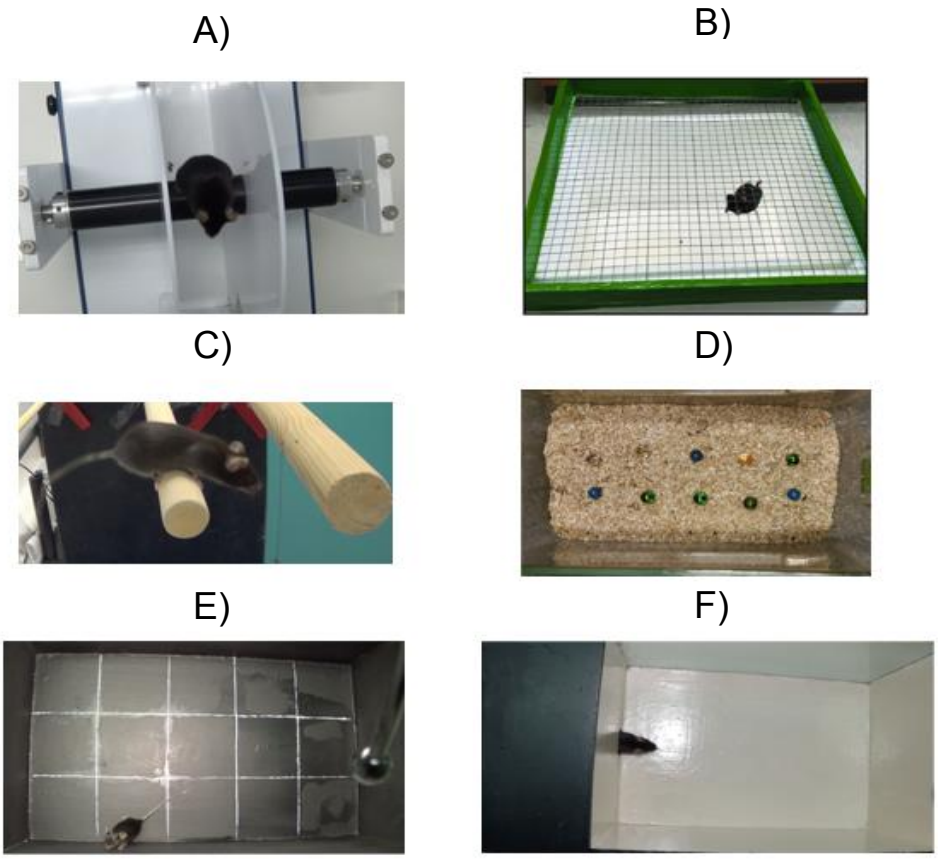

Figure 2.1 Motor, compulsive-like behaviour and anxiety behavioural testing

A) Accelerating rotarod; B) Inverted screen; C) Narrow beams; D) Marble burying test; E) Open field test; F) Light-dark box test.

2.3.4.7 Forced alternation test (FA) or spatial preference test

This test was considered as a short memory test. The apparatus was a Y-maze constructed from transparent Perspex and mounted on an opaque square Perspex board (64.5 cm x 56.5 cm). The walls of the Y-maze were 20 cm high and 0.5 cm thick. Each arm was 30 cm long and 8 cm wide. The test consisted of 3 periods: a habituation period (5 minutes), a delay period (1 minute) and a test period (2 minutes). During the exposure training trials, the entrance to one arm (the Novel arm) was blocked using a rectangular piece of Perspex. The Novel arm (NA) was counterbalanced in each group between right and left side. At the start of a trial the mouse was placed at the end of the Start arm (which was always considered as the one closest to the experimenter) and allowed to explore the Start arm and the Other arm. During this period the time spent in each arm and the number of entries to each arm were recorded. An arm entry was defined as when a mouse had placed all four paws into an arm. At the end of the trial the mouse was removed from the maze and returned to its home cage for 1 minute during which the maze was cleaned and the block to the Novel arm removed. At the beginning of the test period the mouse was returned at the end of the Start arm and now allowed to explore the Start, Other, and Novel arms and exploratory behavior assessed for 2 minutes. During the test the time spent in each arm and the number of entries into each arm were recorded. Preference for the Novel arm was calculated as a Preference Index (entries/time in the new-entries/time in the old arm)/(entries/time in the new + entries/time in the old arm) for both the time in arms and number of arm entries (Rubovitch V et al. 2010). Scores greater than 0.5 indicate a preference for the Novel arm (Figure 2.2).

2.3.4.8 Continuous spontaneous alternation (CSA) test

The continuous spontaneous alternation test was used as a test of working memory. The test was conducted in the same Y-maze as described above and it consisted of a single 5 min trial, in which the mouse was allowed to explore all three arms of the Y-maze. The start arm was varied between animals to avoid placement bias. CSA was assessed by scoring the pattern of entries into each arm during the 5 min of the test. Spontaneous alternation performance (SAP) was defined as successive entries into each of the three arms as on overlapping triplet sets (i.e., ABC, BCA, . . .) and scored as percentage of spontaneous alternation (total alternations/total arm entries - 2* 100) (Wietrzych M et al. 2005). The percentage of alternate arm returns (AARs) (i.e. ABA) and same arm returns (SARs) (i.e. AAB) were also scored for each animal in order to assess aspects of attention within spontaneous working memory (Wall PM and Messier C, 2002). Total entries were scored as an index of ambulatory activity in the Y-maze (Figure 2.2).

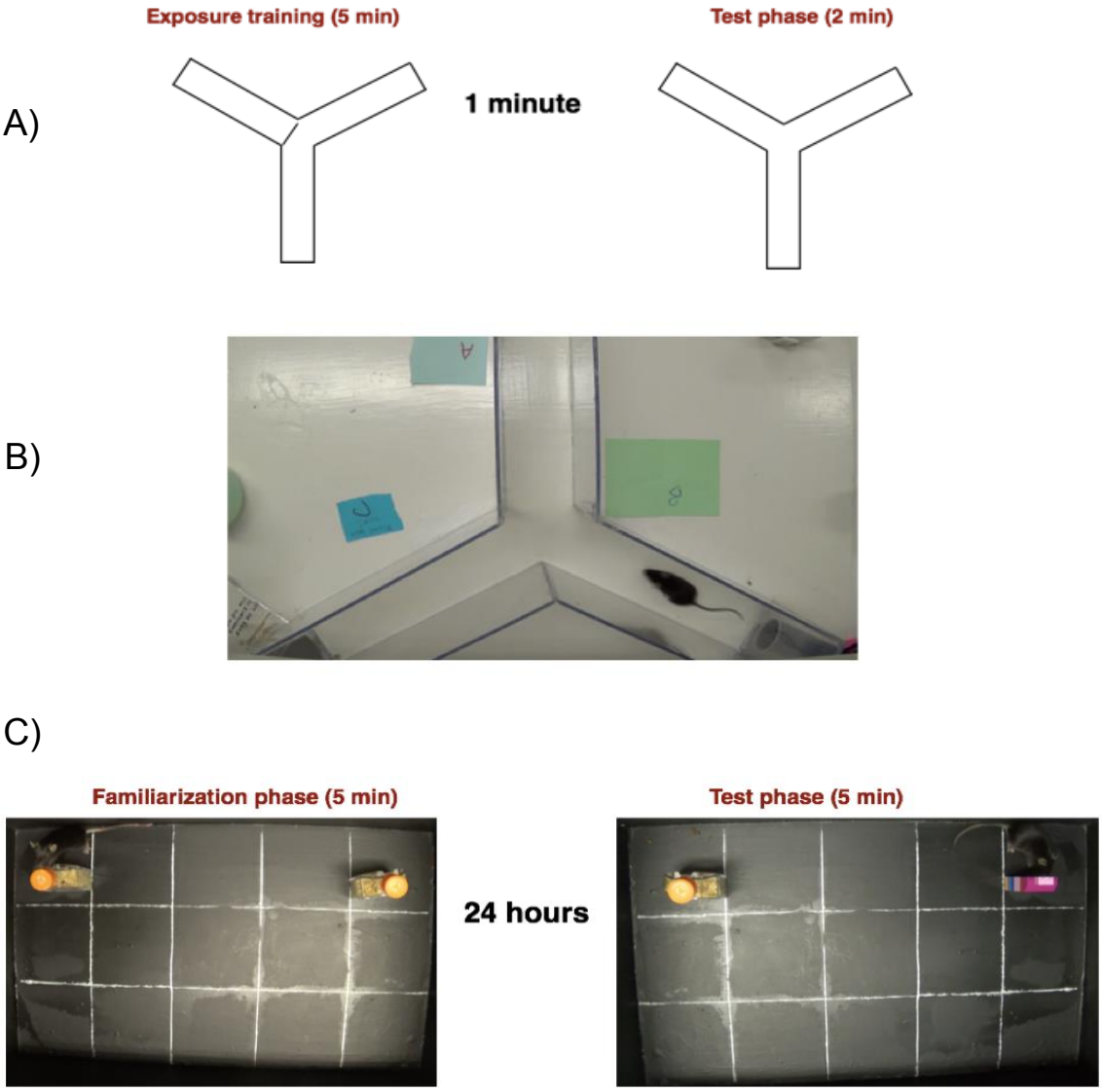

Figure 2.2 Memory tests

A) Forced alternation test: in the habituation period the mouse could explore only two arms of the Y-maze for 5 minutes before being returned to its home cage; after 1 minute delay, the mouse was returned in the Y-maze and allowed to explore all arms; B) Continuous spontaneous alternation test; C) Novel object recognition test: in the familiarization phase the mouse was left to explore two identical objects; after 24 hours one of the object was substituted by a novel object.

2.3.4.9 Novel object recognition (NOR) test

The novel object recognition test was used to evaluate long term memory (Antunes M and Biala G, 2012). The open field arena was used in order to benefit from previous habituation. Towers of Lego bricks (X-cm high and X-cm wide) and Falcon tissue culture flasks filled with bedding (9.5 cm high, 2.5 cm deep and 5.5 cm wide, transparent plastic) were used as objects. Animals were randomly assigned one of those pairs of object for the familiarization phase. The test comprised two phases: a familiarization phase and a test phase performed 24 h later. During the familiarization session two identical objects (either towers of Lego bricks or Falcon tissue culture flasks) were placed in the open field arena, 5 cm away from the walls. The mouse was placed in the open field, its head positioned opposite the objects, and left free to explore for 5 minutes. The test phase was identical to the familiarization phase but one of the familiar object was replaced in the same position with a new object. The position of the novel object (left or right) was randomized between each mouse. The test was video recorded and subsequently analysed for number of visit and time spent with each object during both the familiarization and the test phase. The object exploration was scored whenever the mouse sniffed the object or touched the object while looking at it (i.e., when the distance between the nose and the object was less than 2 cm). Climbing onto the object (unless the mouse sniffs the object it has climbed on) or chewing the object did not qualify as exploration. If an animal had a total objects exploration time < 5 sec during any phase it was excluded from the analysis. The preference for the new object was expressed as Preference index [PI= (time new object - time familiar object)/(time new object + time familiar object)]. This result can varies from +1 to -1 with a positive score indicating preference for the novel object (Antunes M and Biala G, 2012) (Figure 2.2).

2.3.4.10 Reciprocal social interaction test (RSI)

This is a social interaction test where two mice of the same treatment group, unknown to each other, are allowed to interact freely (Barkus C et al. 2012). The apparatus was the same used for the open field as the mice were already habituated to it. Two animals from the same treatment group but housed in different cages, tightly matched for weight (within 5% difference), were exposed to each other for 5 minutes. The test was video recorded and scored offline for the latency to start the interaction and for time spent in social and non-social behaviours and number of social and non-social events. Social behaviours included sniffing, grooming and following closely; non-social behaviours included self-grooming, rearing and freezing. The time active and inactive was also measured.

2.3.4.11 Olfaction test (OT)

An olfaction test was performed in order to assess olfaction deficits that could interfere with the results from the social interaction tests. The open field arena was used in order to benefit from previous habituation. A small plastic container with an odour (1 mL of vanilla or citrus food flavouring) was placed at two corners. The test comprised 3 phases: a sample phase (5 minutes), a delay period (5 minutes) and a test phase (5 minutes). During the first phase, the same odour was placed in both containers (half of the mice for treatment group would smell one odour, while the other half would smell the other). After this, the mice would return to the home cage for 5 minutes. During the test phase, a container with the alternate (new) odour was placed in one of the corners. The location of the new odour was counterbalanced across treatment groups. The test was video recorded and subsequently analysed for number of visit and time spent with each odour.

2.3.5 Blood and brain tissue processing

At day 11, eleven animals (6 CASPR2-IgG and 5 HC-IgG injected) were randomly selected and sacrificed by CO2. Blood samples were collected by cardiac puncture from 10 animals, centrifuged and sera stored at -20 °C. Brains were harvested and split sagittally. For each animal half brain was embedded with freezing media and snap frozen in chilled isopentane for histological analysis whereas the other half-brain was snap frozen without embedding for protein extraction. The remaining eight animals (4/group) were used for morphological and immunofluorescence analysis of the brain. Mice were deeply anesthetized with isoflurane followed by pentobarbital injection (100 mg kg−1 i.p) and transcardially perfused with 50 ml of phosphate-buffered saline (PBS) followed by 50 ml of ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Brains were removed and post-fixed for 24 hours (h) at room temperature (RT), cryoprotected with 30% sucrose in PBS and snap frozen in ice-cold isopentane. All brains were stored at -80°C.

2.3.6 Morphological and immunofluorescence analysis of the brain and image processing

For morphological and immunofluorescence analysis, perfused fixed brains were cut at 50 µm thickness using a Leica CM1900 cryostat in 10 series of free-floating sections. Confocal images were taken of immunofluorescently labeled brain sections using a Zeiss LSM 710 confocal microscope and images were analysed with FiJi software (Open Source HR Software).

2.3.6.1 IgG deposition in the brain and CASPR2 expression

To look at the similarity between CAPSR2 staining and IgG staining, two consecutive series were fixed with 4% PFA, washed 3 times in PBS, blocked for 1 hr in PBS 10% NGS and incubated respectively with anti-human IgG antibodies (Biotium, USA, 20022, 1:500) and with rabbit monoclonal anti-CASPR2 antibodies (Abcam, EPR8738, ab137052, 1:500) in blocking solution overnight at 4°C. We used two consecutive sections instead of a single one to avoid the interactions between secondaries we noted in preliminary experiments. In the same experiments, we also excluded the presence of interactions between the rabbit CASPR2 antibodies, the secondary anti rabbit antibodies and the human IgG already present in the tissue (data not shown). The day after, the latter sections were washed 3 times in PBS and incubated with goat anti-rabbit Alexa Fluor® 568 secondary (1:500) for 1 hr at RT. All sections, included the one incubated with anti-IgG, were washed 3 times in PBS and coverslips were mounted using fluorescent mounting media containing DAPI (1:1000). For each staining, images were acquired using a confocal microscope. For quantitative analysis of the mean fluorescent intensity, 32 single plain pictures (4 from the dentate gyrus, 4 from the CA3, 6 from the CA1 areas of the hippocampus, 6 from the somatosensory cortex, 6 from the thalamus, 6 from the cerebellum) were taken from 2 sections per animal. Mean fluorescence intensity was analysed with FiJi software (Open Source HR Software) and results plotted with GraphPad 6 as the mean of the intensity per each area per mouse.

2.3.6.2 Morphometric studies

The presence of gross morphological alterations was evaluated on Nissl-stained sections. The cresyl violet solution (Sigma C5042) that stains Nissl bodies (granular endoplasmic reticulum and ribosomes) allows observation of neuronal soma. Sections were mounted on super frost plus slides (vWR), left to dry at RT, fixed with 4% PFA for 10 minutes, rinsed twice in PBS and once in deionized water for 5 minutes and immersed in cresyl violet solution (Sigma C5042) for 10 minutes. Slides were than dehydrated by immersion in ethanol solutions of increasing concentrations until 100%, cleared with xylene and coverslipped with DPX mounting medium. Slides were scanned with the Aperio AT2 scanner and analysed with the e-pathology Aperio ImageScope image analysis system from Leica Biosystems. The sums of the area of a series of sections (12 sections), from the appearance of the frontal pole cortex to the most posterior part of cerebellum, was multiplied for the number of sections and the thickness

of the section (50 µm) to obtain the total brain volume on the coronal plain. The same formula was used to calculate the cerebellum and hippocampus volumes (at least 4 sections). Thickness of the following structures were measured (25-50 measurements per area): anterior cingulate, motor, piriform and somatosensory cortices, neuronal layers of dentate gyrus, CA3 and CA1, CA4, CA3 and CA1 fields, granular and molecular layer of the cerebellum.

2.3.6.3 Neuron and astrocyte counts

Neurons and astrocytes were identified by immunofluorescence using a mouse anti-NeuN (Chemicon, MAB377; 1:500) and a polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) antibody (Dako, Z0334; 1:500) respectively. Free-floating sections were fixed with 4% PFA, washed with PBS then blocked with 10% NGS in PBS-Triton-X-100 (0.3%) for an hour then incubated overnight with primary antibodies at 4°C. The next day the sections were washed in PBS then incubated for two hours at RT with goat anti-mouse (568) and goat anti-rabbit (488) Alexa-Fluor secondary antibodies from life technologies at 1:500 dilutions in blocking solution. Sections were subsequently washed in PBS, mounted on slides after a brief wash in TNS (pH = 7.4) and counterstained with DAPI mounting medium, left to dry then sealed and stored protected from the light at 4°C for confocal imaging. Cerebellar sections were stained similarly. In this case, however, primary antibodies included also guinea pig anti-calbindin D28K antibody (Millipore, AB1778; 1:200) for the identification of Purkinje cells. Secondaries antibodies included goat anti-mouse (648), goat anti-rabbit (488/568), goat anti-guinea pig (555). Neuronal and astrocyte cell densities were determined in the hippocampal fields (CA4, CA3, CA1), the somatosensory cortex and the piriform cortex. Confocal images were taken across the z-plane spanning the entire hippocampus in all cases in both hemispheres. Six images were taken per hemisphere for each cortical region. Eighteen stacks were taken every 2 µm z-plane for the hippocampus. For the neuronal density, cells were counted in every fifth image in the same stack. For astrocytes density, images were z-projected, cells counted, and the density calculated as number of cells per volume of area. In the cerebellum, confocal images were taken across the cerebellar lobules. Fifteen stacks were taken for each case every 3 µm z-plane. To avoid bias related to the different distribution of Purkinje cells across photographs the Purkinje cell density was calculated as linear density (number of calbindin positive cells per mm). At least 100 cells were counted for each case over 5,3 mm length. Astrocytes density was assessed in the molecular layer of the cerebellum as mean fluorescence intensity of the GFAP staining. For the neuronal density in the molecular layer, images were z-projected on FiJi and NeuN positive cells counted.

2.3.6.4 C-fos expression

To assess the expression of neuronal c-fos, free flowing sections were rinsed in TNS, fixed with 4% PFA for 15 minutes than washed 3 times in PBS, blocked for one hour in 10% NGS 0.3% PBS-T and incubated with rabbit anti-c-fos (Abcam, ab190289; 1:100) and mouse anti-NeuN (Chemicon, MAB377; 1:500) in blocking solution overnight at 4°C. The day after sections were washed and incubated with goat anti-rabbit (488) and goat anti-mouse (568) Alexa-Fluor® secondary antibodies at 1:500 dilution in blocking solution in the dark at room temperature for two hours. Sections were subsequently washed in PBS, mounted on slides after a brief TNS wash (pH = 7.4) and counterstained with DAPI mounting medium, left to dry then sealed and stored at -20°C for confocal imaging. Quantification of c-fos expressing neurons (defined as c-fos/NeuN positive cells) was performed in the somatosensory, entorhinal and piriform cortex, the CA4, CA3 and CA1 fields of the hippocampus, the amygdala, the torso-medial and lateral nuclei of the hypothalamus. For each hemisphere, twelve z-stacks were taken in the hippocampus (3 for the CA4, 4 for the CA3 and 6 for the CA1), four in the somatosensory area, 3 in the piriform cortex, 4 in the amygdala, 2 in the entorhinal and 4 z-stacks (2 per subarea) were taken in the hypothalamus. The z-step interval was 2 µm within a 50 µm depth. An average density was obtained (cells/mm^3) for each area.

2.3.6.5 Microglia counts and morphological analysis

Microglial cells were identified by the combined expression of Iba1 and CD68 markers. Free floating sections were fixed with 4% PFA, washed with PBS then blocked with 10% NGS in PBS-T 0.3% for an hour then incubated overnight with a rat anti-CD68 (BioRad, MCA1957; 1:400) and a rabbit anti-Iba1 (Wako chemicals, 019-19741) primary antibodies in blocking solution at 4°C. The sections were washed the next day with PBS-T 0.3% then incubated for two hours at room temperature with goat anti-rat (488) and goat anti-rabbit (568) Alexa-Fluor® secondary antibodies at 1:500 dilution in blocking solution in the dark. Sections were subsequently washed in PBS, mounted on slides after a brief TNS wash (pH = 7.4) and counterstained with DAPI mounting medium, left to dry then sealed and stored at -20°C for confocal imaging. Quantification of reactive microglia (defined as CD68/Iba-1 positive cells) was performed in the somatosensory and piriform cortex, the CA4, CA3 and CA1 fields of the hippocampus and in the granular and molecular layers of the cerebellum. For each hemisphere, seventeen z-stacks were taken in the hippocampus (3 for the CA4, 4 for the CA3 and 10 for the CA1), nine in the somatosensory area, 4 in the piriform cortex and 5 z-stacks per layer were

taken in the cerebellum. The z-step interval was 2 µm and microglial cells were counted within a 50 µm depth. An average density was obtained (cells/mm^3) for each area.
Microglial morphology was assessed in confocal z-stacks detecting fluorescence on Iba-1 expressing cells in the hippocampus and in the molecular layer of the cerebellum as previously described (Coutinho E et al. 2017). Soma size (μm^2) and total cell body size (μm^2) were measured and the soma/total cell body size ratio calculated and used as a marker of microglia activation. The length of the longest ramification (max ramification length) was recorded manually in Fiji.

2.3.6.6 Astrocyte complement C3 expression and morphology

Double staining for complement C3 fraction and GFAP was used to evaluate astrocytes activation. Free floating brain sections were mounted on SuperFrost plus slides. When dry, sections were washed with TNS, immersed for 10 minutes in a boiled solution of citrate EDTA buffer and then left in the same solution for 20 minutes on ice. Sections were washed three times in PBS-T 0.3% and incubate with rat anti-C3 (Abcam, EPR19394, ab200999, 1:100) and rabbit anti-GFAP (Dako, 1:500) primary antibodies overnight at 4°C. The day after, sections were washed in PBS-T 0.3% and incubated with goat anti-rat (488) and goat anti-rabbit (568) Alexa-Fluor secondary antibodies at 1:500 dilution in 5% NGS PBS-T 0.3% solution in the dark at room temperature for one hour. Slides were than washed in PBS-T 0.3% and TBS, counterstained with DAPI mounting medium, left to dry then sealed and stored at -20°C for confocal imaging. For each hemisphere, three z-stacks were taken in the hippocampus, somatosensory cortex and cerebellum at 40X magnification with a z-step of 2 µm within a 50 µm depth. Images were analyzed using Fiji. After z-projecting and automatic thresholding, the composed images were split in three channels. Images were magnified. Single astrocyte cells were manually selected, and for each cell the area on the GFAP and on the C3 channels measured. For each cells the C3 expression was calculated as C3/GFAP stained cell areas ratio. 200 cells/group were analyzed in the hippocampus, 81 cells/group in the somatosensory cortex and 100 cells/group in the cerebellum, and the results plotted as both cells average and 4 animals/group average. Astrocytes morphology was assessed on the same z-stacks detecting fluorescence in GFAP expressing cells using the same script as for assessing microglia morphology (Coutinho E at al. 2017). The total cell body size (μm^2) was measured and the number of ramifications and the length of the longest ramification (max ramification length) were recorded manually in Fiji.

2.3.7 Immunoblot analysis

For total brain protein extraction, frozen brain tissue (3 brains and cerebellar hemispheres per mice/group) was homogenate in lysis buffer (50 mM TRIS-HCL, 150 mM NaCl, 0.1% SDS, 1% triton-X 100, pH 7.4) supplemented with protease inhibitors (1:100) with a mechanical homogenizer. The homogenate was than spinned at 17000 rpm for 20 minutes at 4°C. Supernatant was collected and protein concentration measured by a Pierce™ BCA protein assay kit (23225).

2.3.7.1 Western blot analysis of CASPR2 expression

For western blot (WB) analysis, NuPAGE sample reducing agent (10x; Invitrogen, NP0009) and LDS sample buffer (4x; Invitrogen, NP0008) were added to 10 to 30 µg of an appropriate amount of solubilized tissue, boiled for 10 minutes and the proteins separated into 3-8%Tris acetate SDS polyacrylamide gels (Invitrogen, NP0322) and transferred with dry blotting on nitrocellulose membranes. Membranes were blocked in 5% non-fat skimmed milk in PBS 0.1% Tween 20 solution and incubated overnight at 4°C with rabbit anti CASPR2 (Abcam, EPR8738, ab137052, 1:1000) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (Abcam, [EPR16891] ab181602 1:2000). Membranes were washed in PBS-Tween 0.1% and incubated with secondary antibodies in blocking solution for 1 h at room temperature (anti-rabbit IgG HRP 1:1000). Signals were detected by enhanced chemiluminescence (Amersham GE Healthcare) and captured on autoradiography film (GE Healthcare). All studies were done in duplicate or triplicate. Films were digitally scanned, and signals quantified using Fiji ImageJ software. The signal intensity of each antigen was normalized to that of GAPDH in the same lane. The mean OD intensity of signal in CASPR2-IgG and HC-IgG treated animals was compared in GraphPad Prism 6.

2.3.7.2 Cytokine and chemokine array

Brain cytokine expression was analyzed using a Mouse Cytokine Antibody Array (22 Targets) (Abcam, ab133993) as per manufacturer instructions. Briefly, membranes were blocked in blocking buffer for 30 minutes at room temperature than incubated with 250 µg of proteins from the brain lysates in blocking buffer overnight at 4°C. The day after, membranes were thoroughly washed and incubated with biotin-conjugated anti-cytokines antibodies overnight at 4°C. Following washing, streptavidin-HRP was applied for 2 hours at room temperature.

Immunoreactivity was then visualized using enhanced chemiluminescence reagent. X-ray film was than scanned and densitometric analysis performed using Fiji. Positive controls were included on each membrane and used for results normalization. The mean intensity of the normalized signal in HC-IgG injected mice was defined as 1 and the other intensities expressed relatively to this value.

2.4 Statistics

A student's t test or a Mann-Whitney was used to compare the mean of two groups, depending if the data distribution was normal or not respectively. Holm–Sidak correction was applied to correct for multiple comparisons. Chi square or Fisher's exact test were applied to determinate the relations between categorical data. One-way ANOVA was performed when more than two groups were compared. For weight changes over time a two-way ANOVA was used with post hoc analysis. Behavioural data were analyzed by one-way ANCOVA, using the baseline behavior as a covariate. *Post hoc* analysis was performed when appropriate. Significance for all experiments was placed at $p<0.05$. Statistical tests were carried with GraphPad prism version 6 (GraphPad software, San Diego, California, USA) or IBM SPSS statistics version 20.0 (SPSS Inc., Chicago, USA). Graphs were plotted using Graph Pad prism version 6. Data is shown as the mean ± SEM if not otherwise specified.

Chapter 3. Antibodies in cohorts of patients with central nervous system disorders and controls

3.1 Introduction

As discussed in the Introduction, the discovery of syndromes associated with the presence of antibodies directed against neuronal surface antigens (NSA-Abs) has helped to define a clear aetiology and a rationale for a specific therapy in several cases of autoimmune encephalitis. However, patients with *"a minora"* phenotypes, with a more restricted clinical presentation, have been reported in every neurological field from epilepsy to neurodegenerative disorders. As a result, an effort has been made to identify the clinical features that may suggest an antibody-mediated disorder in those cases which do not have a "classical" presentation but could still benefit from the immunotherapy.

On the other hand, the search for antibodies has also been extended to several disorders for which the aetiology is still obscure but in which an autoimmune aetiology is strongly suggested by circumstantial evidences (i.e. Narcolepsy type 1). These efforts, have, as a counterpart, raised new questions, particularly about the role and relevance of the antibodies in "discrete" neurological syndromes, their pathogenicity and, of course, their pathophysiological mechanisms. Moreover, the discovery of antibodies against neuronal-surface antigens (NSA) in a small proportion of healthy controls (Dahm L et al. 2014) or patients with other confirmed neurological diagnosis (Rossi M et al. 2015) or transiently during the course of a neurological disease (Wright S et al. 2016), has led to the hypothesis that these antibodies might be clinically irrelevant or a secondary phenomenon.

The purpose of this study was, therefore, to evaluate the frequencies of NSA-Abs in different CNS neurological disorders and in controls with PNS disorders or none, and to try to establish a correlation with specific clinical phenotypes and, thereby, shed light on their significance. Three groups of patients were studied: narcolepsy type 1 (NT1) as an example of a highly specific disease, epilepsy patients representing a potentially diverse group, mostly with unknown and possibly autoimmune aetiologies, and patients with neurodegeneration who represented a diverse group of patients with brain pathology of other primary causes. For antibody screening, three approaches were used: two screening approaches combining search for unknown antigens by immunohistochemistry (IHC) on rat brain sections and immunofluorescence on dissociated neurons cultures, and a candidate antigen approach using cell-based assays (CBAs) for specific antigens. The clinical data were subsequently collected

from medical records available at the UOC Clinica Neurologica di Bologna. All methods are available in Chapter 2.

3.2 Screening of antibodies in narcolepsy type 1

This cohort included 59 patients (22 F, 37 M), aged 31.10 ± 17.71 years (range 8-69) at sampling. Data are summarised in Table 3.1. Based on the age at sampling, the cohort included 22 children (age < 17 years). The typical association of reduced CSF HCRT1 levels and positivity for the allele DQB1*0602 was observed in 54 patients, whereas one was HLA negative with normal HCRT-1 levels, two were HLA negative with reduced HCRT-1, and two were HLA positive with normal HCRT-1 in the CSF. Patients' age at excessive daytime sleepiness (EDS) onset was 20.4 ± 13.7 years (range 6-62) and age of cataplexy onset was 22 ± 14.4 years (range 6-62). Patients with age at onset over 40 years were considered as late onset (n=6). EDS usually presented before cataplexy and the time gap between EDS and cataplexy onset was 28.9 ± 51.8 months (range 0-276). On the basis of the time lapse between EDS and cataplexy onset, we divided NT1 patients into subjects with acute onset (cases with gap between symptoms ≤ 6 months) and those with a more chronic evolution (progressive). Twenty-eight patients (15 children) were considered as acute onset.

The timing of sampling in relation to symptom onset was highly variable (time lapse from EDS onset 128.3 ± 131.6 months, range 0-528; time lapse from cataplexy onset 108.9 ± 133.6 months, range 1-504) but 22 patients (11 acute) were sampled within 1 year from onset.

After antibody screening, more detailed clinical information regarding additional symptoms, brain imaging, and CSF analysis were collected and correlation with the antibody status performed. First, we screened for the presence of general reactivity, subsequently we looked for specific antigens by CBAs.

3.2.1 Screening for unknown antigens

Immunohistochemistry (IHC) on sagittal rat brain sections showed the presence of antibody binding to the neuropil in 10 (16.9%) NT1 patients (Figure 3.1). The binding was generally diffuse and concentrated on the hippocampus, cortex, cerebellum and thalamus; no new patterns, potentially specific to this disease, were identified.

Table 3. 1 Demographic and clinical features of the NT1 cohort

N. of subjects	59			
Female:Male	22:37			
Children:adults	22:37			
Age at sampling (mean ± SD) (years)	31.1 ± 17.7			
Age EDS onset (mean ± SD) (years)	20.4 ± 13.7			
Age cataplexy onset (mean ± SD) (years)	22 ± 14.4			
Time lapse between EDS onset-sampling (mean ± SD) (months)	128.3 ± 131.6			
Time lapse between cataplexy onset-sampling (mean ± SD) (months)	108.9 ± 133.6			
Acute:progressive	29:30			
Late onset (>50 y)	6			
Sampled close to onset (1 year)	22			
Post-vaccine/H1N1 cases	2			
HCRT-1 levels	Reduced	Reduced	Normal	Normal
HLA-DQB1*0602	+	-	-	+
Number	55	1	1	2

EDS: excessive daytime sleepiness; HCRT-1: hypocretin 1; SD: standard deviation.

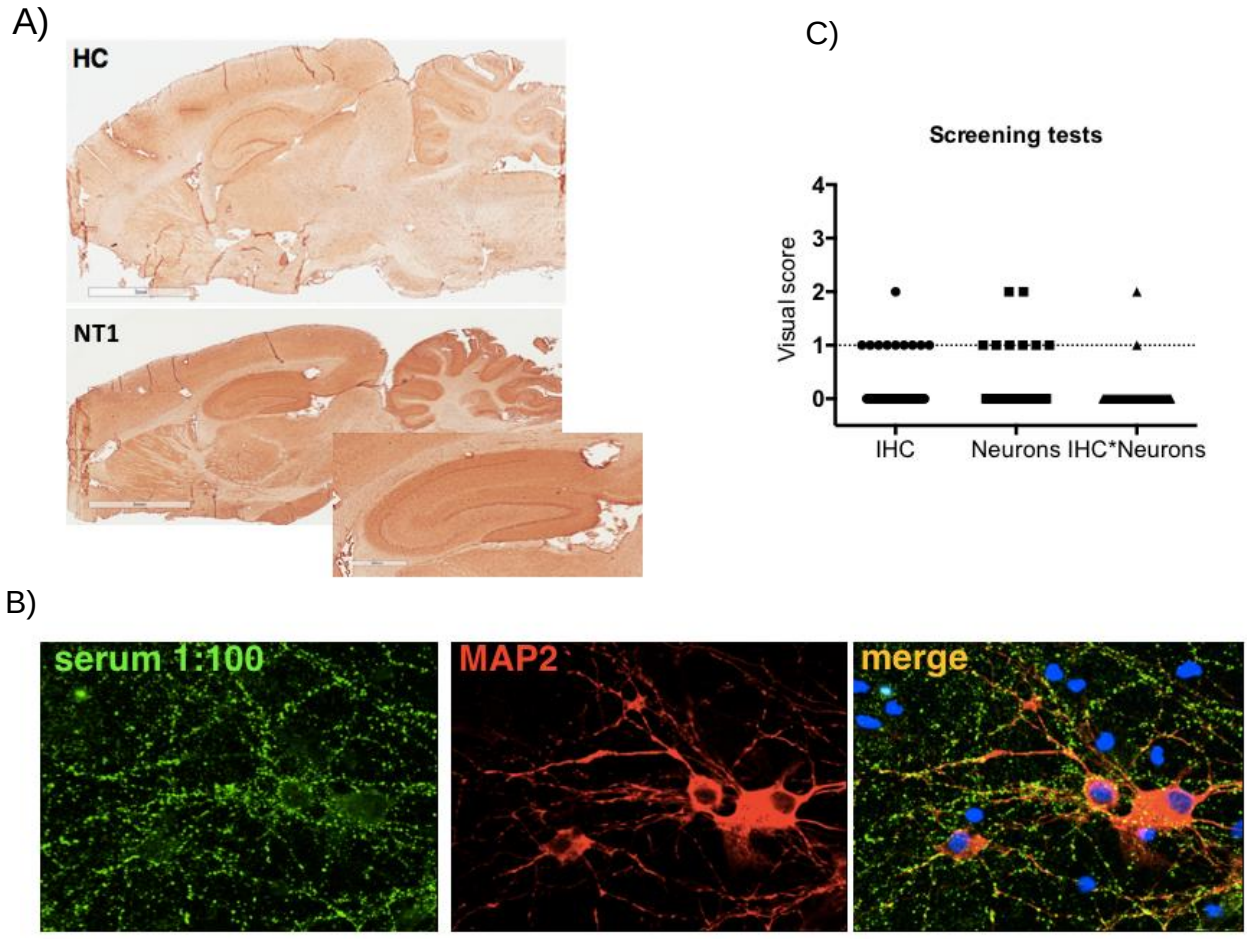

Figure 3.1 Screening for unknown antigens in NT1 patients

A) Representative images of immunohistochemistry (IHC) from a healthy control (HC) and a patient with NT1 showing neuropilar reactivity. B) Representative images of neuronal staining. C) Score results.

Immunofluorescence on rat hippocampal neurons showed punctate staining, suggestive of the presence of an antibody against a neuronal surface antigen, in 8 patients (13.5%) (Figure 3.1). Among these reactive sera, 5 (8.4%) were positive on both tissue and neurons, 5 (8.4%) were positive only on tissue and 3 (5%) positive only on neuronal cultures.

3.2.2 Antibody screening for known antigens on CBAs

The 59 samples from this cohort were screened by CBA for NMDAR, CASPR2, LGI1, AMPAR, $GABA_BR$, $GABA_AR$, D2R, GlyR, Neurexin 1α (NRX1) (Zandian A et al. 2017) and HCRTR2 (Giannoccaro MP et al. 2017) antibodies. Overall, 15 patients had positive antibodies: 8 against NMDAR, 1 against LGI1, 1 against GlyR, 1 against $GABA_AR$, 1 against NRX1 and 3 against HCRTR2. Titres were generally low: 1:20 (n=6), 1:100 (n=1) to 1:500 (n=1) for NMDAR, 1:200 for GlyR, 1:500 for NRX1, 1:100 to 1:200 for HCRTR2 (Figure 3.2). Titres were not determined for $GABA_AR$ and LGI1 antibodies. Among the CBA positive patients 3 (1 LGI1-, 1 GlyR- and 1 $GABA_AR$-Abs) were positive also on brain sections and neuronal cultures, 1 (NMDAR) was positive also on neuronal cultures and 2 (NMDAR) were positive also on immunohistochemistry. The absence of a perfect correspondence between the three different techniques is not surprising as CBAs are known to be more sensitive for most neuronal antibodies. Overall, only 2 patients showed reactivity on both neurons and brain sections in the absence of antibodies against known antigens on CBAs.

Patients with NMDAR antibodies (5 M, 3 F) included 4 adults and 4 children. Age at EDS (P=0.4) and cataplexy onset (P=0.37), age at sampling (P=0.40), time lapse between EDS (P=0.7) and cataplexy onset (P=0.88) and sampling, Epworth sleepiness scale (ESS) (P=0.26) and HCRT-1 CSF levels (P=0.81) were not different from the rest of the patients (independent samples t test; Table 3.2). However, the presence of NMDAR antibodies was associated with an acute onset of the disease (P= 0.022, Fisher's exact test, two tailed) and unexpectedly, these antibodies were inversely associated with the presence of hypnagogic hallucinations (P=0.019, Fisher's exact test, two tailed) (Table 3.3 A and B).

Patients with HCRTR2 antibodies were 2 M and 1 F, and all three had prominent psychiatric symptoms. One male patient, aged 38 years, HLA positive, with undetectable CSF HCRT-1, presented at age 15 years with EDS; cataplexy onset was at 27 years. Since disease onset he had an associated psychosis. The female patient, aged 23 years, HLA negative and with normal CSF HCRT-1, presented with EDS onset at age 16 years followed 2 years later by cataplexy. Recently she displayed severe paranoia and psychotic symptoms. The last patient

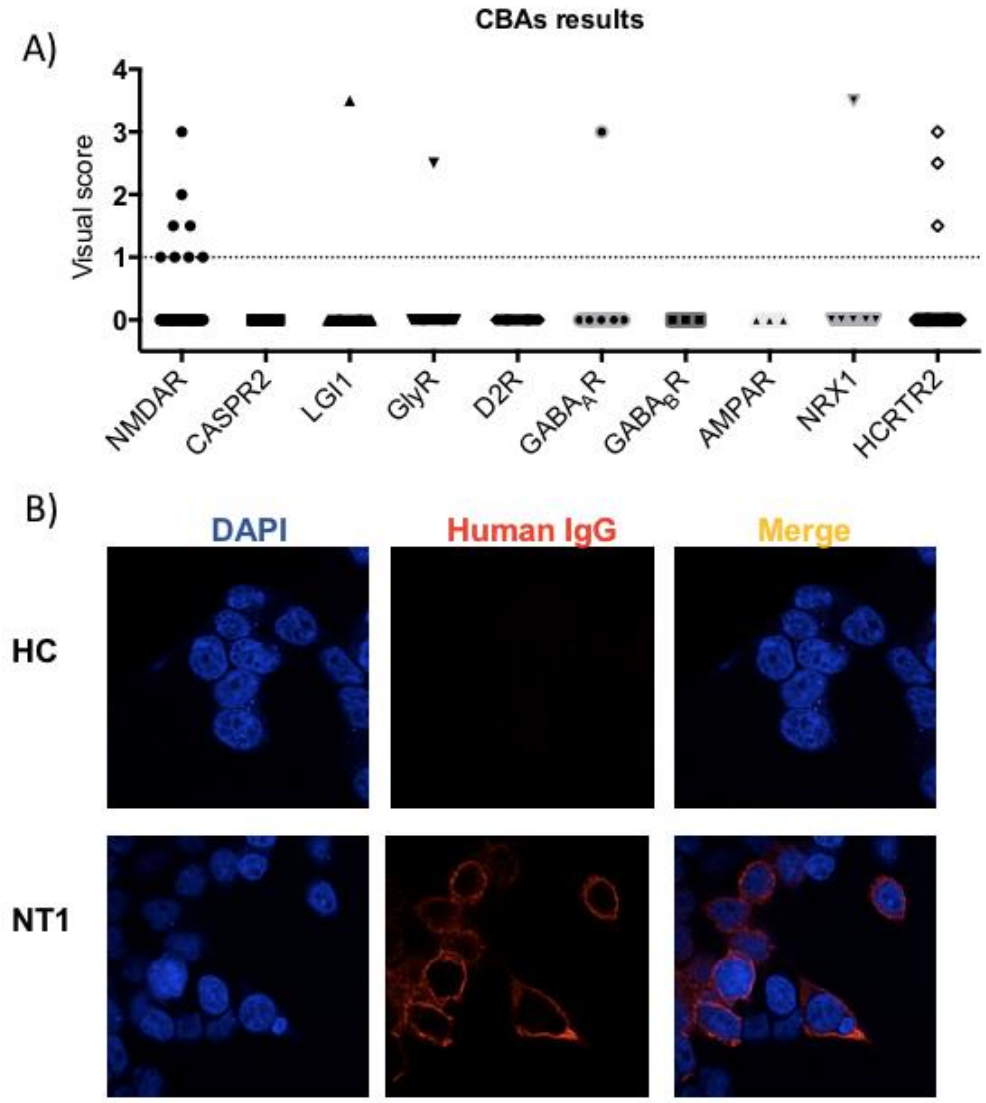

Figure 3.2 Screening for target antigens by CBAs in NT1 patients

A) Score results for different antigens; B) Representative images of a CBA from a negative (HC) and a positive (NT1) patient.

.

Table 3. 2 Demographic features of patients with NMDAR-Abs

	NMDAR positive (n=8)	NMDAR negative (n=51)	p-value
Gender (M, F)	5,3	32,19	>0.999 $
Age EDS onset (years)	16.6 ± 8.1	21 ± 14.14	0.40 ^
Age cataplexy onset (years)	17.7 ± 9.2	22.6 ± 15	0.37 ^
Time lapse EDS-cataplexy onset (months)	13.6 ± 38.1	31.3 ± 53.5	0.37 ^
Age at sampling (years)	26.5 ± 17.5	31.8 ± 17.7	0.40 ^
Time lapse EDS onset-sampling (months)	115.5 ± 134.5	130.3 ± 132.4	0.77 ^
Time lapse cataplexy onset-sampling (months)	102.2 ± 123.6	110 ± 136.3	0.88 ^
HCRT-1 CSF levels	27.1 ± 41.4	32.7 ± 65.7	0.81 ^
ESS	18.1 ± 3.6	16.4 ± 3.9	0.26 ^

$: Fisher's exact test, two tailed; ^: independent samples t-test; CSF: cerebrospinal fluid; EDS: excessive daytime sleepiness; Seaworth Sleepiness Scale; F: female; HCRT-1: hypocretin 1; M: male.

Table 3. 3 Association between NMDAR-positivity in the CBA and acute onset of narcolepsy symptoms (A) and absence of hypnagogic hallucinations (B).

A) Association between NMDAR positivity and acute onset	**NMDAR CBA (Fc) Positive: Negative**
Acute onset (n=28)	7 : 21
Progressive onset(n=31)	1 : 30
P= 0.022 Fisher's exact test, two tailed	
B) Association between NMDAR positivity and absence of hypnagogic hallucinations	**NMDAR CBA (Fc) Positive: Negative**
Hypnagogic hallucinations (n=38)	6 : 15
No hallucinations (n=21)	2 : 36
P= 0.019 Fisher's exact test, two tailed	

was a 17-year old male, HLA positive but with normal CSF HCRT-1 levels. He presented an acute onset of EDS and cataplexy at age 16 years; recently he reported a worsening of auditory hallucinations and mild behavioural problems. No patients had H1N1 infection/vaccination; all had elevated anti-streptolysin O (ASO) titres.

3.2.3 Clinical features of NT1 patients showing any antibody reactivity

Overall 22 patients (37.3%) showed the presence of some reactivity (found either by CBAs, immunohistochemistry or immunofluorescence on neurons). We than analysed if patients with antibody reactivity showed any specific clinical feature. Examined clinical features are described in Table 3.4.

No differences were found in demographics between patients with or without any reactivity (Table 3.5). However, the presence of any antibody reactivity was more frequent in patients with an altered blood-brain barrier (BBB) (P=0.048, Fisher's exact test, two tailed), as expressed by an altered CSF/sera albumin concentration ratio, and less frequent hypnagogic hallucinations (P=0.050, Fisher's exact test, two tailed). The association with an altered BBB was even stronger after exclusion of patients showing isolated reactivity (i.e. only on neurons or tissue) (P=0.018, Fisher's exact test, two tailed). No other associations were found with acute onset, other clinical features (presence of sleep paralysis, other sleep disturbances, psychiatric disorders, other immunological disorders), brain MRI features or other CSF alterations (Table 3.6).

The cohort included 9 patients with NT1 and psychosis. Clinical features are summarised in table (Table 3.7). Surprisingly, none of them had NMDAR-Abs, but, although the frequency of antibody reactivities was not different from the rest of the cohort, 4/9 cases with psychosis showed some evidence of antibody reactivity (1 on immunohistochemistry, 1 for LGI1 and 2 for HCRTR2, as already discussed).

3.3 Screening for antibodies in the Epilepsy cohort

This cohort included 73 patients (6 children) (34 F, 39 M), with clearly defined temporal lobe epilepsy, with or without family history. Age at sampling was 31.7 ± 14.6 years (range 4-82); the age at onset and, therefore, the time lapse between sampling and onset, was not available. After screening for antibodies, clinical information about family history, response to antiepileptic drugs (AEDs), and the presence of temporal lesion or mesial temporal sclerosis

Table 3. 4 Examined clinical features in NT1 patients

Clinical features	N. of data available	N. of patients (%)
Sleep features		
ESS	58	n.a.
Paralysis	59	34 (57.6)
Hypnagogic Hallucinations	59	38 (64.4)
• visual	55	31 (56.4)
• auditory	55	14 (25.5)
• somesthetic	55	14 (25.5)
• multimodal	55	19 (34.5)
RBD	59	21 (35.6)
Other sleep disorders	59	21 (35.6)
Psychiatric disorders		
Psychosis	59	9 (15.3)
Depression	59	18 (30.5)
Anxiety	59	6 (10.2)
Anti streptolysin O titre	48	18 (37.5)
MRI		
Altered	49	10 (20.4)
• white matter abnormalities	49	5 (10.2)
• other lesions	49	5 (10.2)
CSF data		
altered proteins	44	12 (27.3)
altered white cells	43	3 (7)
altered IgGs CSF index	35	8 (22.9)
altered blood-brain barrier index	34	6 (17.6)
OBs		
• absent	42	32 (76.6)
• present	42	5 (11.9)
• mirror pattern	42	4 (9.5)
• mixed	42	1 (2.4)

ESS: Epworth Sleepiness Scale; RBD: REM sleep behaviour disorder; MRI: magnetic resonance imaging; CSF: cerebrospinal fluid; OBs: oligoclonal bands

Table 3. 5 Demographic and clinical features of patients with any reactivity

	Seropositive (n=22)	Seronegative (n=37)	p-value
Gender (M:F)	13:9	24:13	0.78 $
Age EDS onset (years)	21.4 ± 14.7	19.8 ± 13.3	0.67 ^
Age cataplexy onset (years)	23.9 ± 15.5	20.8 ± 13.8	0.47 ^
Time lapse EDS-cataplexy onset (months)	35.5 ± 66.3	24.9 ± 41.4	0.45 ^
Age at sampling (years)	31.5 ± 18.8	30.8 ± 17.2	0.87 ^
Time lapse EDS onset-sampling (months)	122.1 ± 143.2	132 ± 126.1	0.78 ^
Time lapse cataplexy onset-sampling (months)	91.2 ± 133.7	119.4 ± 134.3	0.43 ^
HCRT-1 CSF levels	47.8 ± 91.2	22.2 ± 33.9	0.21 ^
ESS	17.5 ± 4.1	16 ± 3.8	0.16 ^

$: Fisher's exact test, two tailed; ^: independent samples t-test; CSF: cerebrospinal fluid; EDS: excessive daytime sleepiness; ESS: Epworth Sleepiness Scale; F: female; HCRT-1: hypocretin 1; M: male.

Table 3. 6 Contingency table correlating clinical features and antibody positivity

	Seropositive (n=22)	Seronegative (n=37)	p-value
HCRT-1 CSF levels (low:normal)	20:2	36:1	0.54
HLA DQB1*06:02	20:2	36:1	0.54
Onset (acute:progressive)	10:12	16:21	0.43
Sampling from onset (close:far)	10:12	12:25	0.40
Hypnagogic hallucinations (yes:no)	8:13	23:11	0.050
Sleep paralysis (yes:no)	9:13	25:12	0.059
RBD (yes:no)	8:14	13:24	> 0.999
Psychiatric disorders (yes:no)	13:9	17:20	0.42
Other autoimmune diseases (yes:no)	4:18	4:33	0.45
Brain MRI (altered:normal)	6:14	4:25	0.27
CSF analysis data			
Blood brain barrier (altered:normal)	4:6	2:22	0.048*
CSF proteins (increased:normal)	5:11	7:21	0.73
CSF WBC (increased:normal)	0:16	3:24	0.28
CSF IgGs index (altered:normal)	0:10	8:17	0.073

**: significant value (Fisher's exact test, two tailed); WBC: white blood cells*

Table 3. 7 Clinical features of patients with NT1 and psychosis

	Case 17	Case 19	Case 25	Case 39	Case 40	Case 41	Case 61	Case 50	Case 62
Age at sampling	22	13	15	38	15	12	22	14	23
Sex	M	F	M	M	F	M	M	M	F
Age EDS onset (y)	15	11	9	15	14	9	11	12	16
Age cataplexy onset (y)	15	12	15	27	14	9	15	13	17
Type lapse sampling-cataplexy onset (m)	7	1	1 month	11	1	3	7	1	6
Acute onset	Yes	No	Yes	No	Yes	Yes	No	No	No
HCRT-1 levels (pg/ml)	0	10.2	10.8	0	13.3	0	0	23.1	410
HLA-DQB1*0602	+	+	+	+	-	+	+	+	-
Paralysis	+	-	+	+	-	+	+	+	+
Hypnagogic hallucinations	+	+	-	+	+	+	+	+	+
Auditory hallucinations	+	+	-	+	+	+	+	-	-
RDB	-	-	-	+	+	+	+	+	-
Age psychosis onset	21	12	15	16	13	11	21	16	25
Brain MRI	Norm.	N.A.	Norm.	Norm.	Norm.	Norm.	Norm.	Norm.	Norm.
CSF analysis	Altered protein levels	N.A.	Altered IgG index	N.A.	N.A.	N.A.	Normal	Altered IgG index	N.A.
IHC	+	-	-	-	+	-	-	-	-
Neurons	-	-	-	-	+	-	-	-	-
CBAs	-	-	-	HCRTR2	LGI1	-	-	-	HCRTR2
Any reactivity	+	-	-	+	+	-	-	-	+

CSF: cerebrospinal fluid; EDS: excessive daytime sleepiness; IHC: immunohistochemistry; m: months; MRI: magnetic resonance imaging; N.A. not available; norm: normal; y: years; + present; - absent.

(MTS) on brain MRI were retrieved and correlated with the antibody status. These data are presented below. No information on the specific type or frequency of seizures, EEGs or CSF data were available.

3.3.1 Screening for unknown antigens

Neuropil binding was observed by immunohistochemistry in 14 patients (19.1%). The pattern of staining was diffuse and did not suggest a specific reactivity for this cohort. Neuronal cell binding was observed with 11 sera (15.1%) (Figure 3.3). Among these reactive sera, 7 (10.1%) were positive on both tissue and neurons, 7 (10.1%) were positive only on tissue and 5 (5.8%) only on neuronal cultures.

3.3.2 Antibody screening for known antigens

The 73 samples were screened by CBA for NMDAR, CASPR2, LGI1, AMPAR, $GABA_BR$, $GABA_AR$, GlyR, IgLON5 and HCRTR2 antibodies. Given the reported association between GAD65 antibodies and epilepsy, these were also tested by radioimmunoassay (RIA). Fourteen patients (19.1%) had specific antibodies: 2 to GAD65, 2 to GlyR, 5 to CASPR2, 4 to $GABA_AR$, 3 to $GABA_BR$ (Figure 3.3). Two showed multiple reactivities: 1 to CASPR2 and GAD65 and 1 to $GABA_AR$ and $GABA_BR$. Overall, 4 patients (1 GlyR, 1 CASPR2, 2 $GABA_AR$) showed reactivity on neurons and tissue, and 4 (1 GlyR, 1 CASPR2, 1 $GABA_BR$, 1 GAD65) also by immunohistochemistry. Only three patients had undetermined reactivities on both tissue and neurons.

Features of positive patients are described in Table 3.8. No difference in age at sampling were found between patients with different specific antibodies ($F(4, 11)=2.064$, $P= 0.15$, one-way ANOVA). No specific features were associated with specific antibodies (Table 3.8). However, the presence of $GABA_BR$ antibodies was associated with AED resistance ($P=0.026$, Fisher's exact test, two-tailed) (Table 3.8B).

Table 3. 8 Clinical features of Epilepsy patients with specific antibodies

	CASPR2 +	$GABA_AR$ +	$GABA_BR$ +	GlyR +	GAD65 +
Number of patients (%)	5 (6.8)	4 (5.4)	3 (4.1)	2 (2.7)	2 (2.7)
Sex (F:M)	3:2	1:3	1:2	1:1	1:1
Age at sampling	24.6 ± 10.4	44.2 ± 7.4	31.3 ± 15.8	34 ± 9.8	27.5 ± 4.9
Family history (n of patients)	1	0	2	0	0
MTS (n of patients)	2	1	2	0	1
Temporal lesion (n of patients)	2	1	2	0	0
AED resistance (n of patients)	3	3	3	0	2

AED: antiepileptic drugs; MTS: medial temporal sclerosis.

Table 3. 8B Association between $GABA_BR$ antibodies and resistance to anti-epileptic therapies (AEDs)

Association between $GABA_BR$ positivity and AED resistance	**$GABA_BR$ CBA (Fc) Positive: Negative**
AEDs resistance (n=22)	3 : 19
AEDs response (n=50)	0 : 50
P= 0.026 Fisher's exact test, two tailed	

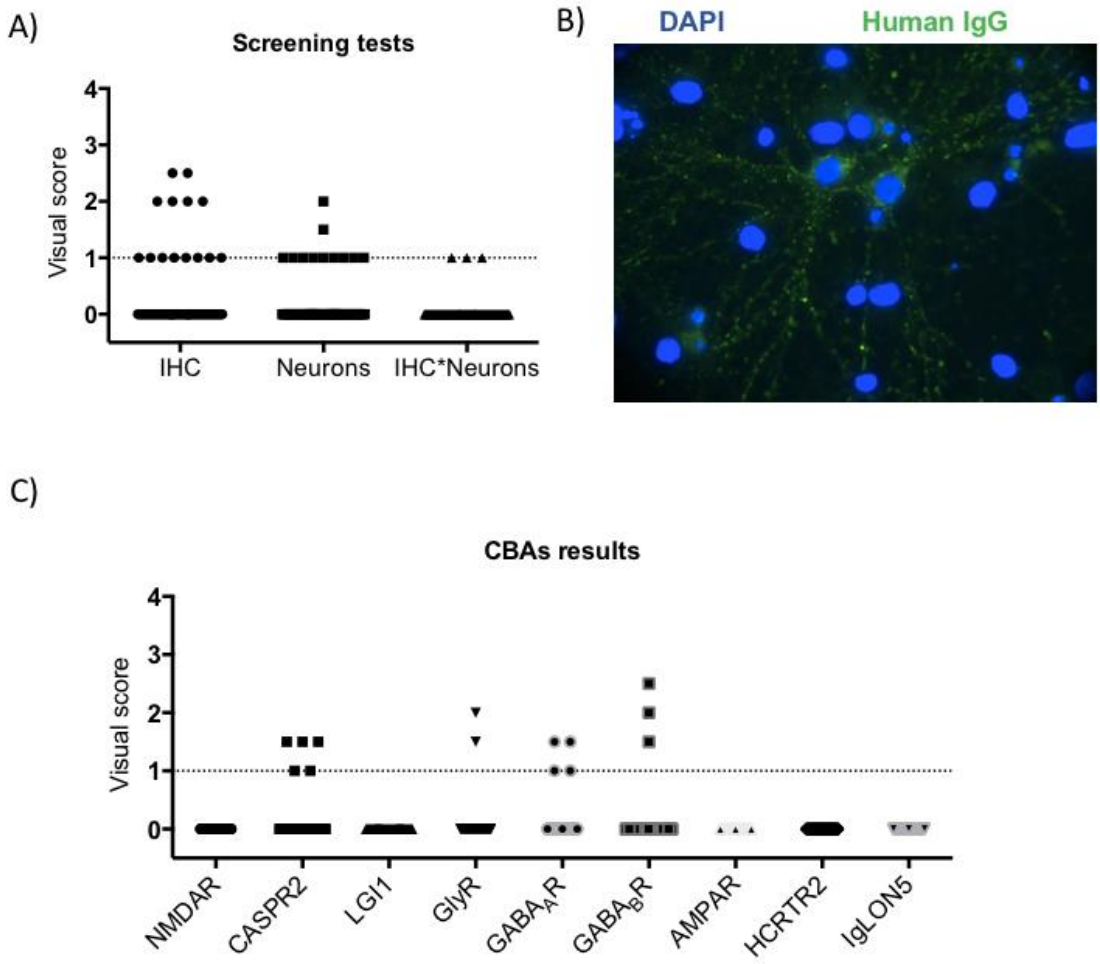

Figure 3.3 Results of antibody screening in Epilepsy patients

A) IHC and neuronal score results; B) Representative images of a low positive on neuronal cultures; C) Score results of CBAs.

3.3.3 Clinical features of patients showing any antibody reactivity

Overall, 24 patients (32.9%) demonstrated some antibody reactivity (either on CBAs, RIA, immunohistochemistry on rat brain tissue or immunofluorescence on neurons).

Epilepsy patients' clinical features are described in Table 3.9. Patients with a family history were significantly younger at sampling than other patients ($t(68)=2$, $P=0.048$, independent samples t-test). The presence of temporal lesions was more common in female patients ($P= 0.014$, Fishers' exact test, two-tailed) and, as expected, mesial temporal sclerosis (MTS) associated closely with other temporal lesions on the brain MRI ($P < 0.0001$, Fishers' exact test, two-tailed).

Patients with antibody reactivity were significantly younger at sampling compared to seronegative patients ($t(71)=2$, $P=0.04$, independent samples t-test) (Table 3.10), and again, antibody reactivity was correlated with resistance to AEDs ($P=0.016$, Fisher's exact test, two tailed) (Table 3.10), and this association remained after excluding patients with only single, undefined, reactivities ($P=0.028$, Fisher's exact test, two tailed) (i.e. positive only on tissue or neurons).

There were no differences in other clinical features, including gender distribution, presence of temporal lesions or mesial temporal sclerosis (MTS) on brain MRI between seropositive and seronegative patients (Table 3.10).

5.4. Screening for antibodies in the Neurodegeneration cohort

This cohort included serum samples from 61 patients (21 F, 40 M), aged 67.9 ± 10.1 years (range 46-84) at sampling, diagnosed with a broad range of neurodegenerative disorders (Figure 3.4), according to current clinical diagnostic criteria. If the clinical criteria for a specific disease were not met, the patients were defined as undetermined dementia (UD) or undetermined parkinsonism (UP). The diagnoses included progressive supranuclear palsy (PSP, n= 5), cortico-basal degeneration (CBD, n= 4), multiple system atrophy (MSA, n= 3), Parkinson's disease (PD, n= 2), PD with dementia (PDD, n= 7), PD with dysautonomia (PDDy, n= 2), Alzheimer's disease (AD, n = 6), Creutzfeldt-Jakob disease (CJD, n= 3), behavioral variant fronto-temporal dementia (FTD, n= 4), dementia with Lewy body (DLB, n=3), UD, n= 14 and UP, n=7. Age at onset was 62.5 ± 13.4 years (range 16-81) and the time lapse between onset and sampling was 71.1 ± 112.7 months (range 1-732).

Table 3. 9 Demographic and clinical features of Epilepsy cohort patients

Feature (n of patients)	Sex (M:F)	Age at sampling	Family history (yes:no)	Temporal lesions (yes:no)	MST (yes:no)	AEDs resistency (yes:no)
Family history						
Yes (n = 24)	12:12	26.8 ± 12.4	-	8:16	3:16	7:17
No (n = 46)	26:20	34.1 ± 15.4	-	17:29	18:27	15:31
p-value	*0.62 $*	*0.048*^*	-	*0.79 $*	*0.082 $*	*>0.999 $*
AEDs resistency						
AEDs resistant (n= 22)	14:8	30.9 ± 15.3	7:15	8:14	9:9	-
AEDs responders (n = 50)	24:26	32.2 ± 14.5	17:31	17:33	12:36	-
p-value	*0.30 $*	*0.73 ^*	*>0.999 $*	*>0.999 $*	*0.075 $*	-
Temporal lesions						
Yes (n = 25)	8:17	33.2 ± 14.2	8:17	-	17:5	8:17
No (n = 47)	30:17	31.1 ± 15	16:29	-	4:40	14:33
p-value	*0.014*$*	*0.56 ^*	*0.79 $*		*<0.0001*$*	*>0.999 $*
MST						
Yes (n = 21)	7:14	34.6 ± 14.7	3:18	17:4	-	9:12
No (n = 45)	27:18	32.2 ± 14.9	16:27	5:40	-	9:36
p-value	*0.064 $*	*0.54 $*	*0.082 $*	*<0.0001*$*	-	*0.075 $*

**: significant value; $: Fishers' exact test, two tailed; ^: independent samples t test; AEDs: antiepileptic drugs; MST: mesial temporal sclerosis.*

Table 3. 10 Contingency table correlating clinical features and antibody positivity

	Seropositive (n=24)	Seronegative (n=49)	p-value
Gender (M:F)	13:11	26:23	> 0.999 $
Age at sampling	26.7 ± 12.6	34.1 ± 15	0.040* ^
Family history (yes:no)	8:15	16:31	> 0.999 $
MTS (yes:no)	6:14	15:31	> 0.999 $
Temporal lesion (yes:no)	8:16	17:31	> 0.999 $
Resistency to AEDs (yes:no)	12:12	10:38	0.016* $

**:significant value; $:Fishers' exact test, two tailed; ^: independent samples t test; AEDs: antiepileptic drugs; MST: mesial temporal sclerosis.*

After screening for antibodies, detailed clinical information about disease onset, clinical presentation, additional features and results of imaging, CSF, neuropsychological and neurophysiological testing were collected for associations with the antibody status.

3.4.1 Screening for unknown antigens

Immunohistochemistry on rat brain sections showed a pattern compatible with the presence of antibodies against neuronal antigens in 9 (14.8%) cases. Neuronal punctate binding was observed with 14 (23%) sera (Figure 3.4). Of these positive sera, 5 (8.2%) were positive with both methods, 9 (14.8%) bound only live neurons in cultures and 4 (6.6%) only on tissue.

3.4.2 Antibody screening for known antigens

Samples were screened by CBA for NMDAR, CASPR2, LGI1, AMPAR, $GABA_BR$, $GABA_AR$, GlyR and IgLON5 antibodies. We identified 11 patients (18%) with specific antibodies (Figure 3.4): 4 to GlyR, 1 to CASPR2, 2 LGI1, 3 to $GABA_AR$, one of whom positive also for AMPAR-Abs, 1 to $GABA_BR$. Overall, 3 patients (1 LGI1, 2 $GABA_AR$) showed reactivity on neurons and tissue and 1 ($GABA_AR$) on neurons. Two patients (3.3%) had undetermined reactivities on both tissue and neurons. Four patients (6.6%) remained positive only on tissue and 8 (13.1%) only on neurons. Demographic and clinical data of patients with specific antibodies are in given in Table 3.11.

3.4.3 Clinical features of patients showing any antibody reactivity

Overall 25 patients (40.9%) showed the presence of some reactivity (either on CBAs, immunohistochemistry or immunofluorescence on neurons). The diagnoses of positive patients were: PSP (n=3), MSA (n=1), PD (n=1), PDD (n=2), PDDy (n=1), CJD (n=2), FTD (n=1), DLB (n=3), UD (n=8), UP (n=3). Clinical diagnosis and demographic data are described in Table 3.12. No associations were found between diagnosis at discharge and antibody presence (P=0.15, Pearson Chi-Square), after dichotomizing patients between defined vs undefined diagnoses (P=0.27, Fishers' exact test, two tailed), or between prevalent parkinsonism or cognitive impairment (P>0.999, Fishers' exact test, two tailed). However, the presence of antibody reactivity was associated with subacute onset in patients with prevalent dementia (P=0.029, Fishers' exact test, two tailed) (Table 3.13 A) but not in patients with prevalent

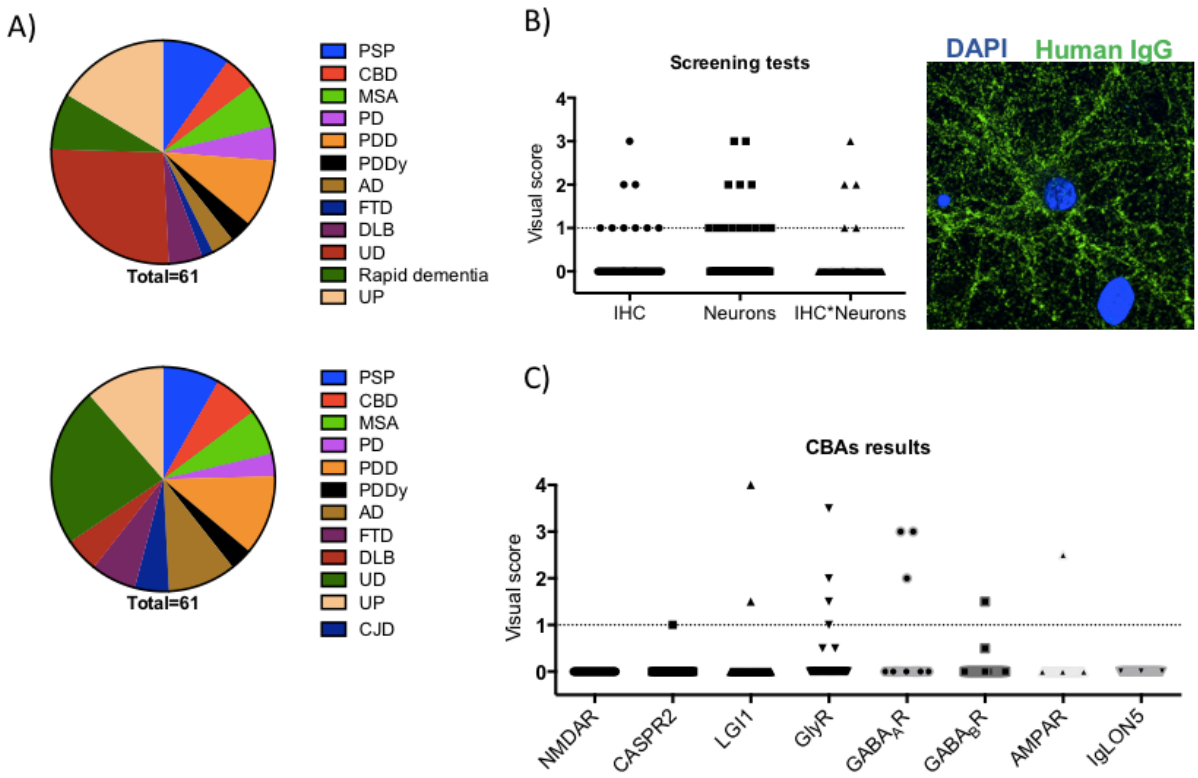

Figure 3.4 Diagnosis and results of antibody screening in Neurodegenerative patients

A) Pie-chart of diagnosis at admission (top); pie-chart of diagnosis at discharge (bottom); B) results of antibody screening by immunohistochemistry (IHC) on rat brain tissue and immunofluorescence on hippocampal rat neurons (left) and representative image of a serum showing reactivity towards an antigen expressed on the surface of live neurons (right); C) Summary of CBA results.

Table 3. 11 Clinical features of patients with Neurodegenerative disorders and specific antibodies

Patient sex, age at sampling	Antibody target	Diagnosis at discharge	Age at onset	Onset	Acute worsening and main feature	Main presentation	Response to symptomatic therapy	Brain MRI	CSF	EEG	MMSE	Other features
F, 68	CASPR2	PSP	64	Chronic	Yes, hypersomnia	Parkinsonism	Yes	Normal	Normal	N.D.	25	RLS
F, 76	$GABA_AR$	PSP and dementia	66	Chronic	No, but step-down	Parkinsonism	Partial	White matter hyperintensity and atrophy	Normal	N.D.	25	RLS, RBD
F, 75	GAB_AAR	Rapidly evolving dementia	71	Subacute	No	Cognitive and behavioural impairment	Partial	White matter hyperintensity and atrophy	Lymphocytosis; OBs (mirror pattern)	Slow	26	AchR+ MG and previous thymo-ma
M, 78	GAB_AAR + AMPAR	Rapidly evolving parkinsonism and ataxia	78	Subacute	No	Parkinsonism and ataxia	No	Compatible with CJD	Increased tau protein and positive 14.3.3	Aspeci-fic	N.D.	Hyperso mnia
M, 79	LGI1	DLB	78	Subacute	No	Parkinsonism, cognitive and psychiatric features	No	White matter hyperintensity and atrophy	Normal	Slow	27	RBD
M, 66	LGI1	Rapidly evolving dementia	66	Subacute	No	Cognitive and behavioural impairment; GTC seizures	N.A.	White matter hyperintensity and atrophy	Increased proteins and altered BBB	Slow and epileptic	24	Status dissocia-tus
M, 65	GlyR	PDD	63	Chronic	No	Cognitive and behavioural impairment	N.A.	White matter hyperintensity and atrophy	Altered BBB	Normal	28	-

Continuation of Table 3.11												
F, 59	GlyR	Undefined dementia	50	Chronic	No	Ataxia and dysautonomia	N.A.	Leukodystrophy	N.D.	Aspecific	N.A.	Seizures; ADLD
M, 70	GlyR	PDD	65	Chronic	No	Cognitive and behavioural impairment	N.A.	Normal	Lymphocytosis	Normal	29	RBD
F, 77	GlyR	Undefined parkinsonism	16	Chronic	No	Parkinsonism	Yes	White matter hyperintensity and atrophy	Normal	N.D.	29	-
F, 53	$GABA_BR$	Undefined dementia	51	Subacute	No	Cognitive and mood disorders; insomnia	N.A.	White matter hyperintensity	Mild lymphocytosis and altered CSF/serum IgG ratio	Slow and aspecific	19	-

ADLD: autosomal dominant leukodystrophy; BBB: blood brain barrier; CJD: Creutzfeldt-Jakob disease; DLB: dementia with Lewy bodies; EEG: electroencephalogram; GTC: generalized tonic-clonic; MG: myasthenia gravis; MMSE: Mini mental State Evaluation; N.A.: Not available; N.D.: Not done; OBs: oligoclonal bands; PDD: Parkinson's disease and dementia; PSP: progressive supranuclear palsy; RBD: REM sleep behavior disorder; RLS: restless legs syndrome.

parkinsonism (P > 0.999, Fishers' exact test, two tailed) (Table 3.13 B).

Overall, irrespective of the clinical diagnosis, the presence of antibody reactivity was associated with a subacute onset or a sudden worsening of symptoms (P=0.005, Fisher's exact test, two-tailed), with the positivity of other antibodies (i.e. anti-nuclear antibodies) (P=0.038, Fisher's exact test, two-tailed) and the presence of sleep disorders (P=0.036, Fisher's exact test, two-tailed). Patients with any reactivity had significantly higher levels of CSF beta-amyloid compared to seronegative cases (P= 0.001, independent sample t test) and lower CSF tau/a-beta ratio IgG (P=0.04, independent sample t test) (Table 3.14 and 3.15).

After exclusion of patients with single undetermined reactivity (i.e. positive only on neurons or tissue), 13 patients (21.3%) still showed Ab-reactivity. This was found to be associated with the presence of cognitive impairment (P=0.006, Chi-square test, two-tailed).

3.5 Control cohorts

The controls included 50 sera from elderly healthy subjects (EHC) over 60 years of age and 159 sera from patients with seronegative myasthenia gravis (SNMG). All sera were anonymised and no information about demographic data were available.

3.5.1 Screening for unknown antigens

No patients from the EHC cohort showed reactivity on tissue or neurons (Figure 3.8). Among the SNMG patients 16 (10%) bound to rat brain sections and 12 (7.5%) bound neurons. Of these only 1 bound both tissue and neurons (Figure 3.5). 13 (8.1%) patients remained positive only on tissue and 10 (6.2%) only on neurons.

3.5.2 Screening for known antigens by CBAs

The control samples were screened by CBA for NMDAR, CASPR2, LGI1, AMPAR, $GABA_BR$, $GABA_AR$ and GlyR antibodies. Among the EHC, only 1 was positive for NDMAR (Figure 3.5). This serum was not positive on tissue or neurons. Among the SNMG, 5 sera were positive: 1 to CASPR2, 2 for $GABA_AR$, 1 for GlyR and 1 for $GABA_BR$. Of these 2 were positive also by IHC (GlyR and CASPR2-Abs) and 1 was positive also on neurons ($GABA_AR$) (Figure 3.5). No patients were positive by all methods. Overall in the SNMG cohort only 1 serum presented an undefined reactivity on both tissue and neurons.

Table 3. 12 Clinical diagnosis and demographic data of Neurodegenerative patients

Diagnosis at discharge	Sex (M:F)	Age at sampling (y) (mean ± SD)	Age at onset (y) (mean ± SD)	Time lapse onset-sampling (m) (mean ± SD)
PSP	2:3	73.2 ± 7.3	68.6 ± 6.9	57.6 ± 36.3
CBD	1:3	74 ± 5.2	72 ± 5	24.7 ± 15.5
MSA	3:1	63 ± 4.9	58.2 ± 3.4	57 ± 38.4
PD	1:1	69.5 ± 7.7	64.5 ± 4.9	60 ± 33.9
PDD	5:2	69.4 ± 12.8	67.8 ± 8.1	92.5 ± 70.5
PDDy	2:0	77.5 ± 3.5	61.5 ± 23.3	132 ± 152.7
AD	6:0	67.8 ± 7.9	64.8 ± 7.9	36 ± 25.1
CJD	2:1	71.6 ± 6.5	71.3 ± 7	5.3 ± 5.7
FTD	4:0	58.5 ± 9	55.7 ± 6.1	36 ± 34.9
DLB	2:1	78.6 ± 2.5	76.6 ± 3.2	24 ± 12
UD	8:6	60.5 ± 9.9	55.2 ± 15	64.2 ± 104.6
UP	3:4	72.7 ± 9.3	56.7 ± 23.2	192 ± 255
Total	*39:22*	*67.9 ± 10.1*	*62.5 ± 13.4*	*71.1 ± 112.7*

Y: years; m: months; PSP, progressive supranuclear palsy; CBD, corticobasal degeneration; MSA, multiple system atrophy; PD, Parkinson's disease; PDD, Parkinson's disease and dementia; PDD, Parkinson's disease and dysautonomia; AD, Alzheimer's disease; CJD, Creutzfeldt-Jakob disease; FTD, fronto-temporal dementia; DLB, dementia with Lewy bodies; UD, undefined dementia; UP, undefined parkinsonism.

Table 3. 13 Association between subacute onset and antibody reactivity in patients with prevalent parkinsonism (A) or dementia (B)

A)

Association between subacute onset and antibody reactivity in patients with prevalent parkinsonism	Antibody Positive: Negative
Subacute onset (n =11)	1 : 2
Chronic progression (n=17)	10 : 15
P> 0.999 Fisher's exact test, two tailed	

B)

Association between subacute onset and antibody reactivity in patients with prevalent dementia	Antibody Positive: Negative
Subacute onset (n =13)	9 : 4
Chronic progression (n=20)	5 : 15
P= 0.029 Fisher's exact test, two tailed	

Table 3. 14 Clinical features of seropositive and seronegative Neurodegenerative patients

	Seropositive (n=25)	Seronegative (n=36)	p-value
Gender (M:F)	14:11	26:10	0.27 $
Age at sampling (y)(mean ± SD)	68.5 ± 11.1	67.5 ± 9.4	0.70 ^
Age at onset (y)(mean ± SD)	61.9 ± 17.1	63 ± 10.3	0.75 ^
Time lapse onset-sampling (m) (mean ± SD)	82 ± 157.2	63.5 ± 68.3	0.53 ^
Family history (yes:no)	6:19	14:22	0.27 $
Subacute onset/acute worsening	13:12	6:30	0.005* $
Main presentation (onset)			
Parkinsonism (yes:no)	7:18	13:23	0.58 $
Seizures (yes:no)	2:23	1:35	0.56 $
Mood disorders (yes:no)	6:19	7:29	0.75 $
Cognitive impairment (yes:no)	10:15	17:19	0.61 $
Neurological examination			
Myoclonus (yes:no)	4: 20	11:24	0.23 $
Ataxia (yes:no)	5:19	3:32	0.25 $
Altered ocular movement (yes:no)	10:14	16:19	0.79 $
Parkinsonism (yes:no)	17:7	25:10	> 0.999 $
Postural instability (yes:no)	4:20	8:27	0.74 $
Pyramidal signs (yes:no)	6:18	6:29	0.52 $
Neuropathy (yes:no)	3:21	1:34	0.29 $
Other clinical features			
Other autoimmune diseases (yes:no)	4:21	6:30	> 0.999 $
History of tumour (yes: no)	3:20	3:32	0.67 $
Sleep disorders	16:9	12:23	0.036* $
Hallucinations (yes:no)	3:22	7:29	0.50 $

**: statistically significant; $: Fishers' exact test, two tailed; ^: independent samples t test; y: years; m: months.*

Table 3. 15 Laboratory, imaging, neurophysiological and neuropsychological features of seropositive and seronegative Neurodegenerative patients

	Seropositive (n=25)	Seronegative (n=36)	p-value
Presence of other antibodies (yes:no)	6:2	5:13	0.038*$
CSF findings			
OBs (yes:no:mirror)	2:0:9	1:2:20	0.27 $
Lymphocytosis (yes:no) **(mean value ± SD)**	3:15 9.5 ± 28.8	5:28 7.4 ± 25.1	0.78 ^
Increased proteins (yes:no) **(mean value ± SD)**	5:13 44.8 ± 15	6:26 43.7 ± 15.3	0.82 ^
Increased IgGs (yes:no) **(mean value ± SD)**	8:7 4.5 ± 1.8	11:13 3.5 ± 1.9	0.13 ^
Altered CSF index (yes:no) **(mean value ± SD)**	2: 11 0.59 ± 0.07	5:18 0.68 ±0.43	0.46 ^
Altered BBB (yes:no) **(mean value ± SD)**	6:6 7.8 ± 3.9	8:15 7.1 ± 4.3	0.64 ^
Increased tau protein (yes:no) **(mean value ± SD)**	6:13 356.5 ± 360.5	10:21 381.9 ± 457.3	0.83 ^
A-beta (mean value ± SD)	808.6 ± 232.2	457.8 ± 209	0.001*^
Altered tau/a-beta ratio (yes:no) **(mean value ± SD)**	3:7 0.38 ± 0.31	7:11 0.77 ± 0.70	0.40* ^
14.3.3 (yes:no)	2:15	1:29	0.54 $
Brain MRI findings			
White matter hyperintensities (yes:no)	15:9	19:17	0.59 $
Atrophy (yes:no)	13:9	21:15	0.47 $
Inflammatory changes (yes:no)	5:17	2:34	0.092 $
other features (yes:no)	0:22	2:34	0.52 $
EEG findings			
slowing	7:4	8:11	> 0.999 $
aspecific	9:4	8:11	0.16 $
epileptic	2:10	1:18	0.54 $
Neuropsychological testing			
MMSE (mean value ± SD)	23.7 ± 5.5	23 ± 5	0.64^
BBMD (mean value ± SD)	0.16 ± 0.9	0.32 ± 1.5	0.69^

**: statistically significant; $: Fishers' exact test, two tailed; ^: independent samples t test. BBMD: brief battery for mental deterioration; CSF: cerebrospinal fluid; EEG: electroencephalogram; MMSE: Mini-Mental State Examination; MRI: magnetic resonance imaging; OBs: oligoclonal bands.*

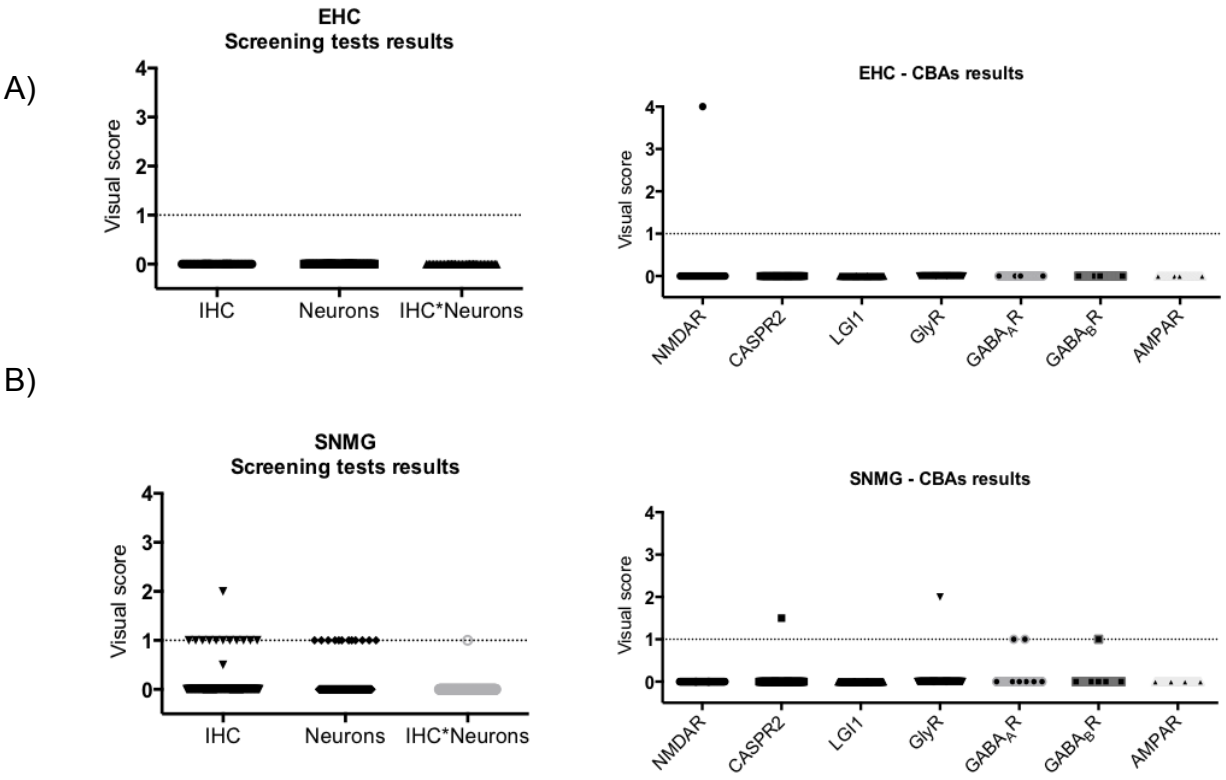

Figure 3.5 Antibody testing results in control cohorts

A) Immunohistochemistry, immunofluorescence on neuronal culture (left) and CBAs results (right) in the elderly healthy controls population; B) Immunohistochemistry, immunofluorescence on neuronal culture (left) and CBAs results (right) in the seronegative myasthenia gravis population.

3.6 Frequency of antibody distribution between cohorts of patients versus controls

The frequency of seropositive cases was compared between patients with CNS disorders (NT1, epilepsy, neurodegenerative) and controls (EHC and SNMG). Results are shown in Table 3.16. The overall frequency of reactivity was higher in the CNS disorders (all patients, n= 193) than in controls (all controls, n= 209) ($P < 0.0001$, Fisher's exact test, two tailed). Irrespective of CBA positivity, positivity on tissue (IHC) ($P=0.006$, Fisher's exact test, two tailed) or neurons ($P < 0.0001$, Fisher's exact test, two tailed) were both associated with CNS disorders, but single positivity on either tissue or neurons were not different between groups. Positivity on CBAs alone ($P < 0.0001$, Fisher's exact test, two tailed) or combined positivity on one or both screening assays were more frequent in CNS patients than controls ($P=0.031$, Fisher's exact test, two tailed). The analysis was, therefore, repeated excluding cases with single undetermined reactivities. The frequency of seropositivity remained significantly higher in the CNS diseases group ($P< 0.001$, Fisher's exact test, two tailed) and binary logistic regression analysis showed a significant predictive value of this variable (Table 3.17).

The frequency of antibodies between each cohort of CNS disorders and controls were then compared. The EHC were compared only with the neurodegenerative patients due to the similar age distribution. Results are in Table 3.18.

NT1 patients were significantly more seropositive (after excluding single reactivities) than SNMG patients ($P < 0.0001$, Fisher's exact test, two tailed) and showed more frequent positivity for NMDAR antibodies ($P < 0.0001$, Fisher's exact test, two tailed).

Epilepsy patients showed more reactivity (after excluding single reactivities) than SNMG patients ($P < 0.0001$, Fisher's exact test, two tailed) and more frequent positivity for CASPR2 antibodies ($P = 0.01$, Fisher's exact test, two tailed) and $GABA_AR$ ($P = 0.009$, Fisher's exact test, two tailed).

Patients with neurodegenerative disorders displayed more reactivity than both EHC and SNMG (Table 5.18) and more frequent positivity for $GABA_AR$ ($P = 0.02$, Fisher's exact test, two tailed) and GlyR ($P = 0.02$, Fisher's exact test, two tailed) antibodies compared to SNMG patients.

Table 3. 16 Comparison of antibody testing between patients with CNS disorders and controls

	CNS disorders patients (n=193)	Controls (n=209)	p-value$
N. of positive on tissue (IHC) (%)	33 (17.5)	16 (7.8)	0.006*
N. of positive on neurons (IF) (%)	33 (17.5)	12 (5.7)	0.000*
N. of isolated positivity on tissue (IHC) (%)	11 (5.7)	13 (6.2)	0.83
N. of isolated positivity on neurons (IF) (%)	14 (7.3)	10 (4.8)	0.40
N. of combined positivity on tissue and neurons (%)	7 (3.6)	1 (0.5)	0.031*
N. of isolated positivity on CBAs (%)	26 (13.5)	3 (1.4)	<0.0001*
N. of combined positivity by all assays (%)	10 (5.2)	0	0.001*
N. of cases with any positivity (%)	66 (34.2)	30 (14.4)	<0.0001*
N. of cases with any double positivity (%)	46 (23.8)	7 (3.3)	<0.0001*
N. of CASPR2 positive cases (%)	6 (3.1)	1 (0.5)	0.058
N. of LGI1 positive cases (%)	3 (1.6)	0	0.10
N. of AMPAR positive cases (%)	1 (0.5)	0	0.47
N. of $GABA_AR$ positive cases (%)	8 (4.1)	2 (1)	0.054
N. of $GABA_BR$ positive cases (%)	4 (2.1)	1 (0.5)	0.20
N. of NMDAR positive cases (%)	8 (4.1)	1 (0.5)	0.016*
N. of GlyR positive cases (%)	7 (3.6)	1 (0.5)	0.031*

**: statistically significant; $: Fisher's exact test, two tailed; CBAs: cell-based assays; IHC; immunohistochemistry; IF: immunofluorescence; N: number.*

Table 3. 17 Logistic regression analysis

Variable	Sig.	Odds ratio	Confidence interval (95%)
IHC only	0.68	1.24	0.53-2.86
Neurons only	0.094	2.05	0.88-4.77
CBA only	0.50	1.73	0.34-8.64
Any combined positive	0.000	7.33	2.4-21.99

Table 3. 18 Comparison of frequency of reactivity between each CNS disorder and controls

	SNMG (n=159)	EHC (n=50)	NT1 patients (n=59)	p-value$	Epilepsy patients (n=73)	p-value$	Neurodegenerative patients (n=61)	p-value$	p-value§
N. of positive on tissue (IHC)	12	0	10	0.07*	14	0.01*	9	0.13	0.003*
N. of positive on neurons (IF)	12	0	8	0.19*	11	0.09	14	0.004*	0.0002*
N. of isolated positivity on tissue (IHC)	13	0	3	0.56	4	0.59	4	0.78	0.12
N. of isolated positivity on neurons (IF)	10	0	2	0.52	4	>0.999	8	0.10	0.007*
N. of isolated positivity on CBAs	2	1	9	0.0002*	13	<0.0001*	11	<0.0001*	0.01*
N. of cases with any positivity	29	1	22	0.01*	24	0.01*	25	0.0008*	<0.0001*
N. of cases with any double positivity	6	1	17	<0.0001*	16	<0.0001*	13	0.0002*	0.002*
N. of CASPR2 positive cases	1	0	0	>0.999	5	0.01*	1	0.48	>0.999
N. of LGI1 positive cases	0	0	1	0.27	0	>0.999	2	0.07*	0.5
N. of AMPAR positive cases	0	0	0	>0.999	0	>0.999	1	0.27	>0.999
N. of $GABA_AR$ positive cases	0	0	1	0.27	4	0.009*	3	0.02*	0.25
N. of $GABA_BR$ positive cases	1	0	1	>0.999	3	0.09	1	0.48	>0.999
N. of NMDAR positive cases	0	1	8	<0.0001*	0	>0.999	0	>0.999	0.45
N. of GlyR positive cases	1	0	1	0.46	1	0.2	4	0.02*	0.12

*$: comparison with SNMG cohort, Fisher's exact test, two tailed; §: comparison with EHC cohort, Fisher's exact test, two tailed; *: significant value CBAs: cell-based assays; EHC: elderly healthy controls; IHC; immunohistochemistry; IF: immunofluorescence; N: number; NT1: narcolepsy type 1; SNMG: seronegative myasthenia gravis.*

3.7 Frequency of antibody distribution between cohorts of patients with CNS disorders

There were no differences in gender distribution between the cohorts, but age at sampling was different ($F(2)=132$, $P<0.0001$, one-way ANOVA). *Post hoc* analysis (Bonferroni) showed that NT1 ($P<0.0001$) and Epilepsy ($P<0.0001$) patients were significantly younger at sampling than Neurogenerative patients, as expected. No differences were found between NT1 and epilepsy patients (Table 3.19).

There were no differences in terms of frequency of general seropositivity among cohorts as found by reactivity on IHC, neurons or CBAs. Surprisingly, NT1 patients showed higher frequencies of NMDAR antibodies compared to epilepsy ($P = 0.001$, Fisher's exact test, two tailed) and neurodegenerative patients ($P = 0.002$, Fisher's exact test, two tailed) (Table 3.20).

3.8 Features of all patients with antibody reactivity

Clinical features of all patients with CNS disorders showing antibody positivity are summarized in Table 3.21. There were no differences in gender distribution between seropositive and seronegative cases nor difference in mean age. This did not change after exclusion of cases with single unspecified reactivities (Table 3.21).

Clinical features of patients showing specific antibody reactivity at CBAs are summarised in Table 3.22. There were no differences in gender distribution among different antibodies. However, age at sampling was significantly different between patients with different antibodies ($F(5,31)=0.29$, $P=0.008$, one-way ANOVA). *Post hoc* analysis showed that patients with NMDAR antibodies were significantly younger than patients with $GABA_AR$-Abs ($P=0.001$) or GlyR-Abs ($P=0.004$). The median age at onset is shown in Table 3.23. Compared to literature data, there was a tendency towards a different age and gender distribution for some antigens.

Among the patients with identified antibody reactivities, for which this information was available, none showed MRI or CSF evidence of inflammation. Overall, only 5 patients, 4 from the neurodegeneration cohort, and 1 from the NT1 met the criteria for possible AE (3 LGI1, 1 $GABA_AR$, 1 $GABA_BR$) (Table 3.24).

Table 3. 19 Demographic data of patients with CNS disorders

	NT1	Epilepsy	Neurodegenerative	p-value
Sex (M:F)	40:21	39:34	37:22	0.31$
Age at sampling	31.1 ± 17.7	31.7 ± 14.6	67.9 ± 10.1	<0.0001*^
*$ Fisher's exact test; ^: one-way ANOVA; *: significant value.*				

Table 3. 20 Comparison of frequency of reactivities among patients with CNS disorders

	NT1 patients (n=59)	Epilepsy patients (n=73)	p-value$	Neurodegenerative patients (n=61)	p-value$	p-value§
N. of positive on tissue (IHC)	10	14	0.65	9	0.80	0.49
N. of positive on neurons (IF)	8	11	0.80	14	0.23	0.37
N. of isolated positivity on tissue (IHC)	3	4	>0.999	4	0.78	>0.999
N. of isolated positivity on neurons (IF)	2	4	0.69	8	0.10	0.007*
N. of isolated positivity on CBAs	9	13	0.81	11	0.80	>0.999
N. of cases with any positivity	22	24	0.85	25	0.46	0.37
N. of cases with any double positivity	17	16	0.42	13	0.4	>0.999
N. of CASPR2 positive cases	0	5	0.06	1	>0.999	0.21
N. of LGI1 positive cases	1	0	0.45	2	>0.999	0.21
N. of AMPAR positive cases	0	0	>0.999	1	>0.999	0.45
N. of $GABA_AR$ positive cases	1	4	0.37	3	0.61	>0.999
N. of $GABA_BR$ positive cases	1	3	0.25	1	>0.999	0.62
N. of NMDAR positive cases	8	0	0.01*	0	>0.999	0.41
N. of GlyR positive cases	1	1	>0.999	4	0.36	>0.999
N. of HCRTR2 positive cases	3	0	0.05	-	-	-
** significant value; Fisher's exact test; $: against NT1 patients; §: against Epilepsy patients; CBAs: cell-based assays; IHC; immunohistochemistry; IF: immunofluorescence; N: number.*						

Table 3. 21 Demographic data of seropositive and seronegative patients with CNS disorders

	Sex (M:F)	Age at sampling (mean ± SD)
Any reactivity		
Seropositive (n=66)	38:28	43.8 ± 23.9
Seronegative (n=127)	78:49	42.5 ± 21.4
p-value	*0.64*	*0.68*
Any reactivity excluding undetermined single reactivities		
Seropositive (n=46)	23:23	42.8 ± 22.2
Seronegative (n=147)	93:54	43 ± 22.3
p-value	*0.12*	*0.95*

Table 3. 22 Demographic data of patients with different specific antibodies reactivities

	Sex (F,M)	**Mean age at sampling ± SD**
CASPR2 (n=6)	4,2	31.8 ± 20
LGI1 (n=3)	1,2	53.3 ± 33.8
$GABA_AR$ (n=8)	4,4	58.3 ± 16.2
$GABA_BR$ (n=4)	2,2	36.7 ± 16.9
NMDAR (n=8)	3,5	26.2 ± 17.5*§
AMPAR (n=1)	0,1	78
GlyR (n= 7)	3,4	57.2± 17.4
**Significantly different from $GABA_AR$; § Significantly different from GlyR*		

Table 3. 23 Comparison between present study's data and literature data

	Present study data			**Literature data**		
	Median age at sampling	Upper limit	Lower limit	Median age	Upper limit	Lower limit
CASPR2 (n=6)	24.5	68	17	66	77	25
LGI1 (n=3)	66	79	15	64	84	31
$GABA_AR$ (n=8)	51	78	36	53	80	2
$GABA_BR$ (n=4)	40.5	53	13	70	75	51
NMDAR (n=8)	23.5	56	9	25	33	16
GlyR (n= 7)	62	77	27	50	75	1

Table 3. 24 AE criteria in patients with specific antibody positivity

Antibody	Acute onset	CSF feature	MRI features	Other features	AE criteria
NMDAR	Yes	Normal	Normal	-	No
NMDAR	No	OBs	Normal	-	No
NMDAR	Yes	Normal	Normal	-	No
NMDAR	Yes	Altered BBB and increased proteins	N.A.	-	No
NMDAR	Yes	N.A.	Mild cerebral and cerebellar atrophy	-	No
NMDAR	Yes	N.A.	n.a.	-	No
NMDAR	Yes	N.A.	Normal	-	No
NMDAR	Yes	N.A.	Normal	-	No
LGI1	Yes	N.A.	Normal	Multimodal hallucinations, including olfactive, and psychosis	Possible
LGI1	Yes	Normal	White matter hyperintensities	Parkinsonism, cognitive and psychiatric features	Possible
LGI1	Yes	Altered BBB and increased proteins	White matter hyperintensities	Cognitive and behavioural impairment; GTC seizures	Possible
GlyR	No	Normal	White matter hyperintensities	-	No
GlyR	No	Altered BBB	White matter hyperintensities	Cognitive and behavioural impairment	No
GlyR	No	N.A.	Leukodystrophy	Ataxia and dysautonomia	No
GlyR	No	Lymphocytosis	Normal	Cognitive and behavioural impairment	No
GlyR	No	Normal	White matter hyperintensities	Parkinsonism	No
$GABA_AR$	No	OBs	White matter hyperintensities	-	No
$GABA_AR$	No	Normal	White matters hyperintensities and atrophy	Cognitive impairment	No
$GABA_AR$	Yes	Lymphocytosis	White matters hyperintensities and atrophy	Cognitive impairment	Possible
$GABA_AR$ +AMPAR	Yes	Normal	Diffuse cortical hyperintensities	Ataxia and parkinsonism	No
CASPR2	No but acute worsening	Normal	Normal	Parkinsonism, hypersomnia	No
$GABA_BR$	Yes	Lymphocytosis and altered CSF IgG index	White matter hyperintensities	Cognitive and mood disorders; insomnia	Possible

AE: autoimmune encephalitis; GTC: generalized tonic-clonic; OBs: oligoclonal bands; N.A.: not available.

3.9 Discussion

In this Chapter, the frequency of neuronal antibodies in patients with different CNS disorders and in both healthy and disease controls was assessed. For each disorder the results were compared with the clinical features in order to see which could predict the seropositive status. Moreover, the frequencies of antibodies between different cohorts were compared.

Several studies have tried to identify specific antibodies in patients with Narcolepsy type 1 with results that indicate the presence of specific reactivities in a variable, and usually small, percentage of cases (Bergman P et al. 2014; Thebault S et al. 2015; Cvetkovic-Lopes V et al. 2010; Ahmed SS et al. 2015). Here, antibodies were found against heterogeneous targets, in 16.9% of NT1 patients. HCRTR2 antibodies had been previously reported in a high proportion of NT1 patients who developed the disease after Pandemrix® vaccination (Ahmed SS et al. 2015). In a previous study, we showed that the frequency of these antibodies in idiopathic NT1 patients was very low (3%; Giannoccaro MP et al. 2017). Here one new case was added, and the previous cases followed up, which confirmed the low frequency of these antibodies in NT1, but interestingly, also the association with atypical clinical features, such as normal HCRT-1 CSF levels and psychiatric disorders. Surprisingly, although the most common antibodies found in this cohort were directed against NMDAR, and their presence was associated with an acute onset of the disease, there were no other distinctive clinical features. These observations suggest a secondary phenomenon occurring as a bystander effect during the neuronal destruction caused by another, possibly T-cell mediated, immune process.

The presence of NMDAR antibodies has previously been described in a few patients with NT1 and associated psychosis (Tsutsui K et al. 2012) but this finding was not replicated in a later study (Dauvilliers Y et al. 2016). Indeed, not only did the NDMAR patients studied here not have psychotic symptoms but the presence of NDMAR antibodies was inversely correlated with the presence of hypnagogic hallucinations.

On the other hand, this cohort included 9 patients with NT1 and psychosis and 4/9 cases showed antibody reactivity (1 by immunohistochemistry, 1 for LGI1 and 2 for HCRTR2, as already discussed). This suggests that antibody screening could be appropriate in patients with atypical features. This is further supported by the fact that other atypical features were observed in these patients, including negative HLA and/or normal HCRT-1 CSF levels.

In the Epileptic cohort antibodies were detected in 32.8% of cases, but specific antibodies were only observed in 13.6%, a percentage lower than other cohorts where the frequency was between 20 and 22% (Dubey D et al. 2017; Vanli-Yavuz EN et al. 2016). As

expected from previous studies (Quek AM et al. 2012; Barajas RF et al. 2010), the seropositive status was associated with AED resistance, particularly in the case of $GABA_BR$ antibodies. This observation is consistent with a pathogenic role for these antibodies at least in some cases. However, the occurrence of seropositivity also in cases with a positive family history for epilepsy and in a patient with an ascertained genetic form of epilepsy due to CASPR2 mutations (GAD65 positive), point to a likely secondary phenomenon.

Due to the limited clinical information available for this cohort, it was not possible to establish other possible relationships that might help identify seropositive cases. Interestingly, seropositive patients were younger than the rest of the population, which is an unexpected finding considering the general increase in antibody reactivity with aging, and the fact that the majority of antibodies against neuronal surface antigens occur in older subjects. This finding, if confirmed, suggests that younger patients with refractory epilepsy of unknown aetiology could be the ones who would benefit the most from an immunotherapy trial.

The most commonly observed antibody in our Epilepsy cohort was CASPR2 (6.8%). This is similar to what observed in a previous study reporting a prevalence of 10% for CASPR2 antibodies in a cohort of 111 patients with mesial temporal lobe epilepsy and hippocampal sclerosis (Vanli-Yavuz EN et al. 2016). However, in our cases CASPR2 antibodies were not associated with temporal lobe lesions or mesial temporal sclerosis.

The highest frequency of reactivity was observed in the Neurodegenerative cohort patients (40.9%), mostly related to the high proportion of sera binding only to neurons. Indeed, only 18% of patients presented specific antibody reactivities. Nevertheless, the proportion of positive cases was higher than previously reported. This could be related to the use of live-cell CBA, which is known to be a more sensitive method than commercial assays using fixed cells (Coban A et al. 2014) or to the use of sera instead of CSFs (Grau-Rivera O et al. 2014).

The most frequently encountered antibodies in this cohort were directed against GlyR. All patients with these antibodies had a chronic disease course and variable clinical phenotypes. Most of the previous studies concentrated on patients with rapidly evolving dementia, as this is one of the most common differential diagnosis of autoimmune encephalitis in elderly patients (Coban A et al. 2014; Grau-Rivera O et al. 2014; Flanagan EP et al. 2010). This cohort included a large range of different neurological conditions and no differences in antibody frequencies were found in patients presenting with prominent dementia compared to patients presenting with prominent parkinsonism. Seropositive status, however, was associated with an acute onset of symptoms and the presence of sleep disorders. Sleep disorders, like hypersomnia, insomnia,

status dissociatus or agrypnia excitata, are common features of patients with autoimmune encephalitis (Liguori R e al. 2001; Stamelou M et al. 2012).

Not surprisingly, seropositivity was also associated with the presence of other antibodies, such as anti-nuclear antibodies, irrespectively of the presence of another autoimmune disease. Interestingly, seropositive patients showed significantly higher levels of A-beta protein in the CSF as well as a lower tau/A-beta ratio, a profile that has been previously associated with Alzheimer's disease (AD) (Tapiola T et al. 2009). However, recently the same findings, with normal levels of CSF total-tau protein, have been reported in patients with chronic insomnia (Chen Dw et al. 2018). Whether our findings correlate with the sleep disorders observed in these patients needs to be investigated. This observation might also have clinical utility, particularly in the context of the differential diagnosis between an autoimmune dementia and AD. This is even more relevant considering that the majority of cases with specific reactivities did not show inflammatory changes either on brain MRI or in the CSF, as previously reported (Escudero D et al. 2017).

Overall, patients with CNS disorders showed more frequently the presence of antibodies against neuronal surface antigens compared to elderly healthy controls and SNMG patients. The differences in antibody reactivities, however, were similar among all cohorts when considering single uncharacterised reactivities found with only one method (i.e. isolated reactivity on tissue or on neurons). These findings are in line with a previous retrospective study comparing the frequency of antibodies in patients and healthy controls reported in the literature (Lang K and Pruss H, 2017) and suggests that, in screening cohorts for the presence of antibodies, positive findings should concentrate on cases showing reactivity on at least two different assays to support their specificity.

Given the small numbers involved, no differences were observed in the frequencies of antibody reactivities among CNS disorder patients, although some specific antibodies were more common in some cohorts, such as NMDAR antibodies in NT1 patients. This suggests that in several neurological conditions at least a proportion of patients might have an autoimmune aetiology. Of course, the role of these antibodies remains unclear. Few sera bound strongly on tissue, neurons or CBAs and CSFs were not available. Moreover, not all cases with positive antibodies on CBAs bound to neurons or tissue. This could reflect the low titres of the antibodies as well as the different serum dilutions used on these assays and the different concentration of the antigens.

Only a few patients in our cohorts met the criteria for possible autoimmune encephalitis. These cases may represent misdiagnosis. The other cases might represent cases with a

"restricted" or "atypical" phenotype. Antibody-associated phenotypes might change in relation with the age of patients. For example, for LGI1 antibodies the typical facio-brachial dystonic seizures were not described in children (López-Chiriboga AS et al. 2018). Indeed, in our cohorts, compared to literature data, there was a tendency towards a different age and gender distribution for some antigens. For example, CASPR2 antibodies were more frequent in female and in younger patients, whereas $GABA_BR$ and GlyR antibodies were observed in older patients compared to the cases reported in the literature.

Antibodies might also be a secondary phenomenon. However, it is important to note that patients with a previous different neurological condition could develop antibodies in the course of their disease, as shown by cases presenting a sudden worsening of symptoms, and they could still benefit from immunotherapy in this situation.

Our study has several limitations. Clinical data were not homogenous among cohorts and in some cases very limited (i.e. for the Epilepsy cohort), and demographic data were not available for the controls. Moreover, the lack of systematic titrations and of CSFs hampered our ability to understand better the role of these antibodies in these patients. Finally, it is important, of course, that in this essentially exploratory study, none of the clinical associations were corrected for multiple comparisons. Despite these limitations, we have shown that antibodies against neuronal surface antigens are more common in patients with CNS conditions than in controls and that some clinical features might help to identify those cases that could benefit for immunotherapy.

In order, to prove pathogenicity of antibodies, it is necessary to perform both *in vitro* and *in vivo* experiments. With the availability of plasma from plasma exchange from a patient with an atypical limbic encephalitis/Morvan syndrome, it was possible to investigate the pathogenic role of CASPR2 antibodies as described in the next two Chapters.

Chapter 4: Behavioural findings in CASPR2-antibody injected mice

4.1 Introduction

As discussed in Chapter 1, antibodies to CASPR2 (CASPR2-Abs) have been associated with limbic encephalitis and a wide range of central and peripheral nervous system disorders. Passive transfer of antibodies to experimental animals is considered the best way to prove pathogenicity of an antibody, but this has not yet been done by the peripheral route for any of the recently-described antibodies to neuronal surface antigens (NSAs).

CASPR2-Abs are typically at higher titres in serum than in CSF. Intraperitoneal injection of purified IgG from two CASPR2-Ab positive patients to mice over 18 days, without attempt to breach the blood-brain barrier (BBB), reduced the thresholds for mechanical stimuli and enhanced the excitability of dorsal root ganglia (DRG) neurons through reductions in Kv1 channel expression, but in that study brain pathology was only marginally investigated (Dawes JM et al. 2018). To explore the effects of CASPR2-Ab in the CNS, we used a similar protocol with 8 daily injections of IgG purified from one patient with autoimmune encephalitis and from one healthy control (as used in previous studies: Coutinho E et al. 2017; Dawes JM et al. 2018). We added a single dose of lipopolysaccharide (LPS) at day 3 to disrupt the BBB. We evaluated the effects of the antibody on mouse behaviors from day 5 of the injections and for three days following the last injection, and then looked for evidence of brain pathology.

4.2 Purification of IgG from CASPR2-Ab patient and healthy individual

Plasma obtained from a patient who underwent plasma exchange during the course of his disease was used in this animal model. The serum of a sex- and age-matched healthy individual was used as control. All methods are described in Chapter 2. Human IgG was purified using the Protein G Sepharose beads, and concentrations measured using a NanoDrop spectrophotometer. The patient with CASPR2-Ab encephalitis was a 70-year old man who presented with cerebellar ataxia and neuropathic pain in his feet and hands, memory complaints (short- and long-term memory, and particularly word-finding difficulties) and short episodes of lack of perception with “goosebumps” suggestive of temporal seizures. Oncological screening excluded the presence of a tumour. A cell-based assay revealed the presence of very high titres of CASPR2 antibodies (1:62500) and he partially responded to plasmapheresis and immunosuppression. The absence of other, concomitant, antibodies and the specificity of the

reactivity against CASPR2 was previously ascertained by Coutinho E et al. (2017), who showed that the binding of this IgG to mouse brain tissue was lost on brain sections from CASPR2 K.O. mice. The healthy control serum was negative for all known antigenic targets.

For the purpose of this thesis, purified IgG from this patient with CASPR2-Ab encephalitis, and from the healthy control, will be designated CASPR2-IgG and HC-IgG, respectively.

4.3 Experimental design

The experimental design is summarised in Figure 4.1. Nineteen C57Bl6 male mice aged 6 weeks (18-22 g) were daily injected intraperitoneally (i.p.) with either CASPR2-IgG (10 mice) or HC-IgG (9 mice) (20 mg/ml on day 0; 12-6 mg/ml afterwards; volume 1-2 ml) for 8 days. At day 3 all animals were injected i.p. with lipopolysaccharide (LPS, E. Coli, 1 mg/Kg). Behavioural testing, including tasks assessing locomotion (open field), strength (inverted screen) and coordination (accelerating rotarod and narrow beam), working memory (continuous spontaneous alternation), short- (forced alternation and) and long-term memory (novel object recognition), anxiety (light-dark box), compulsive-like behaviour (marble burying test), social behavior (reciprocal social interaction tests) and olfaction (olfaction test) was then performed from day 5 to 10. At day 11 animals were sacrificed and sera and brains collected for analysis (details in Chapter 2).

4.3 Weight

Animals were weighted daily during the course of the experiment as a general assessment of well-being. No differences were observed between CASPR2- and HC-IgG injected mice (group $P=0.83$, time x status $P=0.35$, repeated measure ANOVA with Greenhouse-Geisser correction, Figure 4.1).

4.4 Tests of strength and coordination

Strength was assessed by the inverted screen. No difference was observed in the time to fall between groups ($F(1,16)=3.8$, $P=0.06$, one-way ANCOVA), although CASPR2-IgG injected animals showed a trend towards a reduced time to fall compared to HC-IgG injected animals (Figure 4.1).

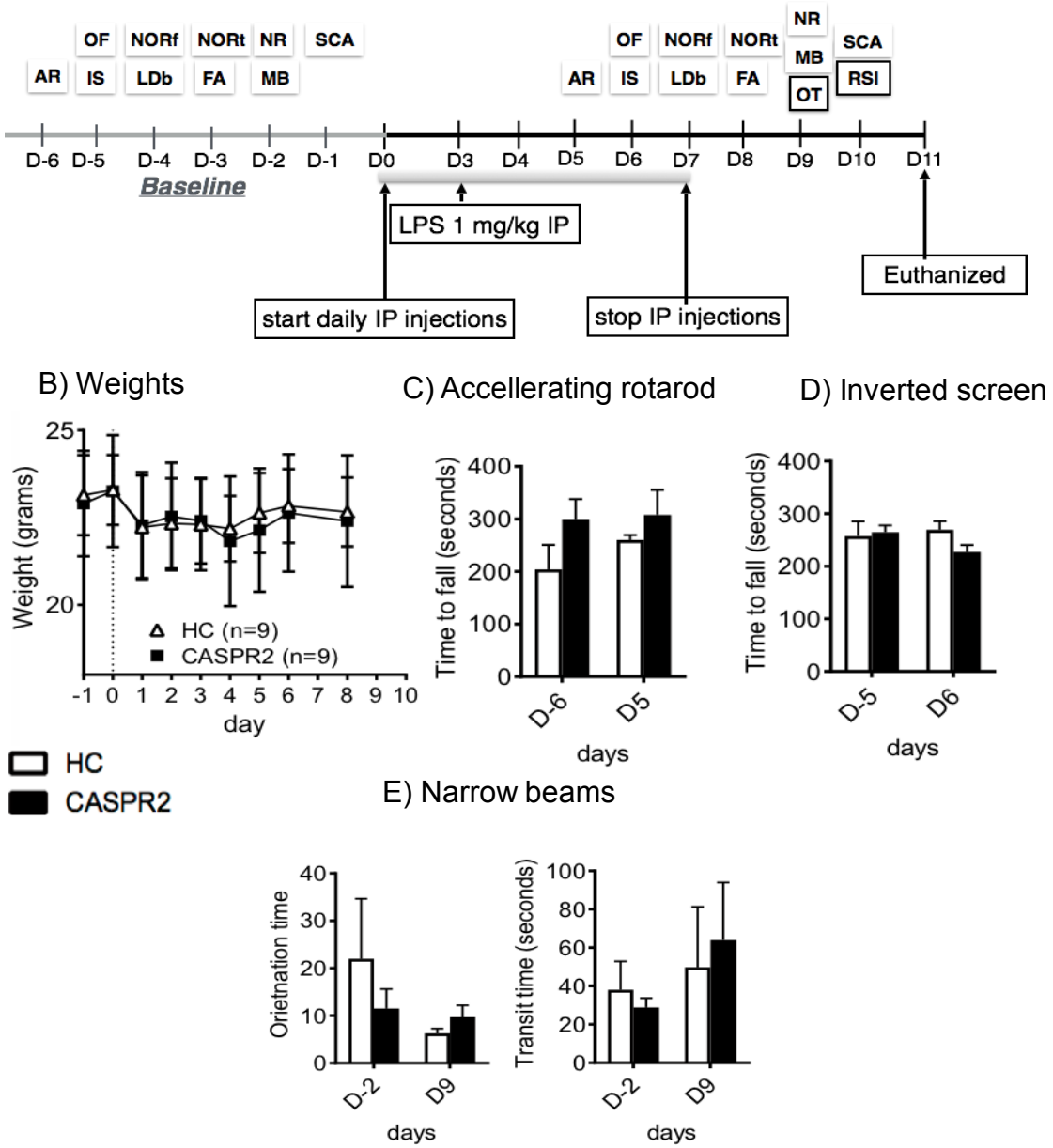

Figure 4.1 Experimental design and locomotor tests results

A) Experimental design. AR= accelerating rotarod; OF= open field; IS= inverted screen; NORf= novel object recognition, familiarization phase; NORt= NOR, test phase; LDb= light-dark box; FA= forced alternation test; NR= narrow beams; MB: marbles burying test; CSA= continuous spontaneous alternation test; OT= olfaction test; RSI= reciprocal social interaction test B) Weights changes over time. Accelerating rotarod (C), inverted screen (D) and narrow beams tests results (E). No differences were observed between HC-IgG and CASPR2-IgG injected mice. Data are expressed as mean ± SEM.

Coordination was evaluated by two tests, the accelerating rotarod and the successive narrow beams test. During the accelerating rotarod no differences were observed in the time to fall between groups ($F(1,16)=0.26$, $P=0.61$, one-way ANCOVA). For the narrow beams we evaluated the results obtained using the smallest rod as no animals fell from the wider ones. No differences were observed between groups in orientation time ($F(1,16)=1.71$, $P=0.20$, one-way ANCOVA) or in transit time ($F(1,16)=1.79$, $P=0.20$, one-way ANCOVA) (Figure 4.1).

4.6 Open field test

The open field test was used to evaluate different behaviours including locomotor activity, anxiety and repetitive behaviour. Locomotor activity was evaluated by the total number of entries in each square, the number of rearing and the total time spent active. No differences were observed between CASPR2- and HC-IgG injected mice in the total number of entries ($F(1,16)=0.48$, $P=0.49$, one-way ANCOVA), number of rearings ($F(1,16)=2.5$, $P=0.13$, one-way ANCOVA) or total time spent active ($F(1,16)=0.85$, $P=0.36$, one-way ANCOVA). Anxiety-like behaviour was evaluated by counting the number of entries in peripheral vs central squares, the total time spent in the periphery or in the center of the arena, and counting the number of foecal boli which signify stress. No difference was observed between groups in the number of peripheral entries ($F(1,16)=0.77$, $P=0.39$, one-way ANCOVA), central entries ($F(1,16)=0.11$, $P=0.73$, one-way ANCOVA), time spent in the periphery ($F(1,16)=0.24$, $P=0.62$, one-way ANCOVA) or in the center of the arena ($F(1,16)=0.24$, $P=0.62$, one-way ANCOVA) and number of foecal boli ($F(1,16)=0.40$, $P=0.53$, one-way ANCOVA). Finally, repetitive behaviour was assessed by analysis of grooming. No differences were observed between groups in the number of grooming events ($F(1,16)=0.39$, $P=0.54$, one-way ANCOVA) or in the time spent grooming ($F(1,16)=1.8$, $P=0.19$, one-way ANCOVA) between the two groups (Figure 4.2).

4.7 Test of anxiety

Beside the open field, anxiety was specifically evaluated by the light-dark box test, which explores the innate preference of mice to explore dark areas. There were no differences between groups in latency to move to the dark side of the box ($F(1,16)=1.05$, $P=0.32$, one-way ANCOVA), time spent in the dark ($F(1,16)=0.13$, $P=0.71$, one-way ANCOVA) or number of crossings between the two chambers ($F(1,16)=0.14$, $P=0.71$, one-way ANCOVA) (Figure 4.3).

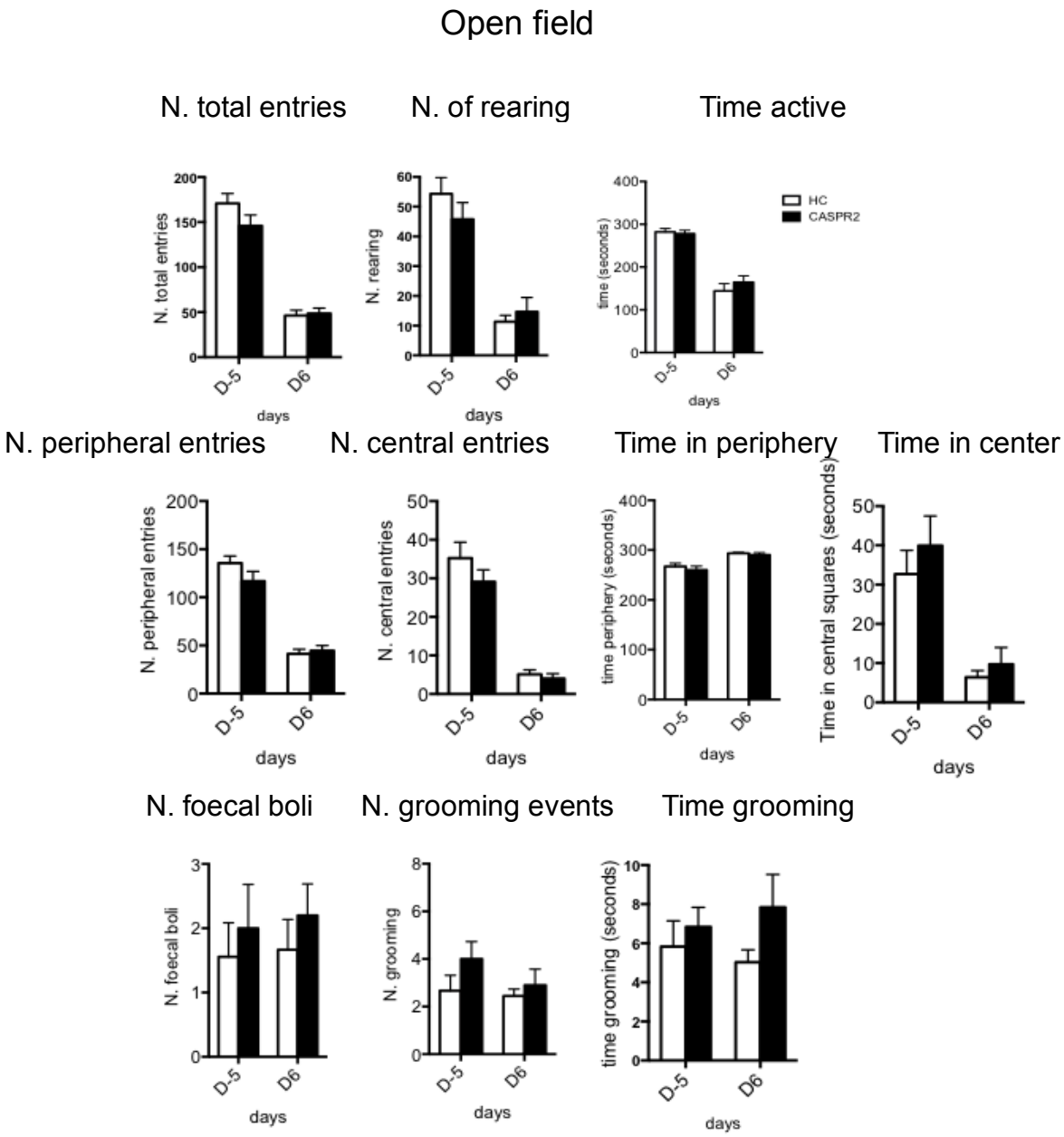

Figure 4.2 Open field test results

No differences were observed between HC-IgG and CASPR2-IgG injected mice in any of the parameters analysed during the open field test. Data are expressed as mean ± SEM.

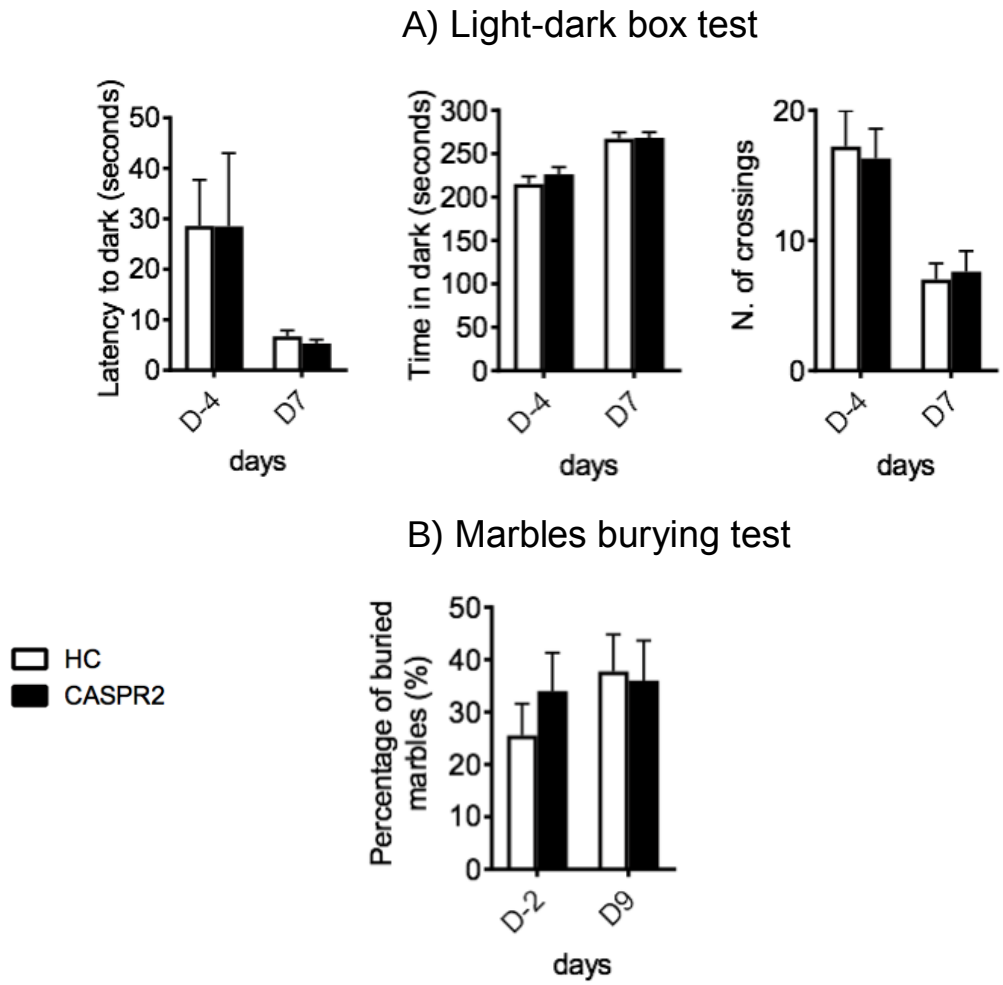

Figure 4.3 Light-dark box and marbles burying test

No differences were observed between HC-IgG and CASPR2-IgG injected mice in anxiety (A) or compulsive-like behaviour (B). Data are expressed as mean ± SEM.

4.8 Test of compulsive-like behavior

Compulsive-like behaviour was assessed using the marble burying test (MBT). No differences were observed between groups in the percentage of marbles buried ($F(1,16)=0.52$, $P=0.47$, one-way ANCOVA) (Figure 4.3).

4.9 Tests of memory

Different memory subtypes were explored using three tests. Both the forced alternation and the novel object recognition tests are based on the innate tendency of mice to prefer novelty. The forced alternation was used as a test of short-term memory. Although CASPR2-IgG injected mice showed a trend towards a reduced preference index for number of entries ($F(1,16) = 3.36$, $P=0.085$, one-way ANCOVA) in the novel arm (NA), no differences were observed between groups in the time spent ($F(1,16)=0.26$, $P=0.61$, one-way ANCOVA) in the new arm. The novel object recognition test was used to evaluate long-term memory. No differences between groups were observed in the preference index for the novel object (NO) ($F(1,16)=2.45$, $P=0.14$, one-way ANCOVA). However, a significant reduction was observed in the percentage of spontaneous alternations ($F(1,16)=5.04$, $P = 0.039$, one-way ANCOVA) in the CASPR2-IgG injected animals compared to controls, despite a similar number of total arms entries between groups ($F(1,16)=0.37$, $P=0.55$, one-way ANCOVA), suggesting a possible working memory impairment in CASPR2-IgG exposed mice. In the same test, there was no difference in the percentage of alternate arm re-entries ($F(1,16)=0.93$, $P=0.34$, one-way ANCOVA) or in the percentage of same arm re-entries ($F(1,16)=3.66$, $P = 0.074$, one-way ANCOVA) (Figure 4.4).

4.10 Olfaction test

As mice rely heavily on smell for orientation and for their social interactions, an olfaction test was performed once at the end of the protocol as a control for the memory test and social interaction experiment. This experiment consisted of a sampling phase, where the same odour was placed in 2 containers and the test mouse allowed to freely explore, a delay period and a test phase, when the mouse was allowed to choose between the old or a novel odour. The mouse was expected to preferentially explore a novel odour, if its olfaction sense was intact. No differences were observed between groups in the time spent ($P=0.36$, independent samples t-test) or in the number of visit to the new odour (NO) ($P=0.67$; independent samples t-test) (Figure 4.4).

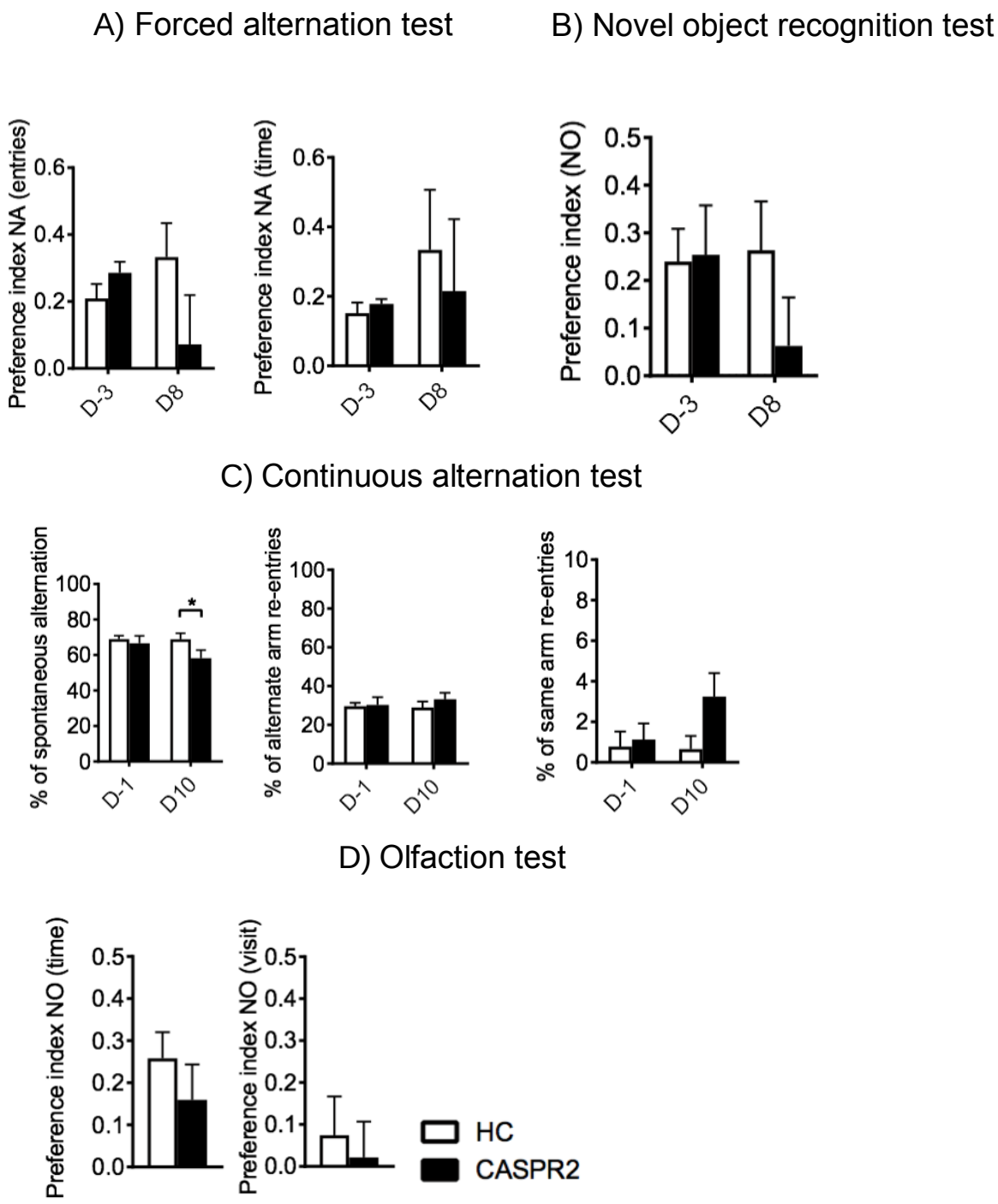

Figure 4.4 Memory tests and olfaction test results

A) In forced alternation, there were no differences in the preference index for number of entries or time spent in the novel arm (NA). B) Novel object recognition test did not show differences between the two groups in the preference index for the novel object (NO) although there was a trend to reduced preference in CASPR2-IgG injected mice. C) Alternations were reduced in CASPR2-IgG injected mice compared with HC-IgG injected mice. D) The olfaction test showed no differences between groups in the time spent or in the number of visits to the novel odour (NO). Data are expressed as mean ± SEM.

4.11 Reciprocal social interaction test

Social behaviour was investigated by the reciprocal social interaction test in pairs of animals of the same group. The animals were tightly matched according to weight (within 5% of the weight), so any variation resulting from this factor was avoided. This meant that only 6 pairs per group could be tested, in order to avoid repeating the test in each animal more than twice. Since this test is based on the interaction between mice that have not been in contact before and given the limited number of animals, the test could only be done once, after the injections. During the test phase, a pair of animals was allowed to interact freely while the interaction was video recorded; later on, both social (licking, sniffing, grooming and following closely the other animal) and non-social behaviours (time spent inactive, number of rearing, self-grooming) were scored offline.

CASPR2-IgG injected mice showed longer latency to start interacting compared to HC-IgG injected mice (U= 5.5, P=0.04; Mann-Whitney U test) but similar interaction time (P=0.58; Mann-Whitney U test) and number of interactions (P=0.18; Mann-Whitney U test). Concerning non-social activities, CASPR2-IgG injected animals showed less exploratory behaviour as indicated by less rearing events ($t(22)=3.01$, P=0.006; independent samples t-test) and longer time spent immobile ($t(22)=3.42$, P=0.002; independent samples t-test), compared to HC-IgG injected animals. No differences, however, were observed between groups in the time spent self-grooming (P=0.97; Mann-Whitney U test) but HC-IgG injected mice showed a higher number of grooming actions ($t(22)=2.61$, P=0.015; independent samples t-test), corresponding to a longer duration of each self-grooming bout in CASPR2-IgG injected mice ($t(22)=2.48$, P=0.002; independent samples t-test) (Figure 4.5). It is noteworthy that all these non-social behaviours were assessed also during the open field and no differences were observed between groups, suggesting a specific influence of the social context.

4.12 Discussion

In this Chapter, the *in vivo* behavioural outcomes of mice injected with CASPR2 IgG were described. In patients with CASPR2 antibodies, there are a wide range of CNS behavioural changes from anxiety and depression to memory impairment, seizures and sleep disorders, representing limbic encephalitis or Morvan Syndrome spectra. Ataxia has also been described in some patients. In addition, genetic defects of CNTNAP2, the gene encoding for CASPR2, have been reported in patients with autism and epilepsy (Friedman JI et al. 2008).

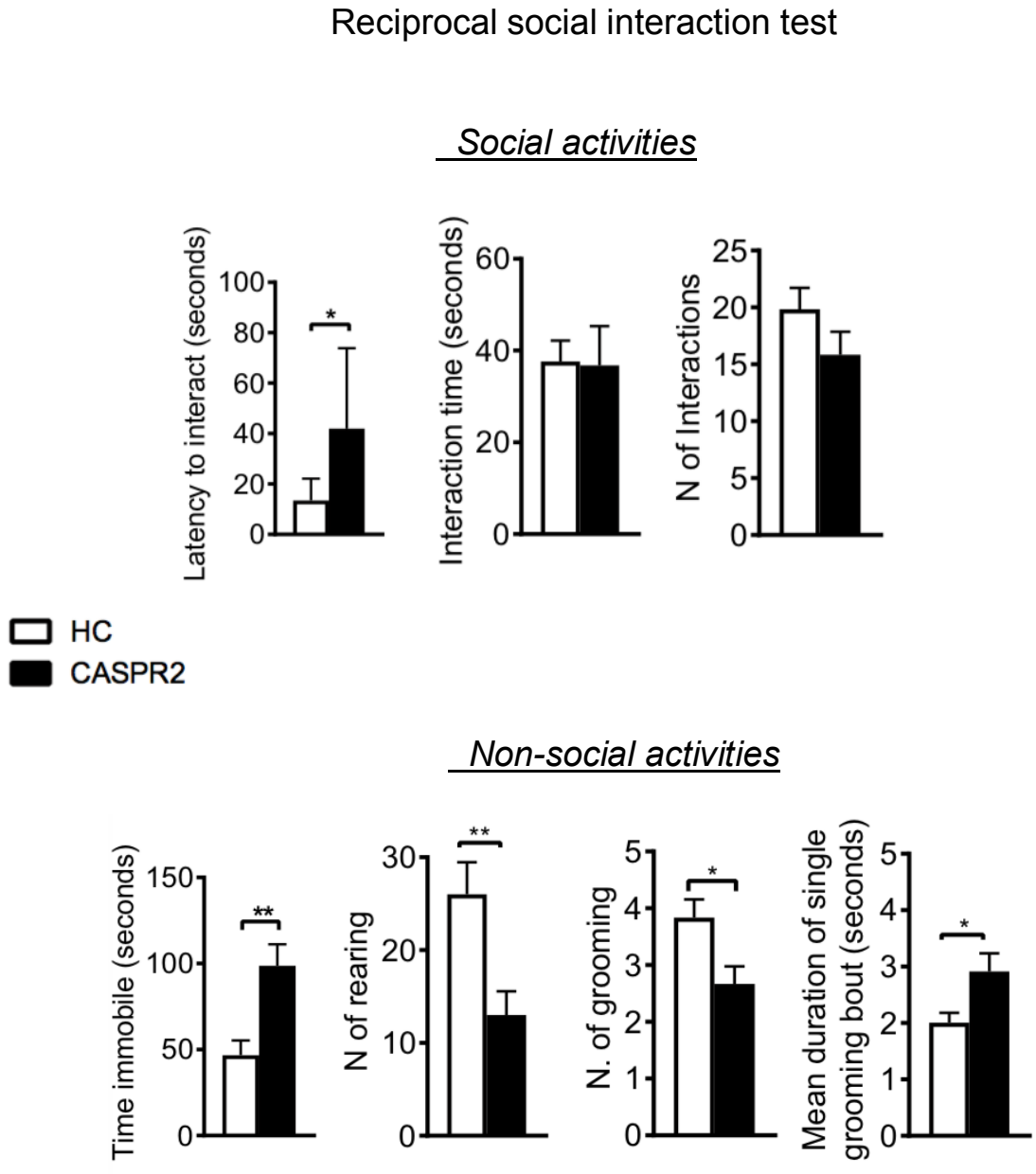

Figure 4.5 Reciprocal social interaction test

In the reciprocal social interaction test there was reduced latency to interact but no differences in the interaction time, or number of interactions between CASPR2-IgG and HC-IgG injected mice. However, in the non-social aspects of the test, CASPR2-IgG injected mice showed increased time spent immobile, reduced rearing, reduced grooming and longer duration of each grooming bout. Data are expressed as mean ± SEM.

Given the difficulty in anticipating the specific deficits that might be caused by CASPR2-Abs in a mouse model, a broad range of functions and behaviours were studied in an exploratory model.

No differences were observed in total body weight, motor tasks, anxiety tests and compulsive-like behavior between groups. However, mice injected with CASPR2-IgG showed less alternation in the continuous spontaneous alternation tests, with similar trends in the forced alternation and novel object recognition tests, suggestive of memory impairment. During the social interaction test CASPR2-IgG injected mice showed longer latency to interact and increased immobility suggestive of anxiety-like behaviour. Since the same non-social activities were normal during other tests (i.e. open field) they are likely to be related to the social novelty context (Meeker H et al. 2013). This interpretation is also supported by the longer grooming bouts during the same test (Kalueff AV and Tuohimaa P, 2004). This needs to be explored in more detail in the future.

Two previous studies assessed the effects of CASPR2 antibodies in different passive transfer animal models. The first one, showed that *in utero* CASPR2-IgG exposed mice displayed pronounced social interaction defects and increased repetitive behaviours such as grooming and digging in the absence of alterations of locomotor or anxiety behaviours. In this model memory or cognitive deficits were not extensively explored, but there was a strong trend for an impairment of working memory in CASPR2-IgG exposed mice, when assessed by spontaneous alternation in the T-maze (Coutinho E et al. 2017). However, this study used a placental passive transfer model, so the mice were exposed to the antibodies early in their neurodevelopment and this is expected to cause different and wider effects compared to the exposure of adult mice. In this respect, indeed, that approach might be expected to recapitulate more closely the effects of CASPR2 mutations.

The second model was focused on the peripheral effects, specifically the presence of pain, of CASPR2-Abs in adult mice and used peripheral CASPR2-IgG injections, very similar to that used here, but in the absence of LPS to open the blood-brain barrier (Dawes JM et al. 2018). Therefore, the authors explored the presence of pain and the presence of alterations in locomotor activity and coordination without exploring CNS functions. Mice exposed to CASPR2-IgG showed mechanical pain-related hypersensitivity. Reassuringly, no differences were observed between groups in the open field and in the accelerating rotarod in that model, similar to the results described here.

Interestingly, the same study compared the effects induced by the antibodies on pain sensitivity with those observed in knockout mice (KO) lacking CASPR2 (CNTNPA2−/−).

These mice demonstrated enhanced pain-related hypersensitivity to noxious mechanical stimuli, although more severe than that obtained with the antibodies, and that was extended to heat and algogens. Nevertheless, either immune or genetic-mediated ablation of CASPR2 enhanced the excitability of dorsal root ganaglia (DRG) neurons through regulation of Kv1 channel expression at the soma membrane. These findings imply that in adult mice, antibodies can recapitulate the effects induced by genetic mutation but in a more limited and less severe manner. CNTNAP2 KO mice were shown to have social deficits, abnormal motor activity, cognitive deficits and seizures (Penagarikano O et al. 2011). More recently, a model showed also the presence of hypoactivity and wake fragmentation with a flattened diurnal activity rhythm (Thomas AM et al. 2017). Performance in memory tasks have shown contrasting results suggesting a lack of spatial learning and memory impairments but a significant impairment in learning (Peñagarikano O et al. 2011), or the presence of significant deficits in spatial working and reference memory (Rendall AR et al. 2016). The reasons for these different findings might be related to the tests used and to the different difficulty of the tasks. However, this suggests that CASPR2 KO mice do not display obvious memory impairment, like the CASPR2-IgG injected mice, which showed only modest changes.

Our approach has some limitations. It is always difficult to establish correlations between animal phenotypes and human disorders and this might explain why we observed such mild changes in our model. The fact that the major differences in behaviour were observed in tests performed at day 10 suggest as well that behavioural changes may need a longer period to fully develop. Indeed, in previous passive transfer models the maximal behavioural effect was observed after 14 days of injections (Dawes JM et al. 2018; Planagumà J et al. 2015). In our study, the limited amount of plasma available for CASPR2-IgG purification did not allowed a longer period of IgG administration.

In conclusion, CASPR2-IgG injected mice showed clear although mild behavioural alterations in the tests performed. A wider and more targeted range of tests needs to be established for further studies. As it stands, it is difficult to interpret the results in the light of the clinical implications but to look further into the effects of the antibodies *in vivo*, we evaluated the presence of neuropathological changes that might relate to these alterations. The results of behavioural and histological changes will be integrated in the general discussion.

Chapter 5. IgG transfer and pathological changes in CASPR2-antibody injected animals

5.1 Introduction

A detailed analysis of the brain was conducted in a randomly selected subgroup of animals at the end of the experiment, in order to look for IgG deposition in the brain and provide a histological substrate for the behaviour abnormalities found in the CASPR2 IgG-injected mice. Firstly, the presence of CASPR2-IgG was assessed in the sera and brain parenchyma of CASPR2-IgG inject animals versus controls. Subsequently, a general morphometric study was conducted. Afterwards, a hypothesis-driven analysis looked for similarities with the histological abnormalities described in the few neuropathological cases of patients with CASPR2-Ab encephalitis reported in the literature; for example, reduced expression of CASPR2, neuronal loss and microglia activation (Sundal C et al. 2017; Liguori R et al. 2001; Kortvelyessy P et al. 2015), as well as the findings of previous animal models such as increased neuronal activity, using c-fos as a surrogate marker (Dawes JM et al. 2018). In addition, changes of CASPR2 expression and levels of cytokines and chemokines were evaluated in brain protein extracts.

5.2 Overview on sera and brain processing

Eleven animals (6 CASPR2-IgG and 5 HC-IgG injected) were randomly selected and sacrificed by CO_2. Blood samples were collected by cardiac puncture, centrifuged and the sera used for testing by cell-based assay for CASPR2-IgG (see Chapter 2). Brains from the same animals were harvested and snap-frozen for protein extraction. Eight animals (4/group) were perfused with phosphate-buffered saline (PBS) followed by ice-cold 4% paraformaldehyde (PFA). Histological studies were performed on PFA-fixed, free-floating, 50 μm coronal sections of the brain. Nissl staining was conducted to assess gross morphological abnormalities and to determine volume and/or thickness of total brain or specific brain regions. The remaining studies were done by immunofluorescence. Protein analysis was conducted on 3 brains per group by western blot for CASPR2 expression and by a commercial array for cytokine/chemokine expression. The statistical analysis compared the results between CASPR2 and HC IgG-injected animals using an N number of 4, if not otherwise specified. All experiments were coded for treatment groups.

5.3 Serum CASPR2 antibodies and brain human IgG deposition

A cell-based assay for CASPR2-Abs confirmed high levels in the CASPR2-IgG injected mice even on Day 11, 4 days after the last IgG injection (Figure 5.1).

The distribution of human IgG was investigated in different brain regions by immunofluorescence (Figure 5.1). Both CASPR2- and HC-IgG injected animals showed the presence of IgG in the brain. However, the level of deposited IgG, as evaluated by fluorescence intensity, was higher in the cortex ($t(6)=2.71$, $P=0.03$, independent samples t-test), hippocampus ($t(6)= 3.03$, $P=0.023$, independent samples t-test) and thalamus ($t(6)= 7.14$, $P=0.0004$, independent samples t-test) of CASPR2-IgG injected mice compared to controls (Figure 5.1). No differences between groups were observed in the cerebellar cortex which was analysed overall, without distinguishing the different layers ($t(6)=1.57$, $P=0.16$, independent samples t-test).

5.4 CASPR2 expression

To look at the similarity between CAPSR2 and IgG staining, two consecutive series were incubated with anti-human IgG antibodies and with rabbit monoclonal anti-CASPR2 antibodies respectively. We used two consecutive sections instead of a single one to avoid the interactions between secondary anti-human and anti-rabbit antibodies we noted in preliminary experiments. In the same experiments, we also excluded the presence of interactions between the rabbit anti-CASPR2 antibodies, the secondary anti-rabbit antibodies and the human IgG already present in the tissue.

We would have expected a reduction of CASPR2 expression as a consequence of the IgG binding. Nevertheless, there were no apparent differences in CASPR2 expression, measured by mean fluorescence intensity between the two groups in the cortex ($t(6)=1.04$, $P=0.33$, independent samples t-test), hippocampus ($t(6)= 1.23$, $P=0.26$, independent samples t-test), thalamus ($t(6)=0.23$, $P=0.82$, independent samples t-test) or cerebellum ($t(6)=0.34$, $P=0.74$, independent samples t-test) (Figure 5.1). Similar results were obtained when total CASPR2 protein was quantified by western blot (WB) in whole brain lysates ($t(4)=1.65$, $P=0.17$, independent samples t-test) (Figure 5.2); indeed, CASPR2 expression showed a trend towards a small increase.

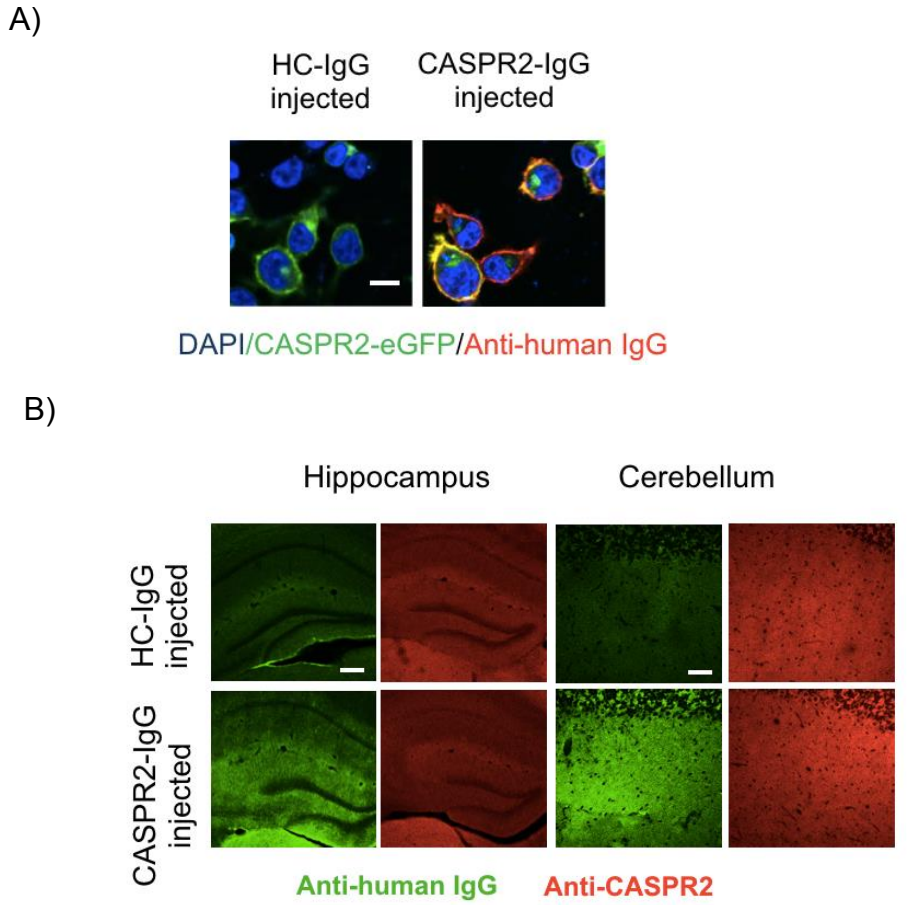

Figure 5.1 Sera and brain IgG and CASPR2 expression

A) Representative images of cell-based assays showing CASPR2-Ab in the serum from a CASPR2-IgG injected mouse but not in the serum from a HC-IgG injected mouse. 63X, scale bar 10 µm. B) Representative images of IgG and CASPR2 expression in perfused fixed brains. C) CASPR2-IgG injected animals had higher levels of IgG in the cortex (Cx), hippocampus (Hip) and thalamus (Th) compared to HC-IgG injected mice, but not in the cerebellum (Cb). No differences were observed in the levels of CASPR2 expression in the same areas (C). 40X, scale bar, hippocampus 200 µm, cerebellum 50 µm. Data are expressed as mean ± SEM.

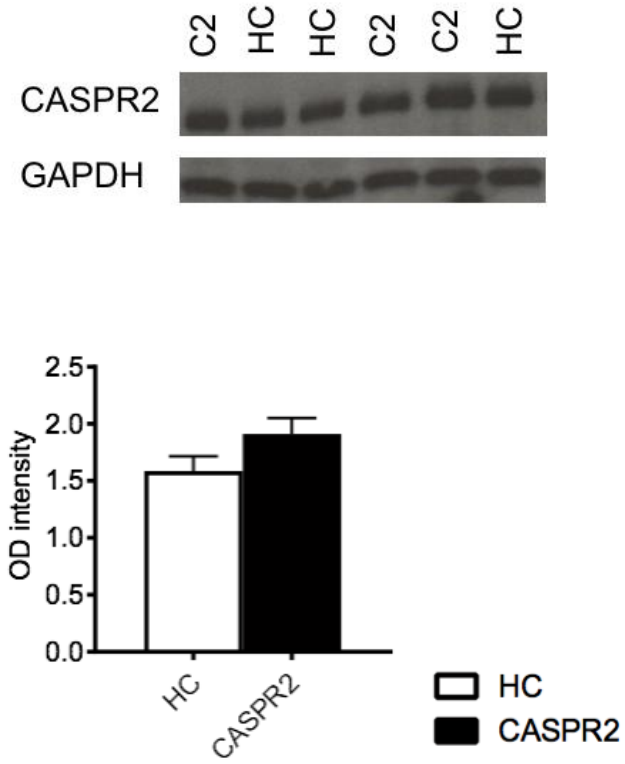

Figure 5.2 Western blot analysis of CASPR2 expression

Western blots of the whole brains from CASPR2-IgG injected mice (C2) showed a trend towards increased CASPR2 expression, normalized by GAPDH, compared to HC-IgG injected mice (HC) (3 brains/group; means of 4 replicates for each brain). Data are expressed as mean ± SEM.

5.5 Morphometric analysis

No gross morphological changes were observed on Nissl-stained sections between the two groups. In particular, there were similar hippocampal ($t(6)=0.5$, $P=0.63$, independent samples t-test), cerebellar ($t(4)=1.19$, $P=0.29$, independent samples t-test) and total brain volumes ($t(4)=1.5$, $P=0.20$, independent samples t-test) between the two groups. Likewise, there were no differences in the thickness of the anterior cingulate ($t(6)=0.25$, $P=0.80$, independent samples t-test), primary motor ($t(6)=0.11$, $P=0.91$, independent samples t-test), piriform ($t(6)=2$, $P=0.07$, independent samples t-test) or somatosensory cortex (overall: $t(6)=0.2$, $P=0.82$; layer I $t(6)=0.7$, $P=0.46$, layers II-IV $t(6)=0.2$, $P=0.83$, layers V-VI $t(6)=0.19$, $P=0.84$, independent samples t-test). No differences were observed in the thickness of the hippocampal cell body layers (dentate gyrus $t(6)=0.04$, $P=0.96$, CA3 $t(6)=0.24$, $P=0.81$, CA1 $t(6)=0.77$, $P=0.46$; independent samples t-test) or fields (CA4 $t(6)=1.2$, $P=0.27$, CA3 $t(6)=0.37$, $P=0.72$, CA1 $t(6)=1.9$, $P=0.10$, independent samples t-test). The thickness of the cerebellar molecular ($t(6)=1.5$, $P=0.16$, independent samples t-test) and granular ($t(6)=0.77$, $P= 0.46$, independent samples t-test) layers was also not different (Figure 5.3).

5.6 Neuronal counts and neuronal activation

To evaluate if CASPR2-Abs were able to cause neuronal death we measured the density of neurons, stained by the NeuN marker. No evidence of neuronal loss was found in the CASPR2-IgG compared to the HC-IgG injected mice in the somatosensory cortex ($t(6)= 1.12$, $P=0.30$, independent samples t-test), in the piriform cortex ($t(6)= 0.54$, $P=0.60$, independent samples t-test) or in the hippocampus (overall $t(6)=1.6$, $P=0.15$; CA4 $t(6)= 0.13$, $P= 0.89$; CA3 $t(6)=0.27$, $P= 0.79$; CA1 $t(6)=1.60$, $P= 0.16$; independent samples t-test). In the cerebellum, however, despite no difference in the count of NeuN positive cells in the molecular layer ($t(6)= 0.86$, $P=0.42$, independent samples t-test), there was a 14.6% reduction in the number of calbindin-positive Purkinje cells ($t(6)=2.45$, $P=0.049$, independent samples t-test) (Figure 5.4).

We used c-fos expression on NeuN stained neurons as a marker of neuronal activity. Compared to the HC-injected mice, mice injected with CASPR2-IgG showed a higher density of c-fos expressing neurons in the entorhinal-piriform cortex ($t(6)=3.11$, $P=0.020$), dorsomedial ($t(6)=2.67$, $P=0.036$) and lateral ($t(6)=2.79$, $P=0.031$, independent samples t-test) hypothalamus. No differences between groups in the density of c-fos expressing neurons were detected in the hippocampus (overall: $t(4)=0.77$, $P= 0.48$; DG $t(6)=0.87$, $P=0.41$; CA3 $t(6)= 1.77$, $P=0.12$; CA1 $t(6)=0.96$, $P=0.37$; independent samples t-test), somatosensory cortex

(t(6)=1.2, P=0.26, independent samples t-test) and amygdala (t(6)=1.03, P=0.34, independent samples t-test) (Figure 5.5).

5.7 Microglia analysis

Microglia are the resident tissue macrophages of the CNS. These cells can transform from a surveillance state to an activated phenotype in response to brain injury. Under normal conditions they are characterised by a small cell body with fine, ramified processes and low expression of surface antigens. In response to brain injury, ischemia and inflammatory stimuli, microglia rapidly transform into an activated phenotype associated with proliferation, migration to the site of injury, elaboration of both neurotoxic and neurotrophic factors and phagocytosis of cellular debris (Garden GA and Moller T, 2006; Hanisch UK and Kettenmann H, 2007; Streit WJ, 2002). Activated microglial cells, as identified by expression of Iba1 and CD68, were measured in the somatosensory and piriform cortices, the hippocampus and the cerebellum. Microglial density was increased in the somatosensory cortex of CASPR2-IgG injected mice (t(6)=2.63, P=0.038, independent samples t-test) and molecular layer of the cerebellum (t(6)=4.99, P=0.002, independent samples t-test) but not in the piriform cortex (t(6)= 1.31, P=0.23, independent samples t-test), the hippocampus (overall t(6)=1.34, P=0.22, CA4 t(6)=0.86, P=0.41, CA3 t(6)=0.59, P= 0.57, CA1 t(6)=1.92, P= 0.10, independent samples t-test) or the granular layer (t(6)= 0.27, P=0.79, independent samples t-test) of the cerebellum (Figure 5.6).

Analysis of microglial morphology was performed on Iba1 positive cells (Figure 5.6) in the hippocampus and in the molecular layer of the cerebellum. When activated, microglia assume an ameboid morphology characterised by a larger soma body and shorter processes. In the hippocampus, microglia from CASPR2-IgG injected mice showed a higher cell soma/cell total body size ratio (t(6)=4.74, P=0.0032, independent samples t-test), less (t(6)=3.27, P=0.017, independent samples t-test) and shorter (t(6)=3.68, P=0.010, independent samples t-test) ramifications than HC-IgG injected mice, compatible with an activated phenotype. Similar results were found in the molecular layer of the cerebellum, with a higher soma/total body area size ratio (t(6)=7.35, P=0.0003, independent samples t-test) and shorter ramification maximal length (t(6)=3.68, P=0.008, independent samples t-test) in CASPR2-IgG than in HC-IgG injected mice (Figure 5.6).

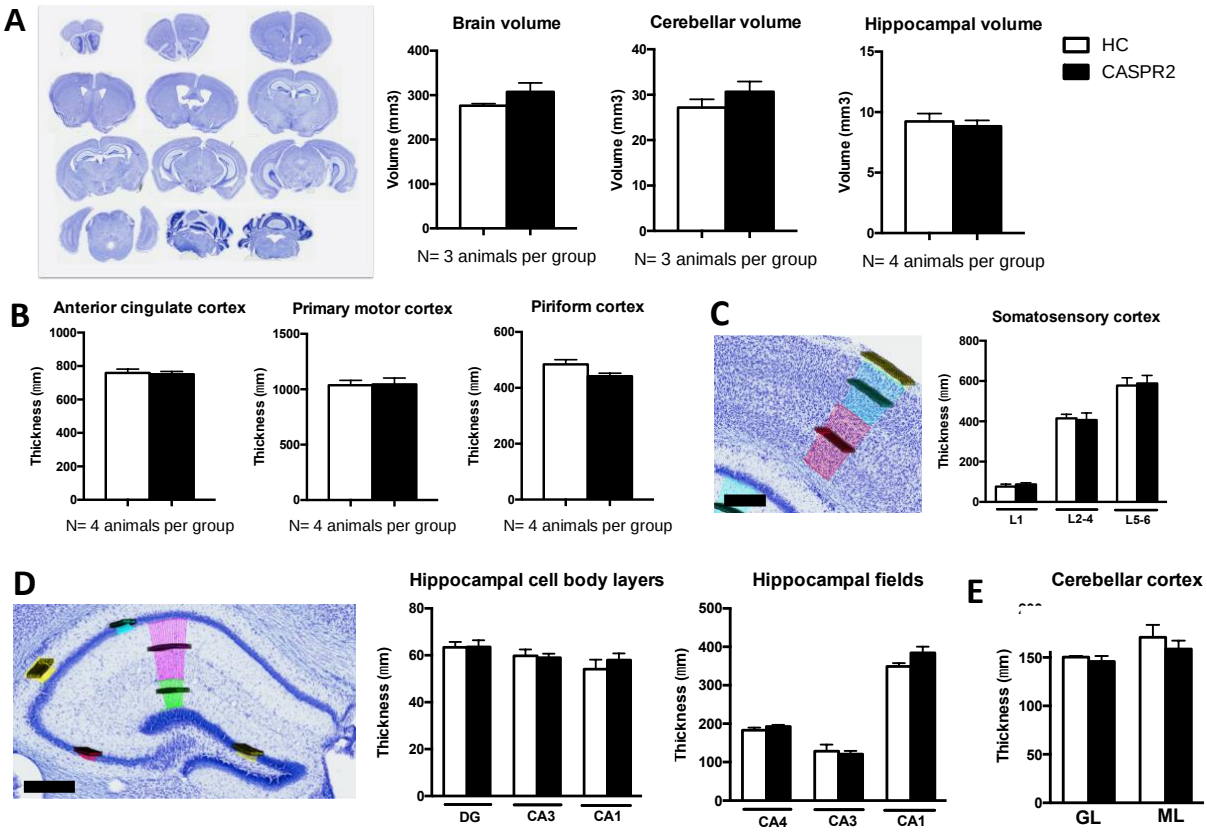

Figure 5.3 Nissl staining

A) Photograph showing the cresyl violet staining of a brain serie (left panel) and volumetric measurements. B) Cortical thickness measurements. C) Photograph showing an example of cortical layers measurement in the somatosensory cortex and quantification of the layers thickness. D) Photograph showing an example of hippocampal cell body layers and fields measurements and their quantification. E) Quantification of the thickness of cerebellar layers. No differences were observed in any parameter between HC-IgG and CASPR2-IgG injected mice. 40X, scale bar, hippocampus 400 µm, cortex 300 µm. Data are expressed as mean ± SEM.

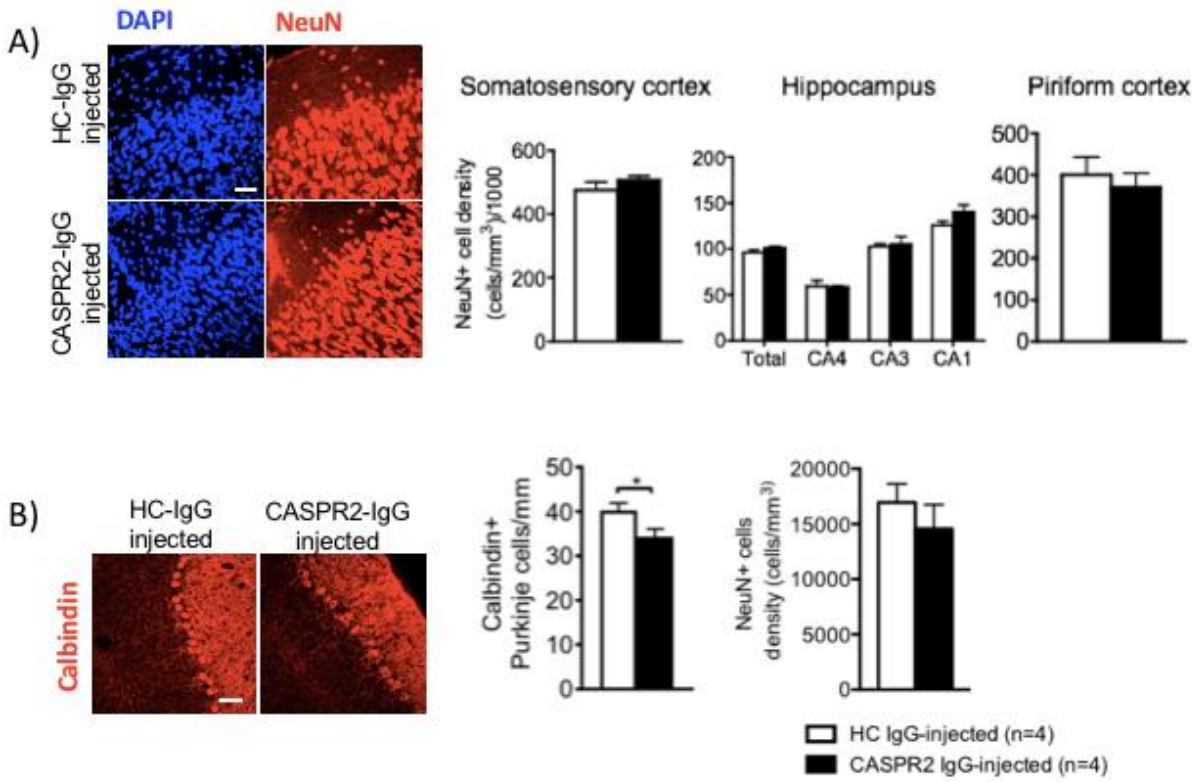

Figure 5.4 Analysis of neuronal count in mouse brains

A) Representative pictures of NeuN expression and quantification of NeuN positive cell densities in three brain regions. There were no differences between CASPR2-IgG and HC-IgG injected brains. B) Calbindin expressing Purkinje cells (PC), but not NeuN expressing neurons, were reduced in the cerebellum of CASPR-IgG injected mice. Data are expressed as mean ± SEM.

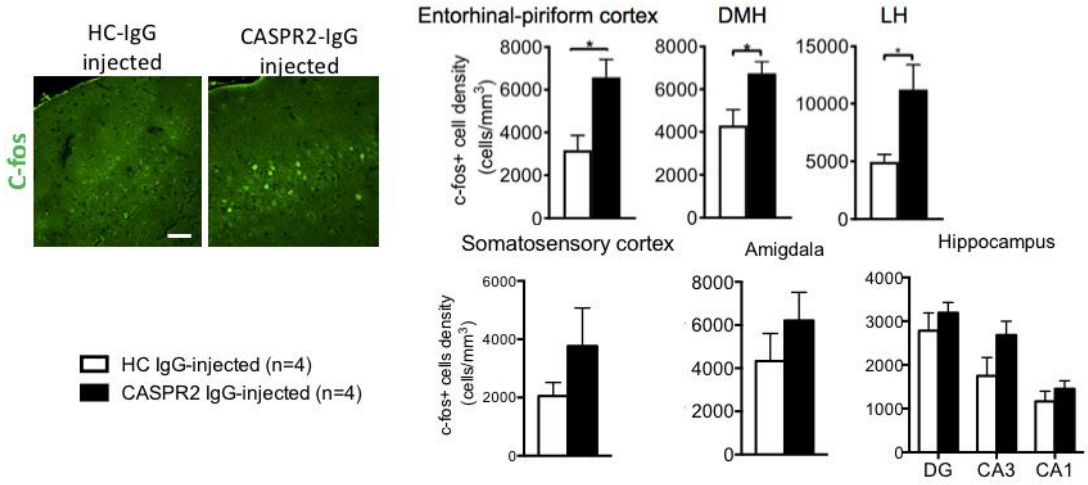

Figure 5.5 C-fos expression

C-fos expression in the entorhinal-piriform cortex, dorsomedial hypothalamus (DMH) and lateral hypothalamus (LH) was higher in the CASPR2-IgG injected compared to the HC-IgG injected mice (Representative images are shown from the entorhinal-piriform cortex). DG= dentate gyrus. 40X, scale bar 50 µm. Data are expressed as mean ± SEM.

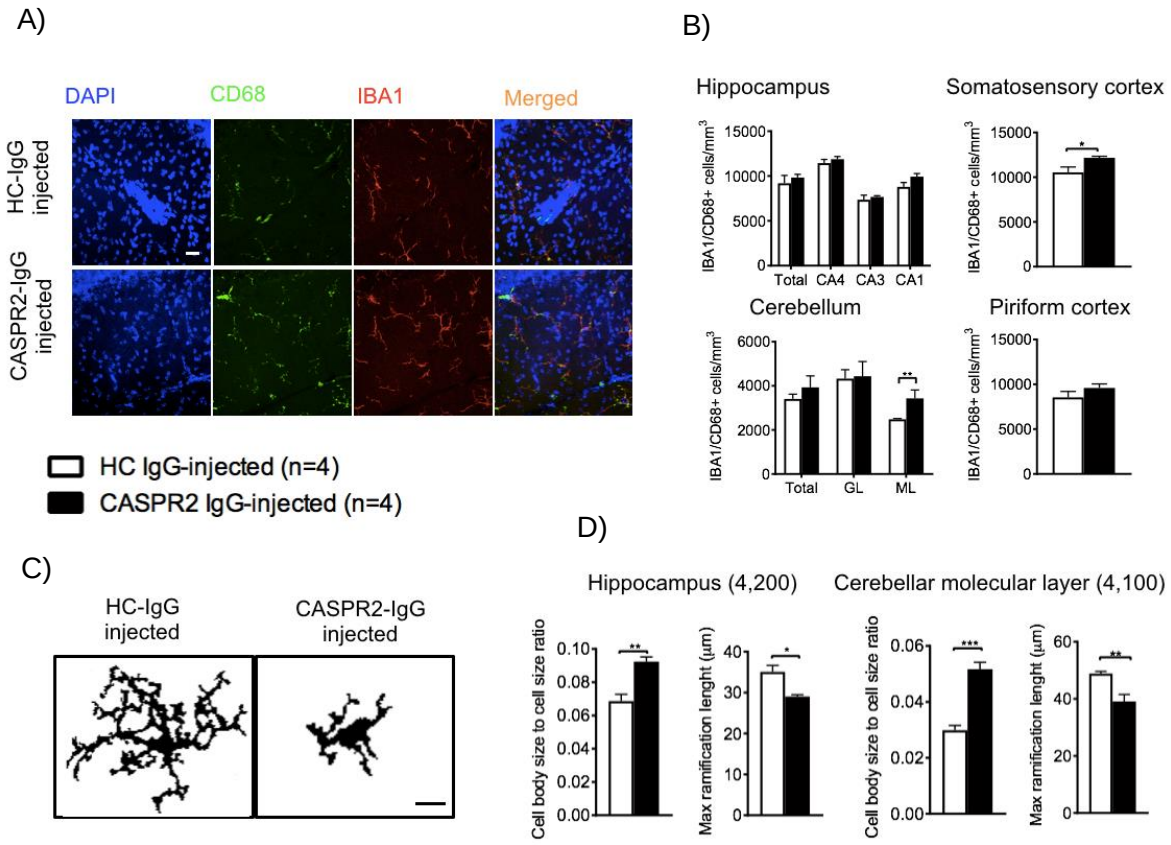

Figure 5.6 Microglial density and morphological analysis

A) Representative images of the molecular layer of the cerebellum showing microglia staining. 40X, scale bar 20 µm. B) CASPR2-IgG injected mice showed higher microglia densities in the somatosensory cortex and in the molecular layer (ML) of the cerebellum but not in the granular layer (GL) or in the hippocampus. N= 4 animals/group. C) Representative images of the z-stack projected Iba1 staining used for morphological analysis. 40X, 10 µm. D) Quantification of morphological data in the hippocampus and molecular layer of the cerebellum showed that microglia from CASPR2-IgG injected mice had a higher cell soma/cell total body size ratio and shorter ramifications than HC-IgG injected mice, compatible with an activated phenotype in both the hippocampus and the cerebellum. N=200 cells from 4 animals/groups were analysed from the hippocampus and 100 from the cerebellar molecular layer. Data are expressed as mean ± SEM.

5.8 Astrocyte analysis

The presence of astrocytosis was assessed by measuring the density of GFAP positive cells. We did not observe an increased density of astrocytes in the somatosensory ($t(6)= 0.46$, $P= 0.66$, independent samples t-test) and piriform cortices ($t(6)=0.45$, $P=0.66$, independent samples t-test) or hippocampus (overall: $t(6)=1.44$, $P=0.19$, CA4 $t(6)=1.69$, $P=0.14$, CA3 $t(6)=0.32$, $P=0.75$, CA1 $t(6)=1.06$, $P=0.32$, independent samples t-test). However, mean fluorescence intensity for GFAP in the molecular layer of the cerebellum showed increased GFAP staining ($t(6)= 2.5$, $P=0.043$, independent samples t-test) in the CASPR2-IgG injected compared to control mice compatible with the presence of a mild reactive gliosis (Figure 5.7).

Since the presence of activated microglia is often associated with a shift of astrocyte morphology towards an activated profile, we investigated the presence of reactive astrocytes by complement C3 expression in the hippocampus, somatosensory cortex and molecular layer of the cerebellum and by morphological analysis. The C3/GFAP co-stained area was raised in CASPR2-IgG injected mice in the hippocampus ($t(398)=4.87$, $P < 0.0001$, 200/cells per group, independent samples t-test), somatosensory cortex ($t(160)=2.41$, $P=0.01$, 81/cells per group, independent samples t-test) and cerebellum ($t(204)=7.14$, $P < 0.0001$, 103/cells per group, independent samples t-test) (Figure 5.7). The morphological analysis on GFAP stained cells showed that astrocytes from CASPR2-IgG injected mice also presented a smaller cell total body size ($U=22052$, $P= 0.0051$, Mann Whitney test U) and shorter ($U=19946$, $P< 0.0001$, Mann Whitney test U) maximal ramification length than HC-IgG injected mice, compatible with an activated phenotype, in the hippocampus (Figure 5.7).

5.9 Cytokine and chemokine expression

As these microglial and astrocyte changes suggested a state of mild neuroinflammation in the CASPR2-IgG injected mice, a commercial cytokine/chemokine array was performed on three whole brain lysates from each group to look for changes in neuroinflammatory markers. A trend towards increased levels was noted for several cytokines and chemokines, and particularly for interleukin (IL)-10, stem cell factor (SCF), and vascular endothelial growth factor (VEGF), but also for granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-colony stimulating factor (GCSF), IL2 and monocyte chemotactic protein 5 (MCP-5), but none reached significance after correction for multiple comparisons (Figure 5.7).

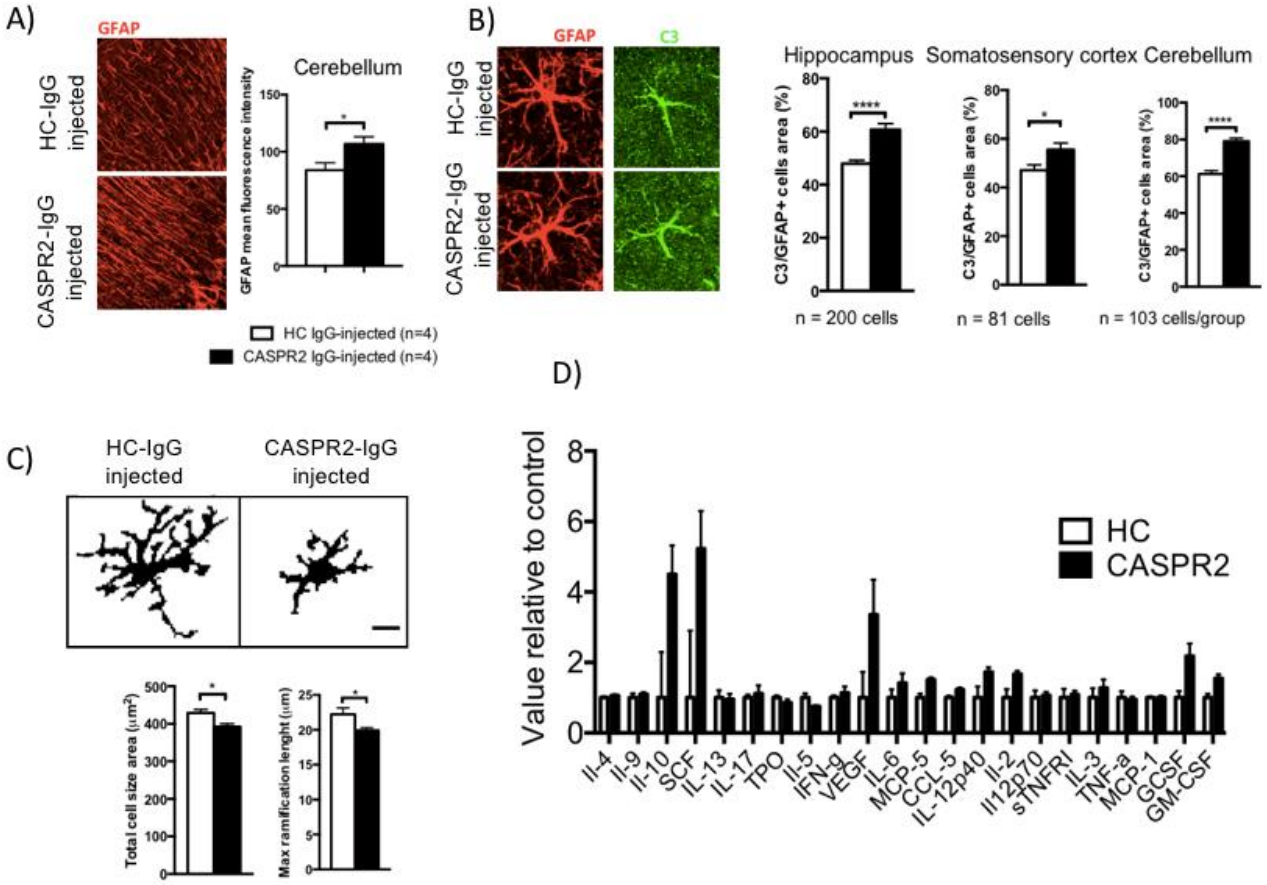

Figure 5.7 Astrocyte morphology and inflammatory markers

A) Representative images of GFAP staining in the molecular layer of the cerebellum and quantification of the mean fluorescence intensity in the same area showing higher GFAP expression in the CASPR2-IgG injected mice. 40X, scale bar 10 µm. B) Representative images of complement C3 expression on GFAP positive cells. Percentage of C3/GFAP area ratio per cell showed increased C3 expression of astrocytes in the hippocampus, somatosensory cortex and cerebellum of CASPR2-IgG injected mice. 40X, scale bar 10 µm. C) Representative pictures of the z-stack projected GFAP staining used for morphological analysis (40X, 10 µm). The astrocytes from CASPR2-IgG injected mice showed a smaller cell total body size and shorter maximal ramification length than HC-IgG injected mice, compatible with an activated phenotype, in the hippocampus. D) Compared to HC-IgG injected mice, CASPR2-IgG exposed animals showed changes in several cytokines and chemokines, but none reached significance after correcting for multiple comparisons.

5.10 Discussion

In this Chapter, the results of brain analysis of mice injected with CASPR2-IgG were presented. After LPS, CASPR2-Abs were able to access and bind to the brain parenchyma. CASPR2-IgG injected mice showed a mild loss of Purkinje cells with cerebellar astrocytosis and increased c-fos expression in the hypothalamus and piriform-entorhinal cortex. Moreover, CASPR2-IgG injected mice displayed increased microglia density and increased numbers of microglia and astrocytes with a reactive phenotype. This observation was supported by a trend toward elevated levels of microglia and astrocyte derived cytokines and chemokines.

In modeling of brain antibody-mediated disease through peripheral administration of the antibodies, it is often necessary to disrupt the BBB integrity in order to allow them to reach their target antigen. Previous studies showed that LPS is able to breach the BBB (Kowal C et al. 2004; Banks WA et al. 2014) and allow peripherally administered antibodies to penetrate the brain parenchyma without causing, *per se*, sustained neurotoxicity (Kowal C et al. 2004). According to these studies, we observed increased human IgG in the brain parenchyma of CASPR2-IgG injected mice. Human IgG was localised mainly in the somatosensory cortex, in the hippocampus and in the thalamus in the perfused brains. Although the preferential localisation of the IgG in the CASPR2-IgG injected mice might be related to a preferential penetration of the antibodies in some regions, as has been observed in a feline model of LGI1 encephalitis (Tröscher AR et al. 2017), it might also be related to a selective opening of the BBB by the LPS. Indeed, a previous study showed that LPS-induced BBB disruption occurred mainly in the frontal cortex, thalamus, pons-medulla and cerebellum (Banks WA et al. 2014).

Once in the brain, antibodies targeting neuronal antigens can cause their effects through different mechanisms, such as internalisation of their target and consequent loss of function, blocking of the interactions with other molecules, inflammation and complement-dependent cytotoxicity with consequent cell lysis (Ludwig RJ et al. 2017). Mechanisms of action of CASPR2-Abs are still largely unclear. CASPR2-Abs are IgG4, often in association with IgG1; they are most likely to act through a disruption of CASPR2 interaction with associated molecules than through internalisation and complement activation. Accordingly, a recent study showed that CASPR2-Abs do not reduce CASPR2 expression on the surface of cultured hippocampal neurons, but they may act by reducing CASPR2 interaction with contactin-2 (Patterson KR et al. 2017). By contrast, a neuropathological study on human tissue showed a reduction of CASPR2 staining in the cortex of a patient with autoimmune encephalitis (Sundal C et al. 2017) and in the previous mouse model we found a reduction in Kv1 channel surface

expression on DRG neurons following CASPR2-Abs treatment, consistent with an increased internalisation of CASPR2 following antibody binding (Dawes JM et al. 2018) as shown previously for many antibodies (e.g. Drachman DB et al 1978; Hughes EG et al. 2010). In our model, CASPR2-Abs did not appear to be associated with a reduction of CASPR2 surface expression although we cannot exclude that, at the time of our observation, a compensatory mechanism had not intervened to compensate for an initial reduction in CASPR2 expression. Indeed, a trend towards higher total CASPR2 levels was noted by western blotting of the brains of CASPR2-IgG injected mice.

Antibodies can change neuronal activity through several mechanisms, including modification of channels and receptor expression or neuroinflammation. C-fos is an immediate early gene product, produced by individual neurons and commonly used as a marker of cell activation. CASPR2-IgG exposed mice showed significantly higher levels of c-fos expression in the entorhinal-piriform cortex and in the dorsomedial and lateral hypothalamus. The expression in the latter area could be related to the sleep-wake cycle. Particularly, c-fos expression has been shown to be increased during the dark period, when the mice are active, and decreased during the light period, when they are asleep (Basheer R et al. 1997) consistently with the presence of wake-active neurons in the lateral hypothalamus (Hsieh KC et al. 2011). Therefore, an increase of c-fos expression during the light period in our animals (euthanized between 1:00 and 5:00 p.m.) might suggest an increased wakefulness, consistent with the insomnia typical of patients with Morvan's Syndrome (Liguori R et al. 2001).

Complement activation has been reported in neuropathological studies of patients with CASPR2-Abs (Körtvélyessy P et al. 2015, Sundal C et al. 2017). Although we did not directly evaluate the presence of apoptotic cells or complement C9 expression, there was no loss of NeuN positive cells in the cortex and hippocampus. In the cerebellum, however, CASPR2-Abs caused a mild loss of Purkinje cells (PC) but not a reduction of NeuN positive cells in the molecular layer. CASPR2-Abs bind to the axons of granule cerebellar neurons in the molecular layer and have been associated with cerebellar ataxia in some patients (Becker E et al. 2013) as well as with morphological changes of PC in a neuropathological case (Sundal C et al. 2017). The loss of PC we observed was modest and this might explain why we did not observe ataxia in mice. It is also possible that the neuronal loss needed more time to manifest extensively and that the behavioural motor tests were performed at a time when the effects were not yet complete. The mechanisms of the Purkinje cell loss are not clear, and they might be related to a direct effect of the antibodies or indirectly to an effect of neuroinflammatory changes.

Indeed, one of the most striking finding in this work is the activation of microglia and astrocytes. Microglia are neuroglia cells that represent the major immune cells in the brain and together with astrocytes are responsible for the innate immunity. Microglia and astrocyte functions are strictly interconnected, and, on the one hand, astrocytes support microglia functions, while on the other, microglia activation is required to initiate and support the astrocytic response (Pascual O et al. 2012). Previous studies showed that, under physiological condition, astrocytes express C3 complement (Lian H et al. 2016; Liddelow SA et al. 2017). In pathological conditions, (Lian H et al. 2016; Liddelow SA et al. 2017) activated microglia are able to induce reactive astrocytes, which up-regulate C3 expression and become responsible for neurotoxicity and synaptic loss. Accordingly, alongside microglia activation and increased density we found increased C3 expression on the surface of astrocytes and higher numbers of activated astrocytes in CASPR2-IgG injected mice. These findings were supported by trends towards elevated levels of cytokines produced by and targeted to astrocytes and microglia, such as GM-CSF, MCP-5 and VEGF. The microglial and astrocyte activation was particularly evident in the cerebellum. Here, reactive microglia have been shown to cause PC death during development (Marin-Teva JL et al. 2004) and in a mouse model of cerebellar degeneration an increase of activated microglia has been associated with PC loss and reactive gliosis (Zaho Z et al. 2011).

Interestingly, microglia activation has been reported in neuropathological cases of patients with CASPR2-Abs encephalitis (Sundal C et al. 2017; Körtvélyessy P et al. 2015). Moreover, microglia activation and increased density was noted in the infralimbic, pre-limbic and somatosensory cortex of animal exposed to CASPR2-Abs *in utero* and this activation persisted in adulthood (Coutinho E et al. 2017). By contrast, the peripheral administration of CASPR2-IgG, without LPS, induced only a mild increase of microglia in the spinal cord and not in the somatosensory cortex of exposed animals (Dawes JM et al. 2018), suggesting that microglia activation might be dependent on the contact with IgG and that, once initiated, this process might be maintained independently from the initial trigger. In our model, LPS allowed the penetration of the IgG antibodies into the brain where they could have bound to the Fc receptors expressed on microglia, but the alternative possibility that the CASPR2-Abs bound to neurons and that this initiated inflammatory reactions that up-regulated microglia would explain better the differences between CASPR2-IgG and HC-IgG injected mice.

Although the mechanisms by which CASPR2 antibodies produce neuropathological changes are not clear, we showed that peripheral injection of CASPR2-IgG can produce, after

a single dose of LPS, extensive CNS changes. This model needs to be further investigated with a wider range of more targeted behavioural tests.

Chapter 6. General discussion

In the last decades, the discovery of antibodies directed against neuronal surface antigens has provided aetiology and treatment for many disorders for which those were previously unknown. In the meantime, several questions have emerged, including the role of these antibodies in patients with different phenotypes or other neurological disorders, and their pathogenicity in these circumstances. Moreover, despite the circumstantial clinical evidence of the pathogenic role of these antibodies in classical autoimmune encephalitis, only in a few cases have these been supported by a definite proof through a passive transfer animal model. The work of this thesis aimed to answer some of these questions by investigating the frequency and role of antibodies directed against neuronal antigens in patients with different central nervous system disorders and in controls, and by establishing a passive transfer animal model of CASPR2 antibody, one of the most commonly encountered antibodies in clinical practice.

The results showed that antibodies against neuronal surface proteins are more frequent in patients with central nervous system disorders compared to healthy controls or patients with peripheral nervous system disorders. An important finding in this respect was that this difference was significant only when CBAs or the combination of positivity by different methods was used, suggesting that the combined approach ensured more specificity. A few studies have also shown antibodies against neuronal antigens are ubiquitous in the sera of both healthy and disease controls when using immunohistology (Levin EC et al. 2010; Nagele RG et al. 2011).

Overall, the frequency of antibodies was similar among patients, despite differences in the distribution of some specific antibodies, which were more prevalent in one cohort compared to others (i.e. NMDAR antibodies in narcoleptic patients). This implies that, irrespective of the clinical diagnosis, a percentage of patients, between 15 and 40%, could potentially benefit from immunotherapy. However, the clinical features of patients with antibody reactivity were only rarely suggestive of a possible autoimmune encephalitis, and typical imaging or CSF inflammatory changes were often absent. Moreover, the majority of patients with specific antibodies did not show the usual clinical, and sometimes not even demographic, features associated with that antibody; this adds difficulties to the interpretation of the results but could reflect the influence of age and gender on the clinical presentation associated with particular antibodies, and partially explain these atypical presentations.

This study has several limitations. Patients and controls were not demographically homogeneous and the clinical information available was sometimes limited or not available at all. Antibodies were not systematically titrated, and subclasses were not determined. Moreover, CSFs were not available for testing; the finding of the antibodies in the CSFs would have supported their pathogenic role, although the pathogenic relationship between serum and CSF antibody is not clear in all patients with autoimmune encephalitis, let alone the disorders that were investigated here. Finally, the retrospective nature of the study means that seropositive patients were not treated and followed up. Therefore, the main question about the significance of these findings remains open.

The results are also complicated by those of others, using the commercially available fixed CBAs. Antibodies against brain proteins were found in healthy individuals (Dahm L et al. 2014; Lang K and Prüss H, 2017) as well as in patients with several neurological or psychiatric presentations (reviewed in Pollak TA et al. 2016; Balint B et al. 2018). Whereas the physiological role of some natural occurring antibodies is partially understood (i.e. cellular debris removal) (Panda S and Ding JL, 2015), for antibodies against specific neuronal antigens this is unknown, nor are the possible circumstances and mechanisms that may turn these potentially 'harmless' antibodies into disease-relevant ones. Dahm L et al. (2014) found no difference in immunoglobulin classes distribution (IgM, IgG and IgA) or antibody titres between patients and controls. Therefore, the role of the blood-brain barrier (BBB) in defining the disease status was investigated. It was found that schizophrenic individuals with a past or present history of BBB disturbance were more likely to have more pronounced neurological symptoms if NMDAR antibody seropositive (Hammer C et al. 2014). It is of interest that in the narcolepsy type 1 patients studied here, seropositive status was associated with BBB disruption.

An important possibility is that the difference between health- or disease-status could be related to different functionality or epitope specificity of the antibodies. Castillo-Gomez E et al. (2017) showed that all NMDAR-Ab positive sera, derived from randomly selected individuals, were able to induce NMDAR internalisation in inducible pluripotent stem cell (IPSC)-derived human cortical neurons. Several different epitopes recognised by NMDAR-Abs were identified, without any consistent functional or epitope pattern related to health/disease state. However, these findings were contradicted by a subsequent study showing different functional effects of NMDAR-Abs from schizophrenic patients versus those found in healthy individuals (Jezequel J et al. 2017). NMDAR-Ab from patients, but not from healthy subjects, were shown to alter the surface dynamics and nanoscale organisation of synaptic NMDAR and its anchoring partner the ephrin-B2 receptor in heterologous cells, cultured neurons and in

mouse brain and preventing long-term potentiation at glutamatergic synapses, while leaving NMDAR-mediated calcium influx intact.

It is clear that more studies are needed to clarify the role of the antibodies in health and disease, mainly to identify the factors associated with the disease status, looking not only at the antibodies *per se* but also at all those factors that could influence their ability to reach their targets (i.e. blood-brain barrier integrity) and those which could modulate the immune response (i.e. HLA status). The implications of these findings could help support the use of immunotherapy in patients that otherwise would have remained untreated and avoid ineffective and potentially harmful treatment in those cases which would not benefit.

Part of the process of investigating antibody-associated diseases is to prove the pathogenicity of disease-relevant antibodies, which was the aim of the second part of this work (Chapters 4,5). Since there was no clear novel antibody emerging from the work in Chapter 3, and because the quantities of IgG needed for pathogenicity studies can only be obtained from plasma exchange, a passive transfer animal model was established to investigate the pathogenic role of CASPR2 antibodies. Mice injected with CASPR2-IgG showed working memory deficits and anxiety-like behaviour during social tasks with a global reduction of locomotor activity. Neuropathology showed that CASPR2-Abs were able to access and to bind to the brain parenchyma. CASPR2-IgG injected mice showed a mild loss of Purkinje cells with cerebellar astrocytosis and increased c-fos expression in the hypothalamus and piriform-entorhinal cortex. Moreover, CASPR2-IgG injected mice displayed increased microglia density and increased microglia and astrocytes with a reactive phenotype. This observation was supported by a trend towards elevated levels of microglia and astrocytes derived cytokines and chemokines. Surprisingly, no changes in CASPR2 expression were observed, suggesting that the main mechanisms of the antibodies might not be the internalisation of the protein but the interaction with its partners, namely Kv 1.1 and Kv 1.2 and contactin-2.

The results strongly suggest that CASPR2-Abs can affect many different brain regions and therefore potentially different functions. CASPR2-Abs have been associated with a broad range of clinical manifestations, many of which, e.g. autonomic and sleep dysfunction, would require additional tests that were not available on the Home Office license. The main finding was a mild memory impairment, which might appear inconsistent with the lack of histological changes in the hippocampus. However, although working memory is related to hippocampal integrity, other brain areas play a role in these tasks (Lalonde R, 2002). Among those the entorhinal cortex, where CASPR2 is highly expressed (Gordon A et al. 2016), is the main source of hippocampal afferents from the neocortex and is involved in different forms of

memory (van Groen T et al. 2003). Indeed, in mouse models of Alzheimer's disease, memory impairment was associated with an early hyper-excitability of the entorhinal cortex (Xu W et al. 2015) which would be consistent with increased neuronal excitability following reduction in Kv1 expression seen in the dorsal root ganglia (DRG) in the peripheral CASPR2-Ab model (Dawes JM et al. 2018). These changes may have been responsible for the higher levels of c-fos expression in the entorhinal-piriform cortex, rather than in the hippocampus.

The long-term effects of CASPR2-Abs are not clear in patients but in general brain MRI studies do not demonstrate brain atrophy. This would be consistent with previous findings (Dawes JM et al. 2018) as CASPR2-Abs did not caused frank neuronal loss in the periphery, and with the findings here where the only neuronal abnormality identified was a mild loss of cerebellar Purkinje cells. More striking was the activation of microglia and astrocytes. Microglial activation has been reported in neuropathological cases of patients with CASPR2-Ab encephalitis (Körtvelyessy P et al. 2015; Sundal C et al. 2017). Moreover, microglial activation and increased density was noted in cortex of animals exposed to CASPR2-Abs *in utero* and this activation persisted into adulthood (Coutinho E et al. 2017). By contrast, the peripheral administration of CASPR2-IgG, without LPS, induced only a mild increase of microglia in the spinal cord but not in the somatosensory cortex of exposed animals (Dawes JM et al. 2018).

This study also has limitations. The use of LPS, could have induced additional inflammation and contributed to determining the site of BBB disruption, leading to or changing some of the antibody effects observed. Indeed, the intracerebroventricular (ICV) injection of the antibodies could have overcome the need to use other molecules to open the BBB, guaranteeing a direct access of the antibodies to the brain; but there is no evidence yet that the ICV approach provides a more realistic or complete model of autoimmune disorders (Planagumà J et al. 2015; Wright S et al. 2015). Indeed, as there is an incomplete understanding regarding the relative roles of CSF and serum antibodies in some of these conditions, a direct comparison between the two approaches could be very interesting. The behavioural studies were broad but did not focus on all the features of CASPR2-related encephalitis, as mentioned above, and these need to be investigated in further studies. The experimental timing we chose could have prevented us from seeing the acute effects of the antibodies while some behavioural and histological effects might have required more time to become fully established.

Finally, other questions remain unanswered such as the mechanisms by which CASPR2-Abs affect CASPR2 function in the CNS and if the glial changes are relevant in the human disease. *In vitro* studies would have been useful to clarify if CASPR2-Abs caused

internalisation of the antigen as seems suggested by studies on DRG neurons (Dawes JM et al. 2018), or an alteration of their interaction with other proteins as suggested by these findings and other studies (Patterson KR et al. 2017). However, due to the lack of time, these studies could not be performed during the course of this PhD.

Many of the recently-described antibody-mediated CNS diseases have higher serum than CSF antibody levels, emphasizing that in many or most cases they are likely to be initiated by a peripheral immune response. It was timely, therefore, to see whether peripheral injection of antibodies could, under conditions where the BBB is briefly compromised, lead to evidence of CNS dysfunction. Despite the mentioned limitations, this study not only supports the pathogenic effect of CASPR2-Abs, but also supports a model where peripherally administered CASPR2-Abs, in the presence of a temporary breach in the BBB, are able to access the brain and produce histological and behavioural changes.

The data collected in this thesis should stimulate further research, both epidemiological and experimental, into the role of potentially pathogenic antibodies against neuronal surface proteins in central nervous system disorders and their mechanisms of action.

Bibliography

Abele M, Weller M, Mescheriakov S, Burk K, Dichgans J, Klockgether T. Cerebellar ataxia with glutamic acid decarboxylase autoantibodies. Neurology 1999; 52:857–859.

Abgrall G, Demeret S, Rohaut B, Leu-Semenescu S, Arnulf I. Status dissociatus and disturbed dreaming in a patient with Morvan syn- drome plus Myasthenia Gravis. Sleep Med 2015; 16: 894–6.

Ahmed SS, Volkmuth W, Duca J, Corti L, Pallaoro M, Pezzicoli A, Karle A, Rigat F, Rappuoli R, Narasimhan V, Julkunen I, Vuorela A, Vaarala O, Nohynek H, Pasini FL, Montomoli E, Trombetta C, Adams CM, Rothbard J, Steinman L. Antibodies to influenza nucleoprotein cross-react with human hypocretin receptor 2. Sci Transl Med. 2015; 7:294ra105.

American Academy of Sleep Medicine. The International Classification of Sleep Disorders: diagnostic and coding manual. 2nd edn. Chicago, IL: AASM, 2005

Angoa-Pérez M, Kane MJ, Briggs DI, Francescutti DM, Kuhn DM. Marble burying and nestlet shredding as tests of repetitive, compulsive-like behaviors in mice. J Vis Exp. 2013; (82):50978.

Angus-Leppan H, Rudge P, Mead S, Collinge J, Vincent A. Autoantibodies in sporadic Creutzfeldt-Jakob disease. JAMA Neurol. 2013; 70(7):919-22.

Antunes M, Biala G. The novel object recognition memory: Neurobiology, test procedure, and its modifications', Cognitive Processing. 2012; 13(2) 93–110.

Ariño H, Armangue T, Petit-Pedrol M, Sabater L, Martinez-Hernandez E, Hara M, Lancaster E, Saiz A, Dalmau J, Graus F. Anti-LGI1-associated cognitive impairment: presentation and long-term outcome. Neurology 2016; 87: 759 –765.

Armangue T, Moris G, Cantarin-Extremera V, Conde CE, Rostasy K, Erro ME, Por- tilla-Cuenca JC, Turon-Vinas E, Malaga I, Munoz-Cabello B, Torres-Torres C, Llufriu S, Gonzalez-Gutierrez-Solana L, Gonzalez G, Casado-Naranjo I, Rosenfeld M, Graus F, Dalmau J. Autoimmune post-herpes simplex encephalitis of adults and teenagers. Neurology 2015; 85: 1736 –1743.

Armangue T, Titulaer MJ, Malaga I, Bataller L, Gabilondo I, Graus F, Dalmau J. Pediatric anti-N-methyl-D-aspartate receptor encephalitis-clinical analysis and novel findings in a series of 20 patients. J Pediatr. 2013;162: 850 – 856.

Avrameas S, Selmi C. Natural autoantibodies in the physiology and pathophysiology of the immune system. J Autoimmun. 2013; 41: 46–9.

Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, Folli F, Richter-Olesen H, De Camilli P. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 1990; 347:51–56.

Balint B, Vincent A, Meinck HM, Irani SR, Bhatia KP. Movement disorders with neuronal antibodies: syndromic approach, genetic parallels and pathophysiology. Brain 2018;141(1):13-36.

Banks WA, Gray AM, Erickson MA, Salameh TS, Damodarasamy M, Sheibani N, Meabon JS, Wing EE, Morofuji Y, Cook DG, Reed MJ. Lipopolysaccharide-induced blood-brain barrier disruption: roles of cyclooxygenase, oxidative stress, neuroinflammation, and elements of the neurovascular unit. J Neuroinflammation. 2015; 12:223.

Barajas RF, Collins DE, Cha S, Geschwind MD. Adult-onset drug-refractory seizure disorder associated with anti-voltage-gated potassium-channel antibody. Epilepsia 2010; 51(3):473-7.

Barkus C, Dawson LA, Sharp T, Bannerman DM. GluN1 hypomorph mice exhibit wide-ranging behavioral alterations. Genes Brain Behav. 2012; 11(3):342-51.

Basheer R, Sherin JE, Saper CB, Morgan JI, McCarley RW, Shiromani PJ. Effects of sleep on wake-induced c-fos expression. J. Neurosci. 1997; 17:9746–9750.

Becker EB, Zuliani L, Pettingill R, Lang B, Waters P, Dulneva A, Sobott F, Wardle M, Graus F, Bataller L, Robertson NP, Vincent A. Contactin-associated protein-2 antibodies in non-paraneoplastic cerebellar ataxia. J Neurol Neurosurg Psychiatry 2012; 83(4):437-40.

Bergman P, Adori C, Vas S, Kai-Larsen Y, Sarkanen T, Cederlund A, Agerberth B, Julkunen I, Horvath B, Kostyalik D, Kalmár L, Bagdy G, Huutoniemi A, Partinen M, Hökfelt T. Narcolepsy patients have antibodies that stain distinct cell populations in rat brain and influence sleep patterns. Proc Natl Acad Sci U S A. 2014; 111(35):E3735-44.

Bettler B, Kaupmann K, Mosbacher J, Gassmann M. Molecular structure and physio- logical functions of GABA(B) receptors. Physiol Rev. 2004; 84: 835– 867.

Berrih-Aknin S, Le Panse R. Myasthenia gravis: a comprehensive review of immune dysregulation and etiological mechanisms. J Autoimmun 2014; 52:90–100.

Bien CG, Mirzadjanova Z, Baumgartner C, Onugoren MD, Grunwald T, Holtkamp I, Senmann S, Kermer P, Melzer N, Naumann M, Riepe M, Schäbitz WR, von Oertzen TJ, von Podewils F, Rauschka H, May TW. Anti-contactin-associated protein-2 encephalitis: relevance of antibody titres, presentation and outcome. Eur J Neurol. 2017; 24(1):175-186.

Binks S, Varley J, Lee W, Makuch M, Elliott K, Gelfand JM, Jacob S, Leite MI, Maddison P, Chen M, Geschwind MD, Grant E, Sen A, Waters P, McCormack M, Cavalleri GL, Barnardo M, Knight JC, Irani SR. Distinct HLA associations of LGI1 and CASPR2-antibody diseases. Brain 2018. doi: 10.1093/brain/awy109. [Epub ahead of print]

Black JL 3rd, Krahn LE, Pankratz VS, Silber M. Search for neuron-specific and non-neuron-specific antibodies in narcoleptic patients with and without HLADQB1*0602. Sleep 2002; 25(7):719-23.

Black JL 3rd, Silber MH, Krahn LE, Fredrickson PA, Pankratz VS, Avula R, Walker DL, Slocumb NL. Analysis of hypocretin (orexin) antibodies in patients with narcolepsy. Sleep 2005; 28(4):427-31.

Bonello M, Jacob A, Ellul MA, Barker E, Parker R, Jefferson S, Alusi S. IgLON5 disease responsive to immunotherapy. Neurol Neuroimmunol Neuroinflamm. 2017; 4(5):e383.

Brenner T, Sills GJ, Hart Y, Howell S, Waters P, Brodie MJ, Vincent A, Lang B. Prevalence of neurologic autoantibodies in cohorts of patients with new and established epilepsy. Epilepsia 2013; 54(6):1028-35.

Brimberg L, Mader S, Jeganathan V, Berlin R, Coleman TR, Gregersen PK, Huerta PT, Volpe BT, Diamond B. Caspr2-reactive antibody cloned from a mother of an ASD child mediates an ASD-like phenotype in mice. Mol Psychiatry 2016; 21(12):1663-1671.

Brüggemann N, Wandinger KP, Gaig C, Sprenger A, Junghanns K, Helmchen C, Münchau A. Dystonia, lower limb stiffness, and upward gaze palsy in a patient with IgLON5 antibodies. Mov Disord. 2016; 31(5):762-4.

Buckley C, Oger J, Clover L, Tüzün E, Carpenter K, Jackson M, Vincent A. Potassium channel antibodies in two patients with reversible limbic encephalitis. Ann Neurol. 2001; 50(1):73-8.

Bufler J, Pitz R, Czep M, Wick M, Franke C. Purified IgG from seropositive and seronegative patients with mysasthenia gravis reversibly blocks currents through nicotinic acetylcholine receptor channels. Ann. Neurol. 1998; 43: 458–464.

Burnet Sir M. Self and not-self: cellular immunology book one. London: Cambridge University Press, 1969: vii.

Carvajal-González A, Leite MI, Waters P, Woodhall M, Coutinho E, Balint B, Lang B, Pettingill P, Carr A, Sheerin UM, Press R, Lunn MP, Lim M, Maddison P, Meinck HM, Vandenberghe W, Vincent A. Glycine receptor antibodies in PERM and related syndromes: characteristics, clinical features and outcomes. Brain 2014; 137:2178-92.

Castillo-Gómez E, Oliveira B, Tapken D, Bertrand S, Klein-Schmidt C, Pan H, Zafeiriou P, Steiner J, Jurek B, Trippe R, Prüss H, Zimmermann WH, Bertrand D, Ehrenreich H, Hollmann M. All naturally occurring autoantibodies against the NMDA receptor subunit NR1 have pathogenic potential irrespective of epitope and immunoglobulin class. Mol Psychiatry. 2017; 22(12):1776-1784.

Castillo P, Woodruff B, Caselli R, Vernino S, Lucchinetti C, Swanson J, Noseworthy J, Aksamit A, Carter J, Sirven J, Hunder G, Fatourechi V, Mokri B, Drubach D, Pittock S, Lennon V, Boeve B. Steroid-responsive encephalopathy associated with autoimmune thyroiditis. Arch Neurol. 2006; 63(2):197-202.

Chang T, Alexopoulos H, McMenamin M, Carvajal-González A, Alexander SK, Deacon R, Erdelyi F, Szabó G, Lang B, Blaes F, Brown P, Vincent A. Neuronal surface and glutamic acid decarboxylase autoantibodies in Nonparaneoplastic stiff person syndrome. JAMA Neurol. 2013 Sep 1;70(9):1140-9. Erratum in: JAMA Neurol. 2014; 71(2):244.

Chang T, Alexopoulos H, Pettingill P, McMenamin M, Deacon R, Erdelyi F, Szabó G, Buckley CJ, Vincent A. Immunization against GAD induces antibody binding to GAD-independent antigens and brainstem GABAergic neuronal loss. PLoS One 2013; 8(9):e72921Erratum in: PLoS One. 2013;8(10).

Chen DW, Wang J, Zhang LL, Wang YJ, Gao CY. Cerebrospinal Fluid Amyloid-β Levels are Increased in Patients with Insomnia. J Alzheimers Dis. 2018; 61(2):645-651.

Chitravas N, Jung RS, Kofskey DM, Blevins JE, Gambetti P, Leigh RJ, Cohen ML. Treatable neurological disorders misdiagnosed as Creutzfeldt-Jakob disease. Ann Neurol. 2011; 70(3):437-44.

Çoban A, Ismail Küçükali C, Bilgiç B, Yalçınkaya N, Haytural H, Ulusoy C, Turan S, Çakır S, Uçok A, Ünübol H, Hanagasi HA, Gürvit H, Tüzün E. Evaluation of incidence and clinical features of antibody-associated autoimmune encephalitis mimicking dementia. Behav Neurol. 2014; 2014:935379.

Corsellis JA, Goldberg GJ, Norton AR. "Limbic encephalitis" and its association with carcinoma. Brain 1968; 91(3):481-96.

Costa M, Saiz A, Casamitjana R, Fernandez Castaner M, Sanmarti A, Graus F, Jaraquemada D. T-cell reactivity to glutamic acid decarboxylase in stiff-man syndrome and cerebellar ataxia associated with polyendocrine autoimmunity. Clin Exp Immunol 2002; 129: 471 – 478.

Coutinho E, Menassa DA, Jacobson L, West SJ, Domingos J, Moloney TC, et al. Persistent microglial activation and synaptic loss with behavioral abnormalities in mouse offspring exposed to CASPR2-antibodies in utero. Acta Neuropathol. 2017; 134: 567–583.

Cvetkovic-Lopes V, Bayer L, Dorsaz S, Maret S, Pradervand S, Dauvilliers Y, Lecendreux M, Lammers GJ, Donjacour CE, Du Pasquier RA, Pfister C, Petit B, Hor H, Mühlethaler M, Tafti M. Elevated Tribbles homolog 2-specific antibody levels in narcolepsy patients. J Clin Invest. 2010; 120(3):713-9.

Dalmau J, Balice-Gordon RJ. Cellular and synaptic mechanisms of anti-NMDA receptor encephalitis. J Neurosci. 2010; 30(17):5866-75.

Dalmau J, Furneaux HM, Rosenblum MK, Graus F, Posner JB. Detection of the anti-Hu antibody in specific regions of the nervous system and tumour from patients with paraneoplastic encephalomyelitis/sensory neuronopathy. Neurology 1991; 41(11):1757-64.

Dalmau J, Gleichman AJ, Hughes EG, Rossi JE, Peng X, Lai M, Dessain SK, Rosenfeld MR, Balice-Gordon R, Lynch DR. Anti-NMDA-receptor encephalitis: case series and analysis of the effects of antibodies. Lancet Neurol. 2008; 7(12):1091-8.

Dalmau J, Lancaster E, Martinez-Hernandez E, Rosenfeld MR, Balice-Gordon R. Clinical experience and laboratory investigations in patients with anti-NMDAR encephalitis. Lancet Neurol. 2011; 10: 63–74.

Dalmau J, Rosenfeld MR. Paraneoplastic syndromes of the CNS. Lancet Neurol. 2008; 7(4):327-40. Review.

Dalmau J, Tüzün E, Wu HY, Masjuan J, Rossi JE, Voloschin A, Baehring JM, Shimazaki H, Koide R, King D, Mason W, Sansing LH, Dichter MA, Rosenfeld MR, Lynch DR. Paraneoplastic anti-N-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma. Ann Neurol. 2007; 61(1):25-36.

Dauvilliers Y, Abril B, Mas E, Michel F, Tafti M. Normalization of hypocretin-1 in narcolepsy after intravenous immunoglobulin treatment. Neurology 2009; 73: 1333-1334

Dauvilliers Y, Carlander B, Rivier F, Touchon J, Tafti M. Successful management of cataplexy with intravenous immunoglobulins at narcolepsy onset. Ann. Neurol. 2004; 56: 905-908.

Dauvilliers Y, Gaig C, Barateau L, Graus F, Iranzo A, Lopez R, Santamaria J. Absence of NMDA receptor antibodies in the rare association between Type 1 Narcolepsy and Psychosis. Sci Rep. 2016; 6:25230.

Dawes JM, Weir GA, Middleton SJ, Patel R, Chisholm KI, Pettingill P, Peck LJ, Sheridan J, Shakir A, Jacobson L, Gutierrez-Mecinas M, Galino J, Walcher J, Kühnemund J, Kuehn H, Sanna MD, Lang B, Clark AJ, Themistocleous AC, Iwagaki N, West SJ, Werynska K, Carroll L, Trendafilova T, Menassa DA, Giannoccaro MP, Coutinho E, Cervellini I, Tewari D, Buckley C, Leite MI, Wildner H, Zeilhofer HU, Peles E, Todd AJ, McMahon SB, Dickenson AH, Lewin GR, Vincent A, Bennett DL. Immune or Genetic-Mediated Disruption of CASPR2 Causes Pain Hypersensitivity Due to Enhanced Primary Afferent Excitability. Neuron 2018; 97(4):806-822.e10.

DeLuca I, Blachere NE, Santomasso B, Darnell RB. Tolerance to the neuron-specific paraneoplastic HuD antigen. PLoS One 2009; 4: e5739, 2009.

Dahm L, Ott C, Steiner J, Stepniak B, Teegen B, Saschenbrecker S, Hammer C, Borowski K, Begemann M, Lemke S, Rentzsch K, Probst C, Martens H, Wienands J, Spalletta G, Weissenborn K, Stöcker W, Ehrenreich H. Seroprevalence of autoantibodies against brain antigens in health and disease. Ann Neurol. 2014; 76(1):82-94.

Deacon RMJ. Measuring Motor Coordination in Mice. Journal of Visualized Experiments 2013; (75): 1–8.

Dhamija R, Renaud DL, Pittock SJ, McKeon A, Lachance DH, Nickels KC, Wirrell EC, Kuntz NL, King MD, Lennon VA. Neuronal voltage-gated potassium channel complex autoimmunity in children. Pediatr Neurol. 2011; 44(4):275-81.

Doss S, Wandinger KP, Hyman BT, Panzer JA, Synofzik M, Dickerson B, Mollenhauer B, Scherzer CR, Ivinson AJ, Finke C, Schöls L, Müller Vom Hagen J, Trenkwalder C, Jahn H, Höltje M, Biswal BB, Harms L, Ruprecht K, Buchert R, Höglinger GU, Oertel WH, Unger MM, Körtvélyessy P, Bittner D, Priller J, Spruth EJ, Paul F, Meisel A, Lynch DR, Dirnagl U, Endres M, Teegen B, Probst C, Komorowski L, Stöcker W, Dalmau J, Prüss H. High prevalence of NMDA receptor IgA/IgM antibodies in different dementia types. Ann Clin Transl Neurol. 2014; 1(10):822-32.

Drachman DB. How to recognize an antibody-mediated autoimmune disease: criteria. Res Publ Assoc Res Nerv Ment Dis. 1990; 68:183-6. Review.

Drachman DB, Angus CW, Adams RN, Michelson JD, Ho man GJ. Myasthenic antibodies cross-link acetylcholine receptors to accelerate degradation. N Engl J Med 1978; 298:1116–22.

Dubey D, Alqallaf A, Hays R, Freeman M, Chen K, Ding K, Agostini M, Vernino S. Neurological Autoantibody Prevalence in Epilepsy of Unknown Etiology. JAMA Neurol. 2017; 74(4):397-402.

Engel AG. Myasthenia gravis and myasthenic syndromes. Ann Neurol 1984; 16:519–34.

Engel AG, Arahata K. The membrane attack complex of complement at the endplate in myasthenia gravis. Ann N Y Acad Sci 1987; 505:326–32.

Ehrlich P. The collected papers of Paul Ehrlich. [vol 2]. New York: Pergamon Press, 1957:253.
Escudero D, Guasp M, Ariño H, Gaig C, Martínez-Hernández E, Dalmau J, Graus F. Antibody-associated CNS syndromes without signs of inflammation in the elderly. Neurology 2017; 89(14):1471-1475.

Finke C, Kopp UA, Pajkert A, Behrens JR, Leypoldt F, Wuerfel JT, Ploner CJ, Prüss H, Paul F. Structural Hippocampal Damage Following Anti-N-Methyl-D-Aspartate Receptor Encephalitis. Biol Psychiatry. 2016;79(9):727-34.

Flanagan EP, Kotsenas AL, Britton JW, McKeon A, Watson RE, Klein CJ, Boeve BF, Lowe V, Ahlskog JE, Shin C, Boes CJ, Crum BA, Laughlin RS, Pittock SJ. Basal ganglia T1 hyperintensity in LGI1-autoantibody faciobrachial dystonic seizures. Neurol Neuroimmunol Neuroinflamm. 2015; 2: e161.

Flanagan EP, McKeon A, Lennon VA, Boeve BF, Trenerry MR, Tan KM, Drubach DA, Josephs KA, Britton JW, Mandrekar JN, Lowe V, Parisi JE, Pittock SJ. Autoimmune dementia: clinical course and predictors of immunotherapy response. Mayo Clin Proc. 2010; 85(10):881-97.

Florance NR, Davis RL, Lam C, Szperka C, Zhou L, Ahmad S, Campen CJ, Moss H, Peter N, Gleichman AJ, Glaser CA, Lynch DR, Rosenfeld MR, Dalmau J. Anti-N- methyl-D-aspartate receptor (NMDAR) encephalitis in children and adolescents. Ann Neurol. 2009; 66: 11–18.

Friedman JI, Vrijenhoek T, Markx S, Janssen IM, van der Vliet WA, Faas BH, Knoers NV, Cahn W, Kahn RS, Edelmann L, Davis KL, Silverman JM, Brunner HG, van Kessel AG, Wijmenga C, Ophoff RA, Veltman JA. CNTNAP2 gene dosage variation is associated with schizophrenia and epilepsy. Mol Psychiatry. 2008; 13(3):261-6.

Fujita K, Yuasa T, Takahashi Y, Tanaka K, Sako W, Koizumi H, Iwasaki Y, Yoshida M, Izumi Y, Kaji R. Antibodies to N-methyl-D-aspartate glutamate receptors in Creutzfeldt-Jakob disease patients. J Neuroimmunol. 2012; 251(1-2):90-3.

Fujita K, Yuasa T, Watanabe O, Takahashi Y, Hashiguchi S, Adachi K, Izumi Y, Kaji R. Voltage-gated potassium channel complex antibodies in Creutzfeldt-Jakob disease. J Neurol. 2012; 259(10):2249-50

Fukata Y, Adesnik H, Iwanaga T, Bredt DS, Nicoll RA, Fukata M. Epilepsy-related ligand/receptor complex LGI1 and ADAM22 regulate synaptic transmission. Science 2006; 313(5794):1792-5.

Gable MS, Gavali S, Radner A, Tilley DH, Lee B, Dyner L, Collins A, Dengel A, Dalmau J, Glaser CA. Anti-NMDA receptor encephalitis: report of ten cases and encephalitis. Eur J Clin Microbiol Infect Dis. 2009; 28: 1421–1429.

Gaig C, Graus F, Compta Y, Högl B, Bataller L, Brüggemann N, Giordana C, Heidbreder A, Kotschet K, Lewerenz J, Macher S, Martí MJ, Montojo T, Pérez-Pérez J, Puertas I, Seitz C, Simabukuro M, Téllez N, Wandinger KP, Iranzo A, Ercilla G, Sabater L, Santamaría J, Dalmau J. Clinical manifestations of the anti-IgLON5 disease. Neurology 2017; 88(18):1736-1743.

Garden GA, Moller T. Microglia biology in health and disease. J Neuroimmune Pharmacol. 2006; 1:127–137.

Gelpi E, Hoftberger R, Graus F, Ling H, Holton JL, Dawson T, Popovic M, Pretnar- Oblak J, Hogl B, Schmutzhard E, Poewe W, Ricken G, Santamaria J, Dalmau J, comparison with viral Budka H, Revesz T, Kovacs GG. Neuropathological criteria of anti-IgLON5-related tauopathy. Acta Neuropathol. 2016; 132: 531–543.

Geschwind MD, Tan KM, Lennon VA, Barajas RF Jr, Haman A, Klein CJ, Josephson SA, Pittock SJ. Voltage-gated potassium channel autoimmunity mimicking Creutzfeldt-Jakob disease. Arch Neurol. 2008; 65(10):1341-6.

Giannoccaro MP, Crisp SJ, Vincent A. Antibody-mediated central nervous system diseases. Brain and Neuroscience Advances, 2018; doi.org/10.1177/2398212818817497

Giannoccaro MP, Waters P, Pizza F, Liguori R, Plazzi G, Vincent A. Antibodies Against Hypocretin Receptor 2 Are Rare in Narcolepsy. Sleep 2017; 40(2).

Giometto B, Marchiori GC, Nicolao P, Scaravilli T, Lion A, Bardin PG, Tavolato B. Sub-acute cerebellar degeneration with anti-Yo autoantibodies: immunohistochemical analysis of the immune reaction in the central nervous system. Neuropathol Appl Neurobiol. 1997; 23(6):468-74. Erratum in: Neuropathol Appl Neurobiol. 1998; 24(1):2.

Gordon A, Salomon D, Barak N, Pen Y, Tsoory M, Kimchi T, Peles E. Expression of Cntnap2 (Caspr2) in multiple levels of sensory systems. Mol Cell Neurosci. 2016; 70:42-53.

Grau-Rivera O, Sánchez-Valle R, Saiz A, Molinuevo JL, Bernabé R, Munteis E, Pujadas F, Salvador A, Saura J, Ugarte A, Titulaer M, Dalmau J, Graus F. Determination of neuronal antibodies in suspected and definite Creutzfeldt-Jakob disease. JAMA Neurol. 2014; 71(1):74-8.

Graus F, Illa I, Agusti M, Ribalta T, Cruz-Sanchez F, Juarez C. Effect of intraventricular injection of an anti-Purkinje cell antibody (anti-Yo) in a guinea pig model. J Neurol Sci. 1991; 106(1):82-7.

Graus F, Titulaer MJ, Balu R, Benseler S, Bien CG, Cellucci T, Cortese I, Dale RC, Gelfand JM, Geschwind M, Glaser CA, Honnorat J, Hoftberger R, Iizuka T, Irani SR, Lancaster E, Leypoldt F, Pruss H, Rae-Grant A, Reindl M, Rosenfeld MR, Rostasy K, Saiz A, Venkatesan A, Vincent A, Wandinger KP, Waters P, Dalmau J. A clinical approach to diagnosis of autoimmune encephalitis. Lancet Neurol. 2016; 15: 391– 404.

Greenlee JE, Burns JB, Rose JW, Jaeckle KA, Clawson S. Uptake of systemically administered human anticerebellar antibody by rat Purkinje cells following blood-brain barrier disruption. Acta Neuropathol. 1995; 89(4):341-5.

Hacohen Y, Absoud M, Hemingway C, Jacobson L, Lin JP, Pike M, Pullaperuma S, Siddiqui A, Wassmer E, Waters P, Irani SR, Buckley C, Vincent A, Lim M. NMDA receptor antibodies associated with distinct white matter syndromes. Neurol Neuroimmunol Neuroinflamm. 2014; 1(1):e2.

Haitao R, Yingmai Y, Yan H, Fei H, Xia L, Honglin H, Chaiyan L, Stöcker W, Liying C, Hongzhi G. Chorea and parkinsonism associated with autoantibodies to IgLON5 and responsive to immunotherapy. J Neuroimmunol. 2016; 300:9-10.

Hammer C, Stepniak B, Schneider A, Papiol S, Tantra M, Begemann M, Sirén AL, Pardo LA, Sperling S, Mohd Jofrry S, Gurvich A, Jensen N, Ostmeier K, Lühder F, Probst C, Martens H, Gillis M, Saher G, Assogna F, Spalletta G, Stöcker W, Schulz TF, Nave KA, Ehrenreich H. Neuropsychiatric disease relevance of circulating anti-NMDA receptor autoantibodies depends on blood-brain barrier integrity. Mol Psychiatry. 2014; 19(10):1143-9.

Hang LM, Nakamura RM. Current concepts and advances in clinical laboratory testing for autoimmune diseases. Crit Rev Clin Lab Sci. 1997; 34: 275–311.

Hanisch UK, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci. 2007; 10:1387–1394.

Hauser SL, Waubant E, Arnold DL, Vollmer T, Antel J, Fox RJ, Bar-Or A, Panzara M, Sarkar N, Agarwal S, Langer-Gould A, Smith CH. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. N Engl J Med. 2008; 358:676 – 688.

Hoch W, McConville J, Helms S, Newsom-Davis J, Melms A, Vincent A. Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. Nat Med. 2001; 7(3):365-8.

Hoftberger R, Titulaer MJ, Sabater L, Dome B, Rozsas A, Hegedus B, Hoda MA, Laszlo V, Ankersmit HJ, Harms L, Boyero S, dF A, Saiz A, Dalmau J, Graus F. Encephalitis and GABAB receptor antibodies: novel findings in a new case series of 20 patients. Neurology 2013; 81: 1500 –1506 .

Hoftberger R, van Sonderen A, Leypoldt F, Houghton D, Geschwind M, Gelfand J, Paredes M, Sabater L, Saiz A, Titulaer MJ, Graus F, Dalmau J. Encephalitis and AMPA receptor antibodies: novel findings in a case series of 22 patients. Neurology 2015; 84: 2403–2412
.
Honnorat J, Trouillas P, Thivolet C, Aguera M, Belin MF. Autoantibodies to glutamate decarboxylase in a patient with cerebellar cortical atrophy, peripheral neuropathy and slow eye movements. Arch Neurol 1995; 52:462–468.

Honorat JA, Komorowski L, Josephs KA, Fechner K, St Louis EK, Hinson SR, Lederer S, Kumar N, Gadoth A, Lennon VA, Pittock SJ, McKeon A. IgLON5 antibody: Neurological accompaniments and outcomes in 20 patients. Neurol Neuroimmunol Neuroinflamm. 2017; 4(5):e385.

Hsieh KC, Gvilia I, Kumar S, Uschakov A, McGinty D, Alam MN, Szymusiak R. c-Fos expression in neurons projecting from the preoptic and lateral hypothalamic areas to the ventrolateral periaqueductal gray in relation to sleep states. Neuroscience. 2011; 188:55-67.

Hughes EG, Peng X, Gleichman AJ, Lai M, Zhou L, Tsou R, Parsons TD, Lynch DR, Dalmau J, Balice-Gordon RJ. Cellular and Synaptic Mechanisms of Anti-NMDA Receptor Encephalitis. J Neurosci. 2010; 30(17):5866-75.

Irani SR, Alexander S, Waters P, Kleopa KA, Pettingill P, Zuliani L, Peles E, Buckley C, Lang B, Vincent A. Antibodies to Kv1 potassium channel-complex proteins leucine-rich, glioma inactivated 1 protein and contactin-associated protein-2 in limbic encephalitis, Morvan's syndrome and acquired neuromyotonia. Brain 2010; 133(9):2734-48.

Irani SR, Bera K, Waters P, Zuliani L, Maxwell S, Zandi MS, Friese MA, Galea I, Kullmann DM, Beeson D, Lang B, Bien CG, Vincent A. N-methyl-D-aspartate antibody encephalitis: temporal progression of clinical and paraclinical observations in a predominantly non-paraneoplastic disorder of both sexes. Brain 2010; 133(Pt 6):1655-67.

Irani SR, Michell AW, Lang B, Pettingill P, Waters P, Johnson MR, Schott JM, Armstrong RJ, S Zagami A, Bleasel A, Somerville ER, Smith SM, Vincent A. Faciobrachial dystonic seizures precede Lgi1 antibody limbic encephalitis. Ann Neurol. 2011; 69(5):892-900.

Irani SR, Pettingill P, Kleopa KA, Schiza N, Waters P, Mazia C, Zuliani L, Watanabe O, Lang B, Buckley C, Vincent A. Morvan syndrome: clinical and serological observations in 29 cases. Ann Neurol. 2012; 72(2):241-55.

Irani SR, Stagg CJ, Schott JM, Rosenthal CR, Schneider SA, Pettingill P, Pettingill R, Waters P, Thomas A, Voets NL, Cardoso MJ, Cash DM, Manning EN, Lang B, Smith SJ, Vincent A, Johnson MR. Faciobrachial dystonic seizures: the influence of immuno- therapy on seizure control and prevention of cognitive impairment in a broadening phenotype. Brain 2013; 136: 3151–3162.

Iranzo A, Graus F, Clover L, Morera J, Bruna J, Vilar C, Martínez-Rodriguez JE, Vincent A, Santamaría J. Rapid eye movement sleep behavior disorder and potassium channel antibody-associated limbic encephalitis. Ann Neurol. 2006; 59(1):178-81.

Jain A, Lancaster E, Dalmau J, Balice-Gordon RJ. Autoantibodies in the CSF of anti- GABA receptor encephalitis patients block activation of GABA receptors in vitro. Ann Neurol. 2015; 78: S77.

Janeway CA Jr, Travers P, Walport M, et al. “The structure of a typical antibody molecule“. Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science; 2001.

Jeffery OJ, Lennon VA, Pittock SJ, Gregory JK, Britton JW, McKeon A. GABAB recep- tor autoantibody frequency in service serologic evaluation. Neurology 2013; 81: 882–887.

Jézéquel J, Johansson EM, Dupuis JP, Rogemond V, Gréa H, Kellermayer B, Hamdani N, Le Guen E, Rabu C, Lepleux M, Spatola M, Mathias E, Bouchet D, Ramsey AJ, Yolken RH,

Tamouza R, Dalmau J, Honnorat J, Leboyer M, Groc L. Dynamic disorganization of synaptic NMDA receptors triggered by autoantibodies from psychotic patients. Nat Commun. 2017; 8(1):1791.

Joubert B, Saint-Martin M, Noraz N, Picard G, Rogemond V, Ducray F, Desestret V, Psimaras D, Delattre JY, Antoine JC, Honnorat J. Characterisation of a subtype of autoimmune encephalitis with anti-contactin-associated protein-like 2 antibodies in the cerebrospinal fluid, prominent limbic symptoms and seizures. JAMA Neurol. 2016; 73 (9) :1115-24.

Kaech S, Banker G. Culturing hippocampal neurons. Nat Protoc. 2006; 1(5):2406-15.

Kalachikov S, Evgrafov O, Ross B, Winawer M, Barker-Cummings C, Martinelli Boneschi F, Choi C, Morozov P, Das K, Teplitskaya E, Yu A, Cayanis E, Penchaszadeh G, Kottmann AH, Pedley TA, Hauser WA, Ottman R, Gilliam TC. Mutations in LGI1 cause autosomal-dominant partial epilepsy with auditory features. Nat Genet. 2002; 30(3):335-41.

Kalueff A V., Tuohimaa P. Grooming analysis algorithm for neurobehavioural stress research. Brain Res. Protoc. 2004; 13: 151–158.

Kannoth S, Anandakkuttan A, Mathai A, Sasikumar AN, Nambiar V. Autoimmune atypical parkinsonism - A group of treatable parkinsonism. J Neurol Sci. 2016; 362:40-6.

Kim N, Stiegler AL, Cameron TO, Hallock PT, Gomez AM, Huang JH, Hubbard SR, Dustin ML, Burden SJ. Lrp4 is a receptor for Agrin and forms a complex with MuSK. Cell. 2008; 135(2) : 334-42.

Kim TJ, Lee ST, Moon J, Sunwoo JS, Byun JI, Lim JA, Shin YW, Jun JS, Lee HS, Lee WJ, Yang AR, Choi Y, Park KI, Jung KH, Jung KY, Kim M, Kun Lee S, Chu K. Anti-LGI1 Encephalitis is Associated with Unique HLA Subtypes. Ann Neurol. 2017; 81(2):183-192.

Klang A, Schmidt P, Kneissl S, Bagó Z, Vincent A, Lang B, Moloney T, Bien CG, Halász P, Bauer J, Pákozdy A. IgG and complement deposition and neuronal loss in cats and humans with epilepsy and voltage-gated potassium channel complex antibodies. J Neuropathol Exp Neurol. 2014; 73(5):403-13.

Klooster R, Plomp JJ, Huijbers MG, Niks EH, Straasheijm KR, Detmers FJ, Hermans PW, Sleijpen K, Verrips A, Losen M, Martinez-Martinez P, De Baets MH, van der Maarel SM, Verschuuren JJ. Muscle-specific kinase myasthenia gravis IgG4 autoantibodies cause severe neuromuscular junction dysfunction in mice. Brain 2012; 135(Pt 4):1081-101.

Knudsen S, Mikkelsen JD, Jennum P. Antibodies in narcolepsy-cataplexy patient serum bind to rat hypocretin neurons. Neuroreport 2007; 18 :77 – 79

Kordas G, Lagoumintzis G, Sideris S, Poulas K, Tzartos SJ. Direct proof of the in vivo pathogenic role of the AChR autoantibodies from myasthenia gravis patients. PLoS One. 2014;9(9):e108327. Erratum in: PLoS One 2015; 10(1):e0117673.

Körtvelyessy P, Bauer J, Stoppel CM, Brück W, Gerth I, Vielhaber S, Wiedemann FR, Heinze HJ, Bartels C, Bien CG. Complement-associated neuronal loss in a patient with CASPR2 antibody-associated encephalitis. Neurol Neuroimmunol Neuroinflamm. 2015; 2(2):e75.

Kowal C, DeGiorgio LA, Nakaoka T, Hetherington H, Huerta PT, Diamond B, Volpe BT. Cognition and immunity; antibody impairs memory. Immunity. 2004; 21(2):179-88.

Küçükali Cİ, Haytural H, Benbir G, Coban A, Ulusoy C, Giriş M, Kürtüncü M, Shugaiv E, Karadeniz D, Tüzün E. Reduced serum orexin-A levels in autoimmune encephalitis and neuromyelitis optica patients. J Neurol Sci. 2014 Nov 15;346(1-2):353-5.

Kurtis MM, Toledano R, Garcia-Morales I, Gil-Nagel A. Immunomodulated parkinsonism as a presenting symptom of LGI1 antibody encephalitis. Parkinsonism Relat Disord 2015; 21: 1286–7.

Lai M, Hughes EG, Peng X, Zhou L, Gleichman AJ, Shu H, Mata S, Kremens D, Vitaliani R, Geschwind MD, Bataller L, Kalb RG, Davis R, Graus F, Lynch DR, Balice-Gordon R, Dalmau J. AMPA receptor antibodies in limbic encephalitis alter synaptic receptor location. Ann Neurol. 2009; 65: 424–434.

Lai M, Huijbers MG, Lancaster E, Graus F, Bataller L, Balice-Gordon R, Cowell JK, Dalmau J. Investigation of LGI1 as the antigen in limbic encephalitis previously attrib- uted to potassium channels: a case series. Lancet Neurol. 2010; 9: 776 –785.

Lalonde R. The neurobiological basis of spontaneous alternation. Neurosci. Biobehav. Rev. 2002; 26: 91–104.

Lancaster E, Lai M, Peng X, Hughes E, Constantinescu R, Raizer J, Friedman D, Skeen MB, Grisold W, Kimura A, Ohta K, Iizuka T, Guzman M, Graus F, Moss SJ, Balice- Gordon R, Dalmau J. Antibodies to the GABA(B) receptor in limbic encephalitis with seizures: case series and characterisation of the antigen. Lancet Neurol. 2010; 9: 67–76.

Lang K, Prüss H. Frequencies of neuronal autoantibodies in healthy controls: Estimation of disease specificity. Neurol Neuroimmunol Neuroinflamm. 2017; 4(5):e386.

Latorre D, Kallweit U, Armentani E, Foglierini M, Mele F, Cassotta A, Jovic S, Jarrossay D, Mathis J, Zellini F, Becher B, Lanzavecchia A, Khatami R, Manconi M,Tafti M, Bassetti CL, Sallusto F. T cells in patients with narcolepsy target self-antigens of hypocretin neurons. Nature. 2018; 562(7725):63-68.

Legendre P. The glycinergic inhibitory synapse. Cell Mol Life Sci. 2001; 58(5-6):760-93. Review.

Leite MI, Jacob S, Viegas S, Cossins J, Clover L, Morgan BP, Beeson D, Willcox N, Vincent A. IgG1 antibodies to acetylcholine receptors in 'seronegative' myasthenia gravis. Brain 2008; 131(Pt 7):1940-52.

Levin EC, Acharya NK, Han M, Zavareh SB, Sedeyn JC, Venkataraman V, Nagele RG. Brain-reactive autoantibodies are nearly ubiquitous in human sera and may be linked to pathology in the context of blood-brain barrier breakdown. Brain Res. 2010; 1345:221-32.

Lian H, Litvinchuk XA, Chiang AC, Aithmitti N, Jankowsky JL. Astrocyte-Microglia Cross Talk through Complement Activation Modulates Amyloid Pathology in Mouse Models of

Alzheimer's Disease. 2016; 36: 577–589.

Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, Bennett ML, Münch AE, Chung WS, Peterson TC, Wilton DK, Frouin A, Napier BA, Panicker N, Kumar M, Buckwalter MS, Rowitch DH, Dawson VL, Dawson TM, Stevens B, Barres BA. Neurotoxic reactive astrocytes are induced by activated microglia. Nature 2017; 541(7638):481-487.

López-Chiriboga AS, Klein C, Zekeridou A, McKeon A, Dubey D, Flanagan EP, Lennon VA, Tillema JM, Wirrell EC, Patterson MC, Gadoth A, Aaen JG, Brenton JN, Bui JD, Moen A, Otten C, Piquet A, Pittock SJ. LGI1 and CASPR2 neurological autoimmunity in children. Ann Neurol. 2018; 84(3):473-480.

Ludwig RJ, Vanhoorelbeke K, Leypoldt F, Kaya Z, Bieber K, McLachlan SM, Komorowski L, Luo J, Cabral-Marques O, Hammers CM, Lindstrom JM, Lamprecht P, Fischer A, Riemekasten G, Tersteeg C, Sondermann P, Rapoport B, Wandinger KP, Probst C, El Beidaq A, Schmidt E, Verkman A, Manz RA, Nimmerjahn F. Mechanisms of Autoantibody-Induced Pathology. Front Immunol. 2017; 8:603. Review.

Luo J, Lindstrom J. Antigen-specific immunotherapeutic vaccine for experimental autoimmune myasthenia gravis. J Immunol 2014; 193:5044–55.

Luo J, Taylor P, Losen M, de Baets MH, Shelton GD, Lindstrom J. Main immunogenic region structure promotes binding of conformation-dependent myasthenia gravis autoantibodies, nicotinic acetylcholine receptor conformation maturation, and agonist sensitivity. J Neurosci. 2009; 29:13898–908.

Macdonald RL, Olsen RW. GABAA receptor channels. Annu Rev Neurosci. 1994; 17:569-602.

Mackay G, Ahmad K, Stone J, Sudlow C, Summers D, Knight R, Will R, Irani SR, Vincent A, Maddison P. NMDA receptor autoantibodies in sporadic Creutzfeldt-Jakob disease. J Neurol. 2012; 259(9):1979-81.

Maddison P. Neuromyotonia. Clin Neurophysiol. 2006; 117(10):2118-27. Review.

Majoie HJ, de Baets M, Renier W, Lang B, Vincent A. Antibodies to voltage-gated potassium and calcium channels in epilepsy. Epilepsy Res 2006; 71:135–41.

Malter MP, Frisch C, Zeitler H, Surges R, Urbach H, Helmstaedter C, Elger CE, Bien CG. Treatment of immune-mediated temporal lobe epilepsy with GAD antibodies. Seizure 2015; 30:57-63.

Manto M, Dalmau J, Didelot A, Rogemond V, Honnorat J. In vivo effects of antibodies from patients with anti-NMDA receptor encephalitis: further evidence of synaptic glutamatergic dysfunction. Orphanet J Rare Dis. 2010; 5:31.

Marín-Teva JL, Dusart I, Colin C, Gervais A, van Rooijen N, Mallat M. Microglia promote the death of developing Purkinje cells. Neuron. 2004; 41(4):535-47.

Martínez-Rodríguez JE, Sabater L, Graus F, Iranzo A, Santamaria J. Evaluation of hypothalamic-specific autoimmunity in patients with narcolepsy. Sleep 2007;30(1):27-8.

McKeon A, Lennon VA, Pittock SJ. Immunotherapy-responsive dementias and encephalopathies. Continuum Lifelong Learning Neurol. 2010; 16(2):80-101.

McKeon A, Marnane M, O'Connell M, Stack JP, Kelly PJ, Lynch T. Po- tassium channel antibody associated encephalopathy presenting with a fronto- temporal dementia like syndrome. Arch Neurol. 2007; 64(10):1528-1530.

McKnight K, Jiang Y, Hart Y, Cavey A, Wroe S, Blank M, Shoenfeld Y, Vincent A, Palace J, Lang B. Serum antibodies in epilepsy and seizure-associated disorders. Neurology 2005; 65(11):1730-6.

Meeker HC, Chadman KK, Heaney AT, Carp RI. Assessment of social interaction and anxiety like behavior in senescence-accelerated-prone and -resistant mice. Physiol. Behav. 2013; 118: 97–102.

Mignot E, Hayduk R, Black J, Grumet FC, Guilleminault C. HLA DQB1*0602 is associated with cataplexy in 509 narcoleptic patients. Sleep 1997; 20:1012-20.

Mignot E, Lammers GJ, Ripley B, Okun M, Nevsimalova S, Overeem S, Vankova J, Black J, Harsh J, Bassetti C, Schrader H, Nishino S. The role of cerebrospinal fluid hypocretin measurement in the diagnosis of narcolepsy and other hypersomnias. Arch Neurol. 2002; 59(10):1553-62.

Mikasova L, De Rossi P, Bouchet D, Georges F, Rogemond V, Didelot A, Meissirel C, Honnorat J, Groc L. Disrupted surface cross-talk between NMDA and Ephrin-B2 receptors in anti-NMDA encephalitis. Brain 2012; 135(Pt 5):1606-21.

Mitoma H, Song S-Y, Ishida K, Yamakuni T, Kobayashi T, Mizusawa H. Presynaptic impairment of cerebellar inhibitory synapses by an autoantibody to glutamate decarboxylase. J Neurol Sci. 2000; 175:40–44.

Mohammad SS, Sinclair K, Pillai S, Merheb V, Aumann TD, Gill D, Dale RC, Brilot F. Herpes simplex encephalitis relapse with chorea is associated with autoantibodies to N-methyl-D-aspartate receptor or dopamine-2 receptor. Mov Disord. 2014; 29: 117–122.

Montagna M, Amir R, De Volder I, Lammens M, Huyskens J, Willekens B. IgLON5-associated encephalitis with atypical brain magnetic resonance Imaging and cerebrospinal fluid changes. Front Neurol. 2018; 9:329.

Montagna P, Lugaresi E. Agrypnia Excitata: a generalized overactivity syndrome and a useful concept in the neurophysiopathology of sleep. Clin Neurophysiol. 2002; 113: 552–560.

Nacu A, Andersen JB, Lisnic V, Owe JF, Gilhus NE. Complicating autoimmune diseases in myasthenia gravis: a review. Autoimmunity 2015; 48(6):362-8. Review.

Nagele RG, Clifford PM, Siu G, Levin EC, Acharya NK, Han M, Kosciuk MC, Venkataraman V, Zavareh S, Zarrabi S, Kinsler K, Thaker NG, Nagele EP, Dash J, Wang HY, Levitas A.

Brain-reactive autoantibodies prevalent in human sera increase intraneuronal amyloid-β(1-42) deposition. J Alzheimers Dis. 2011; 25(4):605-22.

Newcomer JW, Farber NB, Olney JW. NMDA receptor function, memory, and brain aging. Dialogues Clin Neurosci. 2000; 2(3):219-32.

Newsom-Davis J, Mills KR. Immunological associations of acquired neuromyotonia (Isaacs' syndrome). Report of five cases and literature review. Brain 1993;116 (Pt 2):453-69.

Niehusmann P, Dalmau J, Rudlowski C, Vincent A, Elger CE, Rossi JE, Bien CG. Diagnostic value of N-methyl-D-aspartate receptor antibodies in women with new-onset epilepsy. Arch Neurol. 2009; 66(4):458-64.

Nishino S, Ripley B, Overeem S, Lammers GJ, Mignot E. Hypocretin (orexin) deficiency in human narcolepsy. Lancet 2000; 355: 39–40.

Nishino S, Ripley B, Overeem S, Nevsimalova S, Lammers GJ, Vankova J, Okun M, Rogers W, Brooks S, Mignot E. Low cerebrospinal fluid hypocretin (Orexin) and altered energy homeostasis in human narcolepsy. Ann Neurol. 2001; 50(3):381-8.

Ohkawa T, Fukata Y, Yamasaki M, Miyazaki T, Yokoi N, Takashima H, Watanabe M, Watanabe O, Fukata M. Autoantibodies to epilepsy-related LGI1 in limbic encephalitis neutralize LGI1-ADAM22 interaction and reduce synaptic AMPA receptors. J Neurosci. 2013; 33(46):18161-74.

Ohkawa T, Satake S, Yokoi N, Miyazaki Y, Ohshita T, Sobue G, Takashima H, Watanabe O, Fukata Y, Fukata M. Identification and characterization of GABA(A) receptor autoantibodies in autoimmune encephalitis. J Neurosci. 2014; 34(24):8151-63.

Olsen AL, Lai Y, Dalmau J, Scherer SS, Lancaster E. Caspr2 autoantibodies target multiple epitopes. Neurol Neuroimmunol Neuroinflamm. 2015; 2(4):e127.

Overeem S, Verschuuren JJ, Fronczek R, Schreurs L, den Hertog H, Hegeman-Kleinn IM, van Duinen SG, Unmehopa UA, Swaab DF, Lammers GJ. Immunohistochemical screening for autoantibodies against lateral hypothalamic neurons in human narcolepsy. J Neuroimmunol. 2006; 174(1-2):187-91.

Owuor K, Harel NY, Englot DJ, Hisama F, Blumenfeld H, Strittmatter SM. LGI1-associated epilepsy through altered ADAM23-dependent neuronal morphology. Mol Cell Neurosci. 2009; 42(4):448-57.

Palmer CL, Cotton L, Henley JM. The molecular pharmacology and cell biology of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. Pharmacol Rev. 2005; 57: 253–277.

Panda S, Ding JL. Natural antibodies bridge innate and adaptive immunity. J Immunol. 2015; 194: 13–20.

Pascual O, Ben Achour S, Rostaing P, Triller A, Bessis A. Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission. Proc Natl Acad Sci U S A. 2012; 109(4):E197-205.

Patrick J, Lindstrom J. Autoimmune response to acetylcholine receptor. Science 1973; 180:871–2.

Patterson KR, Dalmau J, Lancaster E. Mechanisms of Caspr2 antibodies in autoimmune encephalitis and neuromyotonia. Ann Neurol. 2018; 83(1):40-51.

Paxinos G and Watson C. The Rat Brain in Stereotaxic Coordinates, 7th Edition, Elsevier

Peñagarikano O, Abrahams BS, Herman EI, Winden KD, Gdalyahu A, Dong H, Sonnenblick LI, Gruver R, Almajano J, Bragin A, Golshani P, Trachtenberg JT, Peles E, Geschwind DH. Absence of CNTNAP2 leads to epilepsy, neuronal migration abnormalities, and core autism-related deficits. Cell. 2011; 147(1):235-46.

Peng X, Hughes EG, Moscato EH, Parsons TD, Dalmau J, Balice-Gordon RJ. Cellular plasticity induced by anti-alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor encephalitis antibodies. Ann Neurol. 2015; 77: 381–398.

Petit-Pedrol M, Armangue T, Peng X, Bataller L, Cellucci T, Davis R, McCracken L, Martinez-Hernandez E, Mason WP, Kruer MC, Ritacco DG, Grisold W, Meaney BF, Alcala C, Sillevis-Smitt P, Titulaer MJ, Balice-Gordon R, Graus F, Dalmau J. Encepha- litis with refractory seizures, status epilepticus, and antibodies to the GABAA receptor: a case series, characterisation of the antigen, and analysis of the effects of anti- bodies. Lancet Neurol. 2014; 13: 276 –286.

Petit-Pedrol M, Sell J, Planagumà J, Mannara F, Radosevic M, Haselmann H, Ceanga M, Sabater L, Spatola M, Soto D, Gasull X, Dalmau J, Geis C. LGI1 antibodies alter Kv1.1 and AMPA receptors changing synaptic excitability, plasticity and memory. Brain. 2018; 141(11):3144-3159.

Pettingill P, Kramer HB, Coebergh JA, Pettingill R, Maxwell S, Nibber A, Malaspina A, Jacob A, Irani SR, Buckley C, Beeson D, Lang B, Waters P, Vincent A. Antibodies to GABAA receptor α1 and γ2 subunits: clinical and serologic characterization. Neurology 2015; 84(12):1233-41.

Peyron C, Tighe DK, van den Pol AN, et al. Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci. 1998; 18:9996–10015.

Pinatel D, Hivert B, Boucraut J, Saint-Martin M, Rogemond V, Zoupi L, Karagogeos D, Honnorat J, Faivre-Sarrailh C. Inhibitory axons are targeted in hippocampal cell culture by anti-Caspr2 autoantibodies associated with limbic encephalitis. Front Cell Neurosci. 2015; 9:265

Pinching AJ, Peters DK, Newsom David J. Remission of myasthenia gravis following plasma-exchange. Lancet. 1976; 2(8000):1373-6.

Pittock SJ, Yoshikawa H, Ahlskog JE, Tisch SH, Benarroch EE, Kryzer TJ, Lennon VA. Glutamic acid decarboxylase autoimmunity with brainstem, extrapyramidal, and spinal cord dysfunction. Mayo Clin Proc. 2006; 81(9):1207-14.

Planagumà J, Leypoldt F, Mannara F, Gutiérrez-Cuesta J, Martín-García E, Aguilar E, Titulaer MJ, Petit-Pedrol M, Jain A, Balice-Gordon R, Lakadamyali M, Graus F, Maldonado R, Dalmau J. Human N-methyl D-aspartate receptor antibodies alter memory and behaviour in mice. Brain 2015; 138(Pt 1):94-109.

Pollak TA, Beck K, Irani SR, Howes OD, David AS, McGuire PK. Autoantibodies to central nervous system neuronal surface antigens: psychiatric symptoms and psychopharmacological implications. Psychopharmacology (Berl). 2016; 233(9):1605-21.

Provini F, Marconi S, Amadori M, Guaraldi P, Pierangeli G, Cortelli P, Lugaresi E, Montagna P, Tinuper P. Morvan chorea and agrypnia excitata: when video-polysom- nographic recording guides the diagnosis. Sleep Med. 2011; 12: 1041–1043.

Prüss H, Finke C, Höltje M, Hofmann J, Klingbeil C, Probst C, Borowski K, Ahnert-Hilger G, Harms L, Schwab JM, Ploner CJ, Komorowski L, Stoecker W, Dalmau J, Wandinger KP. N-methyl-D-aspartate receptor antibodies in herpes simplex encephalitis. Ann Neurol. 2012; 72(6):902-11.

Quek AM, Britton JW, McKeon A, So E, Lennon VA, Shin C, Klein C, Watson RE Jr, Kotsenas AL, Lagerlund TD, Cascino GD, Worrell GA, Wirrell EC, Nickels KC, Aksamit AJ, Noe KH, Pittock SJ. Autoimmune epilepsy: clinical characteristics and response to immunotherapy. Arch Neurol. 2012; 69(5):582-93.

Rendall AR, Truong DT, Fitch RH. Learning Delays in a mouse model of Autism Spectrum Disorder. Behavioural brain research. 2016; 303:201-207.

Romi F. Thymoma in myasthenia gravis: from diagnosis to treatment. Autoimmune Dis. 2011; 474-512.

Rose NR, Bona C. Defining criteria for autoimmune diseases (Witebsky's postulates revisited). Immunol Today. 1993; 14(9):426-30. Review.

Rossi M, Mead S, Collinge J, Rudge P, Vincent A. Neuronal antibodies in patients with suspected or confirmed sporadic Creutzfeldt-Jakob disease. J Neurol Neurosurg Psychiatry. 2015; 86(6):692-4.

Rubovitch V, Edut S, Sarfstein R, Werner H, Pick CG. The intricate involvement of the Insulin-like growth factor receptor signaling in mild traumatic brain injury in mice. Neurobiol Dis. 2010; 38(2):299-303.

Sabater L, Gaig C, Gelpi E, Bataller L, Lewerenz J, Torres-Vega E, Contreras A, Giometto B, Compta Y, Embid C, Vilaseca I, Iranzo A, Santamaría J, Dalmau J, Graus F. A novel non-rapid-eye movement and rapid-eye-movement parasomnia with sleep breathing disorder associated with antibodies to IgLON5: a case series, characterisation of the antigen, and post-mortem study. Lancet Neurol. 2014; 13(6):575-86.

Sabater L, Planagumà J, Dalmau J, Graus F. Cellular investigations with human antibodies associated with the anti-IgLON5 syndrome. J Neuroinflammation. 2016; 13(1):226.

Saiz A, Arpa J, Sagasta A, Casamitjana R, Zarranz JJ, Tolosa E, Graus F. Autoantibodies to glutamic acid decarboxylase in three patients with cerebellar ataxia, late-onset insulin-dependent diabetes mellitus, and polyendocrine autoimmunity. Neurology 1997; 49:1026–1030.

Saiz A, Blanco Y, Sabater L, Gonzalez F, Bataller L, Casamitjana R, Ramio-Torrenta L, Graus F. Spectrum of neurological syndromes associated with glutamic acid decarboxylase antibodies: diagnostic clues for this association. Brain 2008; 131: 2553–2563.

Salinas GF, Braza F, Brouard S, Tak PP, Baeten D. The role of B lymphocytes in the progression from autoimmunity to autoimmune disease. Clin Immunol 2013; 146: 34– 45.

Scammell TE. The neurobiology, diagnosis, and treatment of narcolepsy. Ann Neurol 2003; 53: 154–66.

Schabitz WR, Rogalewski A, Hagemeister C, Bien CG. VZV brainstem encephalitis triggers NMDA receptor immunoreaction. Neurology 2014; 83: 2309 –2311.

Schulte U, Thumfart JO, Klöcker N, Sailer CA, Bildl W, Biniossek M, Dehn D, Deller T, Eble S, Abbass K, Wangler T, Knaus HG, Fakler B. The epilepsy-linked Lgi1 protein assembles into presynaptic Kv1 channels and inhibits inactivation by Kvbeta1. Neuron 2006; 49(5):697-706.

Scott VE, Muniz ZM, Sewing S, Lichtinghagen R, Parcej DN, Pongs O, Dolly JO. Antibodies specific for distinct Kv subunits unveil a heterooligomeric basis for subtypes of .alpha.-dendrotoxin- sensitive potassium channels in bovine brain. Biochemistry 2002; 33(7):1617-23.

Shepherd JD, Huganir RL. The cell biology of synaptic plasticity: AMPA receptor trafficking. Annu Rev Cell Dev Biol. 2007; 23: 613– 643.

Shillito P, Molenaar PC, Vincent A, Leys K, Zheng W, van den Berg RJ, Plomp JJ, van Kempen GT, Chauplannaz G, Wintzen AR, et al. Acquired neuromyotonia: evidence for autoantibodies directed against K+ channels of peripheral nerves. Ann Neurol. 1995; 38(5):714-22.

Simabukuro MM, Sabater L, Adoni T, Cury RG, Haddad MS, Moreira CH, Oliveira L, Boaventura M, Alves RC, Azevedo Soster L, Nitrini R, Gaig C, Santamaria J, Dalmau J, Graus F. Sleep disorder, chorea, and dementia associated with IgLON5 antibodies. Neurol Neuroimmunol Neuroinflamm. 2015; 2(4):e136.

Simpson, JA. Myasthenia gravis: a new hypothesis. Scott Med J. 1960; 5: 419-439.

Sinha S, Newsom-Davis J, Mills K, Byrne N, Lang B, Vincent A. Autoimmune aetiology for acquired neuromyotonia (Isaacs' syndrome). Lancet. 1991; 338(8759):75-7.

Soltys J, Kusner LL, Young A, Richmonds C, Hatala D, Gong B, Shanmugavel V, Kaminski HJ. Novel complement inhibitor limits severity of experimentally myasthenia gravis. Ann Neurol. 2009; 65(1): 67-75.

Spatola M, Petit-Pedrol M, Simabukuro MM, Armangué T, Castro FJ, Barcelo-Ar- tigues MI, Juli`a Benique MR, Benson L, Gorman M, Felipe A, Caparó-Oblitas R, Rosen- feld MR, Graus F, Dalmau J. Investigations in GABAa receptor antibody-associated encephalitis. Neurology 2017; 88: 1–9.

Sprengel R. Role of AMPA receptors in synaptic plasticity. Cell Tissue Res. 2006; 326: 447–455.

Stamelou M, Plazzi G, Lugaresi E, Edwards MJ, Bhatia KP. The distinct movement disorder in anti-NMDA receptor encephalitis may be related to status dissociatus: a hypothesis. Mov Disord. 2012; 27: 1360–3.

Streit WJ. Microglia as neuroprotective, immunocompetent cells of the CNS. Glia. 2002; 40:133–139.

Suleiman J, Brenner T, Gill D, Brilot F, Antony J, Vincent A, Lang B, Dale RC. VGKC antibodies in pediatric encephalitis presenting with status epilepticus. Neurology 2011; 76(14):1252-5.

Suleiman J, Brilot F, Lang B, Vincent A, Dale RC. Autoimmune epilepsy in children: case series and proposed guidelines for identification. Epilepsia 2013; 54(6):1036-45.

Suleiman J, Wright S, Gill D, Brilot F, Waters P, Peacock K, Procopis P, Nibber A, Vincent A, Dale RC, Lang B. Autoantibodies to neuronal antigens in children with new-onset seizures classified according to the revised ILAE organization of seizures and epilepsies. Epilepsia. 2013; 54(12):2091-100.

Sundal C, Vedeler C, Miletic H, Andersen O. Morvan syndrome with Caspr2 antibodies. Clinical and autopsy report. J Neurol Sci. 2017; 372:453-455.

Tanaka S, Honda Y, Inoue Y, Honda M. Detection of autoantibodies against hypocretin, hcrtrl, and hcrtr2 in narcolepsy: anti-Hcrt system antibody in narcolepsy. Sleep 2006; 29(5):633-8.

Tapiola T, Alafuzoff I, Herukka SK, Parkkinen L, Hartikainen P, Soininen H, Pirttilä T. Cerebrospinal fluid {beta}-amyloid 42 and tau proteins as biomarkers of Alzheimer-type pathologic changes in the brain. Arch Neurol. 2009; 66(3):382-9.

Thannickal TC, Moore RY, Nienhuis R, Ramanathan L, Gulyani S, Aldrich M, Cornford M, Siegel JM. Reduced number of hypocretin neurons in human narcolepsy. Neuron 2000; 27(3):469-74.

Thieben MJ, Lennon VA, Boeve BF, Aksamit AJ, Keegan M, Vernino S. Potentially reversible autoimmune limbic encephalitis with neuronal potassium channel antibody. Neurology 2004; 62(7):1177-82.

Thompson J, Bi M, Murchison AG, Makuch M, Bien CG, Chu K, Farooque P, Gelfand JM, Geschwind MD, Hirsch LJ, Somerville E, Lang B, Vincent A, Leite MI, Waters P, Irani SR; Faciobrachial Dystonic Seizures Study Group. The importance of early immunotherapy in patients with faciobrachial dystonic seizures. Brain 2018; 141(2):348-356.

Thebault S, Waters P, Snape MD, Cottrell D, Darin N, Hallböök T, Huutoniemi A, Partinen M, Pollard AJ, Vincent A. Neuronal Antibodies in Children with or without Narcolepsy following H1N1-AS03 Vaccination. PLoS One 2015; 10:e0129555.

Thomas AM, Schwartz MD, Saxe MD, Kilduff TS. Cntnap2 Knockout Rats and Mice Exhibit Epileptiform Activity and Abnormal Sleep-Wake Physiology. Sleep 2017; 40(1).

Titulaer MJ, McCracken L, Gabilondo I, Armangué T, Glaser C, Iizuka T, Honig LS, Benseler SM, Kawachi I, Martinez-Hernandez E, Aguilar E, Gresa-Arribas N, Ryan-Florance N, Torrents A, Saiz A, Rosenfeld MR, Balice-Gordon R, Graus F, Dalmau J. Treatment and prognostic factors for long-term outcome in patients with anti-NMDA receptor encephalitis: an observational cohort study. Lancet Neurol. 2013; 12(2):157-65.

Tobin WO, Lennon VA, Komorowski L, Probst C, Clardy SL, Aksamit AJ, Appendino JP, Lucchinetti CF, Matsumoto JY, Pittock SJ, Sandroni P, Tippmann-Peikert M, Wirrell EC, McKeon A. DPPX potassium channel antibody: frequency, clinical accompaniments, and outcomes in 20 patients. Neurology 2014; 83(20):1797-803.

Toledano M, Britton JW, McKeon A, Shin C, Lennon VA, Quek AM, So E, Worrell GA, Cascino GD, Klein CJ, Lagerlund TD, Wirrell EC, Nickels KC, Pittock SJ. Utility of an immunotherapy trial in evaluating patients with presumed autoimmune epilepsy. Neurology 2014; 82(18):1578-86.

Toyka KV, Drachman DB, Griffin DE, Pestronk A, Winkelstein JA, Fishbeck KH, Kao I. Myasthenia gravis. Study of humoral immune mechanisms by passive transfer to mice. N Engl J Med. 1977; 296(3):125-31.

Tröscher AR, Klang A, French M, Quemada-Garrido L, Kneissl SM, Bien CG, Pákozdy Á, Bauer J. Selective Limbic Blood-Brain Barrier Breakdown in a Feline Model of Limbic Encephalitis with LGI1 Antibodies. Front Immunol. 2017; 8:1364.

Tsutsui K, Kanbayashi T, Tanaka K, Boku S, Ito W, Tokunaga J, Mori A, Hishikawa Y, Shimizu T, Nishino S. Anti-NMDA-receptor antibody detected in encephalitis, schizophrenia, and narcolepsy with psychotic features. BMC Psychiatry. 2012; 12:37.

Tuzun E, Erdag E, Durmus H, Brenner T, Turkoglu R, Kurtuncu M, Lang B, Akman- Demir G, Eraksoy M, Vincent A. Autoantibodies to neuronal surface antigens in thyroid antibody-positive and -negative limbic encephalitis. Neurol. India 2011; 59: 47–50.

Tuzun E, Zhou L, Baehring JM, Bannykh S, Rosenfeld MR, Dalmau J. Evidence for antibody-mediated pathogenesis in anti-NMDAR encephalitis associated with ovarian teratoma. Acta Neuropathol. 2009; 118: 737–743.

Tzartos S, Hochschwender S, Vasquez P, Lindstrom J. Passive transfer of experimental autoimmune myasthenia gravis by monoclonal antibodies to the main immunogenic region of the acetylcholine receptor. J Neuroimmunol. 1987; 15(2):185-94.

van Groen T, Miettinen P, Kadish I. The entorhinal cortex of the mouse: Organization of the projection to the hippocampal formation. Hippocampus 2003; 13: 133–149.

Vanli-Yavuz EN, Erdag E, Tuzun E, Ekizoglu E, Baysal-Kirac L, Ulusoy C, Peach S, Gundogdu G, Sencer S, Sencer A, Kucukali CI, Bebek N, Gurses C, Gokyigit A, Baykan B. Neuronal autoantibodies in mesial temporal lobe epilepsy with hippocampal sclerosis. J Neurol Neurosurg Psychiatry 2016; 87(7):684-92.

Van Sonderen ARD, Arino H, Petit-Pedrol M, Leypoldt F, Kortvelyessy P, Wandinger KP, Lancaster E, Wirtz PW, Schreurs MW, Sillevis Smitt PA, Graus F, Dalmau J, Titulaer MJ. The clinical spectrum of Caspr2 antibody-associated disease. Neurology 2016; 87: 521– 528.

Van Sonderen ARD, Thijs RD, Coenders EC, Jiskoot LC, Sanchez E, de Bruijn MA, van Coevorden-Hameete MH, Wirtz PW, Schreurs MW, Sillevis Smitt PA, Titulaer MJ. Anti-LGI1 encephalitis: clinical syndrome and long-term follow-up. Neurology 2016; 87: 1449 –1456.

Van Sonderen ARD, Stoop JA, Verdijk RM, Haasnoot GW, Thijs RD, Wirtz PW, Schreurs MWJ, Claas FHJ, Sillevis Smitt PAE, Titulaer MJ. Anti-LGI1 encephalitis is strongly associated with HLA-DR7, and HLA-DRB4. Ann Neurol. 2017; 81: 193–198.

van Sonderen A, Petit-Pedrol M, Dalmau J, Titulaer MJ. The value of LGI1, Caspr2 and voltage-gated potassium channel antibodies in encephalitis. Nat Rev Neurol. 2017;13(5):290-301.

Vianello M, Giometto B, Vassanelli S, Canato M, Betterle C, Mucignat C. Peculiar labeling of cultured hippocampal neurons by different sera harbouring anti-glutamic acid decarboxylase autoantibodies. Exp Neurol. 2006; 202: 514 – 518.

Vincent A, Bien CG, Irani SR, Waters P. Autoantibodies associated with diseases of the CNS: new developments and future challenges. Lancet Neurol 2011; 10: 759-72.

Vincent A, Bowen J, Newsom-Davis J, McConville J. Seronegative generalised myasthenia gravis: clinical features, antibodies, and their targets. Lancet Neurol. 2003; 2:99–106.

Vincent A, Buckley C, Schott JM, Baker I, Dewar BK, Detert N, Clover L, Parkinson A, Bien CG, Omer S, Lang B, Rossor MN, Palace J. Potassium channel antibody-associated encephalopathy: a potentially immunotherapy-responsive form of limbic encephalitis. Brain 2004; 127(3):701-12.

Vincent A, Irani SR. Caspr2 antibodies in patients with thymomas. J Thorac Oncol. 2010; 5(10 Suppl 4):S277-80.

Vitaliani R, Mason W, Ances B, Zwerdling T, Jiang Z, Dalmau J. Paraneoplastic encephalitis, psychiatric symptoms, and hypoventilation in ovarian teratoma. Ann Neurol. 2005; 58(4):594-604.

Wall PM, Messier C. Infralimbic kappa opioid and muscarinic M1 receptor interactions in the concurrent modulation of anxiety and memory. Psychopharmacology 2002; 160(3): 233–244.

Wandinger KP, Saschenbrecker S, Stoecker W, Dalmau J. Anti-NMDA-receptor encephalitis: a severe, multistage, treatable disorder presenting with psychosis. J Neuroimmunol. 2011; 231(1-2):86-91.

Waters PJ, McKeon A, Leite MI, Rajasekharan S, Lennon VA, Villalobos A, Palace J, Mandrekar JN, Vincent A, Bar-Or A, Pittock SJ. Serologic diagnosis of NMO: a multicenter comparison of aquaporin-4-IgG assays. Neurology 2012; 78(9):665-71.

Wietrzych M, Meziane H, Sutter A, Ghyselinck N, Chapman PF, Chambon P, Krezel W. Working memory deficits in retinoid X receptor gamma-deficient mice. Learn Mem. 2005; 12(3):318-26.

Wright S, Geerts AT, Jol-van der Zijde CM, Jacobson L, Lang B, Waters P, van Tol MJ, Stroink H, Neuteboom RF, Brouwer OF, Vincent A. Neuronal antibodies in pediatric epilepsy: Clinical features and long-term outcomes of a historical cohort not treated with immunotherapy. Epilepsia 2016; 57(5):823-31.

Wright S, Hashemi K, Stasiak L, Bartram J, Lang B, Vincent A, Upton AL. Epileptogenic effects of NMDAR antibodies in a passive transfer mouse model. Brain 2015; 138(Pt 11):3159-67.

Xu W, Fitzgerald S, Nixon RA, Levy E, Wilson DA. Early hyperactivity in lateral entorhinal cortex is associated with elevated levels of AβPP metabolites in the Tg2576 mouse model of Alzheimer's disease. Exp. Neurol. 2015; 264: 82–91.

Yoss RE, Daly DD. Criteria for the diagnosis of the narcoleptic syndrome. Proc Staff Meeting Mayo Clinic. 1957; 32:320-28.

Zandian A, Forsström B, Häggmark-Månberg A, Schwenk MJ, Uhlén M, Nilsson P, Ayoglu B Whole-Proteome Peptide Microarrays for Profiling Autoantibody Repertoires within Multiple Sclerosis and Narcolepsy Journal of Proteome Research 2017: 16 (3): 1300-1314.

Zhang B, Luo S, Wang Q, Suzuki T, Xiong WC, Mei L. LRP4 serves as a coreceptor of agrin. Neuron 2008; 60(2):285-97.

Zhao Z, Wang J, Zhao C, Bi W, Yue Z, Ma ZA. Genetic ablation of PLA2G6 in mice leads to cerebellar atrophy characterized by Purkinje cell loss and glial cell activation. PLoS One. 2011; 6(10):e26991.