

UNIVERSITÀ
DI PAVIA

Dipartimento di Biologia e Biotecnologie "L. Spallanzani"

Laurea Magistralis in Neurobiology

Fondazione Istituto Neurologico Nazionale Casimiro Mondino IRCCS

Novel Neuronal Antibodies Identification through Tissue-Based Assay in Autoimmune Encephalitis

Supervisor:

Dott. Matteo Gastaldi

Experimental thesis by

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Academic Year 2025/2026



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Identificazione di nuovi anticorpi neuronali nelle encefaliti autoimmuni mediante immunoistochimica

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Abstract

Autoimmune Encephalitis (AE) is a group of neurological disorders characterised by an abnormal activation of the immune system, leading to the production of antibodies directed against surface neuronal or intracellular neuronal antigens within the central nervous system (CNS). The main clinical features relate to cognition, including cognitive impairment, behavioural changes, and seizure; psychiatric symptoms such as psychosis, aggression, and euphoria, as well as movement disorders, are also common. The diagnosis of AE remains a challenging process, requiring the integration of clinical, neurological, radiological, and laboratory findings: positive or negative test results should always be interpreted in the context of the clinical phenotype, rather than in isolation.

The aim of this study was to characterise the previously uncharacterised immunohistochemical staining pattern observed in the patient cohort, with the ultimate goal of identifying novel neuronal surface antigens through cell-based assay (CBA) analysis.

The study included 54 patients (mean age 52 years), whose biological samples were sent to the Laboratory of Neuroimmunology of "Fondazione IRCCS Istituto Neurologico Nazionale Casimiro Mondino" with suspected AE, who had previously tested positive on immunohistochemistry (IHC) with an uncharacterised pattern and negative on commercial fixed-CBA.

Screening of the cohort by live CBA identified 4 positive samples against three distinct antigens. UNC-07 tested positive for SEZ6L2 (endpoint titre 1:2560, IgG1, confirmed in CSF) and was also positive for mGluR5 (endpoint titre 1:20480, IgG1, confirmed in CSF). UNC-02 and UNC-23 tested positive for SEZ6L2 at a low titre (1:80), not confirmed in CSF, with differing IgG subclasses (IgG1 and IgG2 respectively). UNC-33 tested positive for mGluR1 (endpoint titre 1:2560, IgG1 and IgG2, confirmed in CSF).

To further confirm the presence of neuronal surface antibodies (NS-Abs), the cohort was additionally screened on primary neuronal cultures. Of the 54 patients, 8 tested positive: UNC-07, UNC-14, and UNC-33 were confirmed in both serum and CSF,

while UNC-05, UNC-23, UNC-34, UNC-37, and UNC-50 were positive in only serum (CSF was unavailable for UNC-34, UNC-37 and UNC-50).

These L-CBA findings accounted for only a fraction of the reactivity detected by IHC and, more strikingly, by primary neuronal culture assays, indicating that most of the neuronal surface antibody reactivity in this cohort remains unexplained by the currently available antigen panel. These results point to the presence of still uncharacterised autoantibodies and support the need for further studies to expand the antigen panel, thereby allowing the detection of additional autoantibodies, enlarge the study cohort, and systematically test CSF samples alongside serum, in order to improve the diagnostic yield of AE.

Abstract

Le encefaliti autoimmuni (AE) sono un gruppo di disturbi neurologici determinati da un'anomala attivazione del sistema immunitario, che porta alla produzione di anticorpi diretti contro antigeni neuronali di superficie o intracellulari del sistema nervoso centrale. Le manifestazioni cliniche coinvolgono prevalentemente la sfera cognitiva – deterioramento cognitivo, alterazioni comportamentali e crisi epilettiche – spesso accompagnate da sintomi psichiatrici, quali psicosi, aggressività ed euforia, e da disturbi del movimento. La diagnosi di AE richiede l'integrazione di dati clinici, neurologici, radiologici e di laboratorio: la positività o negatività dei test deve sempre essere valutata in rapporto al quadro clinico, e non valutata singolarmente.

Questo studio ha l'obiettivo principale di caratterizzare pattern di colorazione immunoistochimica finora non caratterizzati, osservati nella coorte di pazienti, e di individuare nuovi antigeni neuronali di superficie mediante cell-based assay (CBA).

Sono stati inclusi 54 pazienti (età media 52 anni), i cui campioni biologici sono stati inviati al Laboratorio di Neuroimmunologia della Fondazione IRCCS Istituto Neurologico Nazionale Casimiro Mondino con sospetta AE, già risultati positivi all'immunoistochimica (IHC) con pattern non caratterizzato e negativi al CBA commerciale su cellule fissate.

Lo screening della coorte mediante CBA su cellule vive (L-CBA) ha permesso di identificare 4 campioni positivi per tre antigeni distinti. UNC-07 è risultato positivo per SEZ6L2 (titolo endpoint 1:2560, IgG1, confermato su liquido cerebrospinale (cerebrospinal fluid CSF)) e, inoltre, per mGluR5 (titolo endpoint 1:20480, IgG1, confermato su CSF). UNC-02 e UNC-23 hanno mostrato positività per SEZ6L2 a basso titolo (1:80), non confermata su CSF, con sottoclassi IgG differenti (rispettivamente IgG1 e IgG2). UNC-33, infine, è risultato positivo per mGluR1 (titolo endpoint 1:2560, IgG1 e IgG2, confermato su CSF).

Per confermare ulteriormente la presenza di anticorpi neuronali di superficie, i campioni sono stati testati su colture neuronali primarie: su 54 pazienti, 8 sono risultati positivi. Tra questi, UNC-07, UNC-14 e UNC-33 sono stati confermati sia su siero che su CSF, mentre UNC-05, UNC-23, UNC-34, UNC-37 e UNC-50 sono risultati positivi esclusivamente su siero (CSF non disponibile per UNC-34, UNC-37 e UNC-50).

I risultati ottenuti con L-CBA hanno spiegato solo una frazione della reattività osservata tramite IHC e, in misura ancora più evidente, tramite i saggi su colture neuronali primarie: un dato che indica come la maggior parte della reattività anticorpale contro antigeni di superficie, in questa coorte, resti ancora non caratterizzata dal punto di vista antigenico. Questi risultati confermano l'esistenza di autoanticorpi non ancora caratterizzati e sottolineano la necessità di ampliare il pannello antigenico permettendo di identificare ulteriori autoanticorpi, espandere la coorte in esame e testare sistematicamente i campioni di CSF in parallelo a quelli di siero, al fine di migliorare la resa diagnostica delle encefaliti autoimmuni.

Introduction

What is Autoimmune Encephalitis?

Autoimmune Encephalitis (AE) is a group of neurological disorders characterised by an abnormal activation of the immune system, leading to the production of antibodies directed against surface neuronal antigens (epitopes of channels, receptors, and other proteins), or intracellular neuronal antigens within the central nervous system (CNS)[1].

Aetiology of AE

Although most cases of AE are idiopathic[2], there are some risk factors founded in certain AE variants. The best-studied one is the presence of an underlying tumor with ectopic expression of neuronal synaptic autoantigens; immune system attacks both tumor cells and cells of the CNS leading to the so-called Paraneoplastic Neurological Syndrome (PNS). The presence of certain heterozygous HLA haplotypes can also be correlated with the presence and the onset of the autoimmune disorders[3,4], as well as prior use of immune checkpoint inhibitors and recent episodes of viral encephalitis, in particular Armangue et al. (2014), in their cohort, founded that about 40% of patients with Herpes Simplex Encephalitis, that were clinically followed since symptoms onset, developed neuronal surface antibodies and that about half of them, developed symptoms of AE[5].

Epidemiology

According to Yucel et al. (2025), the incidence ranges from 7 to 15 cases per 100,000 population[6]. This incidence has increased in recent years, paralleling a rise in infectious events.

Clinical features

The main clinical symptoms of AE are related to cognition and behaviour, particularly characterised by cognitive impairment, behavioural changes and seizure. There can also be psychiatric symptoms like psychosis, aggression, euphoria, and movement disorders[7].

Diagnosis

Based on the diagnostic criteria established by Graus et al. (2016), AE is recognised when three conditions are satisfied.

First, the patient must present a relatively fast-evolving clinical picture (<3 months) involving impaired working memory (manifesting as short-term memory loss), altered mental status, or psychiatric disturbances.

Second, at least one additional finding must be present among the following:

- newly identified focal neurological signs referable to the CNS;
- new-onset seizure activity not attributable to a pre-existing epileptic disorder;
- pleocytosis on cerebrospinal fluid (CSF) analysis, defined as a white blood cell count exceeding 5 cells/mm^3;
- magnetic resonance imaging (MRI) findings consistent with an encephalitic process.

Third, other possible aetiologies must be reasonably ruled out through appropriate clinical and diagnostic workup[8].

Once these criteria are satisfied, prompt initiation of first-line immunotherapy is advised, even in the absence of antibody-confirmed diagnosis[9]. [see Treatment of Autoimmune Encephalitis]

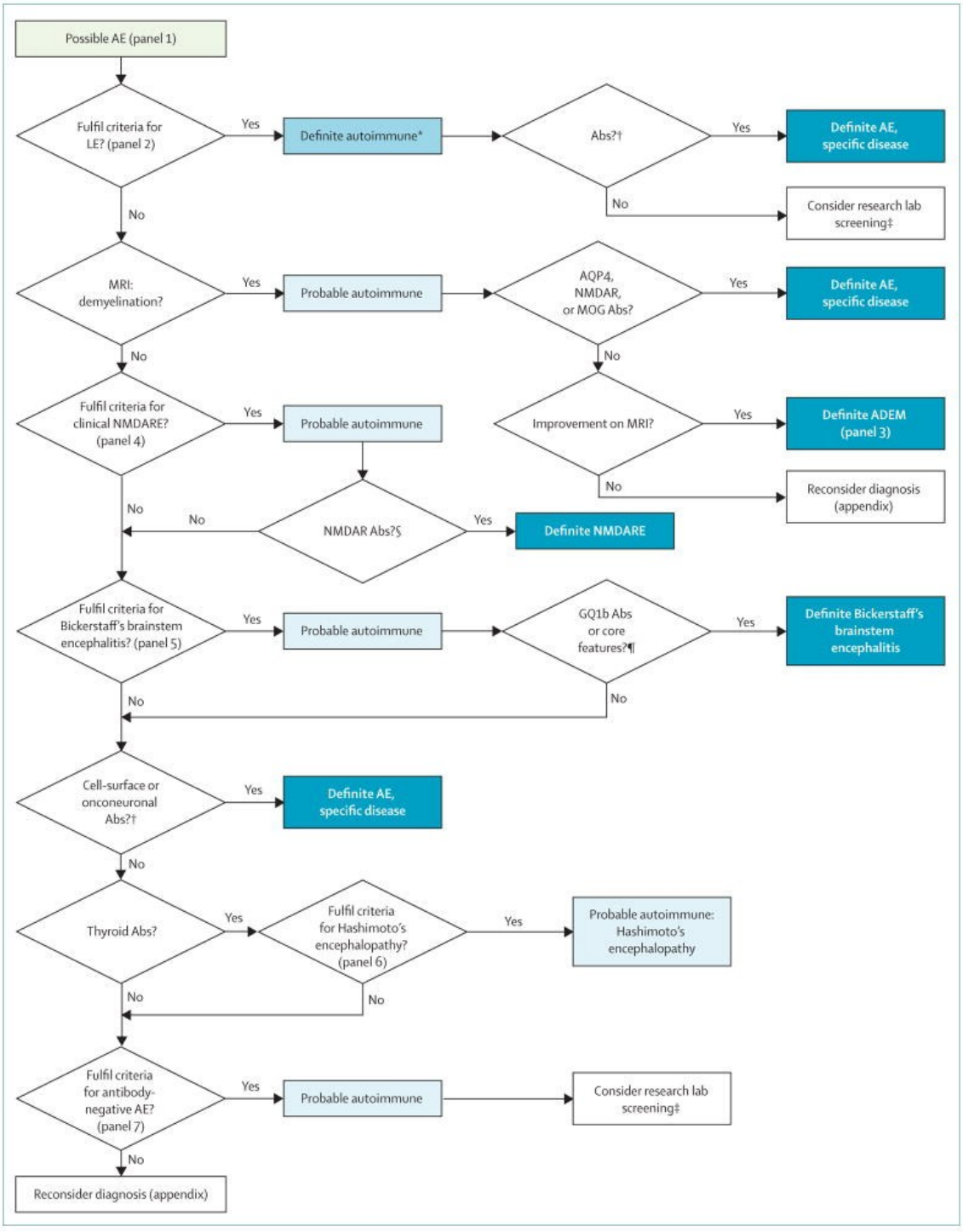


Figure 1. Algorithm for the diagnosis of autoimmune encephalitis[8].

Paraneoplastic Neurological Syndromes

Paraneoplastic Neurological Syndromes (PNS) are a group of neurological disorders characterised by immune system activation, with the production of autoantibodies, in the presence of an underlying malignancy, that triggers the immune response[10].

In 2021 new criteria for PNS were published by Graus et al.: autoantibodies have been divided based on the risk of association with underlying malignancy in high-risk (>70%, previously known as "onconeural antibodies"), intermediate-risk (30-70%) and low-risk (<30%). A new score based on the phenotype and antibody risk (PNS care score) is now used to estimate the risk of having a PNS[11].

Table 1 High-Risk Antibodies (>70% Associated With Cancer)

Antibody (alternative name)	Neurologic phenotypes	Frequency of cancer (%)	Usual tumors	Sex, age-related, and other specificities
Hu (ANNA-1)[8]	SNN, chronic gastrointestinal pseudo-obstruction, EM, and LE	85	SCLC >> NSCLC, other neuroendocrine tumors, and neuroblastoma	LE is usually nonparaneoplastic in patients aged <18 y[18]
CV2/ CRMP5[30,e17,e40,e41]	EM and SNN	>80	SCLC and thymoma	Patients with an associated thymoma are younger and present more frequently MG and less commonly neuropathy
SOX1[36,e42]	LEMS with and without rapidly progressive cerebellar syndrome	>90	SCLC	Stronger correlation with SCLC than with a particular neurologic presentation
PCA2 (MAP1B) [57,e43,e44]	Sensorimotor neuropathy, rapidly progressive cerebellar syndrome, and EM	80	SCLC, NSCLC, and breast cancer	
Amphiphysin[31,e18]	Polyradiculoneuropathy, SNN, EM, SPS	80	SCLC and breast cancer	Associated antibodies commonly coexist. Patients with isolated antiamphiphysin → women, with breast cancer and SPS
Ri (ANNA-2)[20,26]	Brainstem/cerebellar syndrome, OMS	>70	Breast > lung (SCLC and NSCLC)	Breast cancer in women; lung cancer in men
Yo (PCA-1)[21,e16]	Rapidly progressive cerebellar syndrome	>90	Ovary and breast cancers	Almost all female; in men, antigen expression by tumor should be proven
Ma2 and/or Ma[45,e15,e45]	LE, diencephalitis, and brainstem encephalitis	>75	Testicular cancer and NSCLC	Young men → testicular tumors and isolated Ma2 positivity; older patients → SCLC and both Ma1/2 positivity
Tr (DNER)[22,23]	Rapidly progressive cerebellar syndrome	90	Hodgkin lymphoma	
KLHL11[48-50]	Brainstem/cerebellar syndrome	80	Testicular cancer	Young men

Abbreviations: ANNA = antineuronal nuclear antibody; CRMP5 = collapsin response-mediator protein 5; DNER = delta/notch-like epidermal growth factor–related receptor; EM = encephalomyelitis; KLHL11 = Kelch-like protein 11; LE = limbic encephalitis; LEMS = Lambert-Eaton myasthenic syndrome; MAP1B = microtubule-associated protein 1B; MG = myasthenia gravis; NMDAR = NMDA receptor; NSCLC = non–small-cell lung cancer; OMS = opsoclonus-myoclonus syndrome; PCA = Purkinje cell antibody; SCLC = small-cell lung cancer; SNN = sensory neuronopathy; SPS = stiff-person syndrome.

Table 1. Classification of high-risk antibodies[11]

Table 2 Intermediate-Risk Antibodies (30%–70% Associated With Cancer)

Antibody	Neurologic phenotypes	Frequency of cancer (%)	Usual tumors	Sex, age-related, and other specificities
AMPAR[16,17,e46]	Limbic encephalitis	>50	SCLC and malignant thymoma	Paraneoplastic origin is more likely when other onconeuronal antibodies co-occur
$GABA_BR$[e14,15,e2,e3,e47-e49]	Limbic encephalitis	>50	SCLC	Paraneoplastic cases are more commonly observed in elderly men, smokers, with associated anti-KCTD16 antibodies. Most of young patients are not paraneoplastic
mGluR5[38]	Encephalitis	~50	Hodgkin lymphoma	
P/Q VGCC[e50,e51]	LEMS, rapidly progressive cerebellar syndrome	50 (LEMS; nearly 90 for rapidly progressive cerebellar syndrome)	SCLC	Co-occurrence with N-type VGCC antibodies might be slightly more common in paraneoplastic LEMS[e52-e54]
NMDAR[40,43,44]	Anti-NMDAR encephalitis	38	Ovarian or extraovarian teratomas	Tumor (mostly ovarian teratomas) predominates in female aged between 12 and 45 y (50%). Elderly patients have less frequently tumors (<25%), but usually they are carcinomas. Paraneoplastic cases in children are very rare (<10%)
CASPR2[51,52]	Morvan syndrome	50	Malignant thymoma	CASPR2 should be considered as intermediate-risk antibody only in the setting of Morvan syndrome. When associated with other neurologic syndromes, the risk of cancer is very low.

Abbreviations: AMPAR = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; $GABA_BR$ = gamma-aminobutyric acid-b receptor; KCTD16 = potassium channel tetramerization domain containing; LEMS = Lambert-Eaton myasthenic syndrome; mGluR5 = metabotropic glutamate receptor type 5; NMDAR = NMDA receptor; SCLC = small-cell lung cancer; VGCC = voltage-gated calcium channel.

Table 2. Classification of intermediate-risk antibodies[11]

Table 3 Lower-Risk Antibodies (<30% Associated With Cancer)

Antibody	Neurologic phenotypes	Frequency of cancer (%)	Usual tumors	Sex, age-related, and other specificities
mGluR1[e55]	Cerebellar ataxia	30	Mostly hematologic	
$GABA_AR$[39,e56]	Encephalitis	<30	Malignant thymoma	Paraneoplastic origin is less frequent (10%) in children than in adults (60%)
CASPR2[51,52,e57,e58]	LE, acquired neuromyotonia (Isaac syndrome), and Morvan syndrome	<30	Malignant thymoma	Morvan syndrome is more associated (≈50%) with malignant thymoma, whereas LE is almost always nonparaneoplastic
GFAP[e59,e60]	Meningoencephalitis	≈20	Ovarian teratomas and adenocarcinomas	May occur as an immunologic accompaniment in anti-NMDAR encephalitis with ovarian teratomas
GAD65[e61,e62]	LE, SPS, and cerebellar ataxia	<15	SCLC, other neuroendocrine tumors, and malignant thymoma	Paraneoplastic patients are older, more frequently male, with associated neuronal antibodies, and atypical clinical presentations
LGI1[e63-e67]	LE	<10	Malignant thymoma and neuroendocrine	Paraneoplastic cases are mainly observed in patients with Morvan syndrome and both serum LGI1 and CASPR2 antibodies
DPPX[e68,e69]	Encephalitis with CNS hyperexcitability and PERM	<10	B-cell neoplasms	
GlyR[55,56]	LE and PERM	<10	Malignant thymoma and Hodgkin lymphoma	
AQP4[e70]	Neuromyelitis optica spectrum disorder	<5	Adenocarcinomas	Older age, male, and severe nausea/vomiting at onset
MOG[e71-e73]	MOG antibody–associated disease	5 cases reported	Mostly ovarian teratomas	

Abbreviations: AQP4 = aquaporin 4; CASPR2 = contactin-associated protein-like 2; DPPX = dipeptidyl peptidase-like protein; $GABA_AR$ = gamma-aminobutyric-acid-A receptor; GAD = glutamic acid decarboxylase; GFAP = glial fibrillary acidic protein; GlyR = glycine receptor; LE = limbic encephalitis; LGI1 = leucine-rich glioma-inactivated protein 1; mGluR1 = metabotropic glutamate receptor type 1; MOG = myelin oligodendrocyte glycoprotein; NMDAR = NMDA receptor; PERM = progressive encephalomyelitis with rigidity and myoclonus; SCLC = small-cell lung cancer; SPS = stiff-person syndrome.

Table 3. Classification of low-risk antibodies[11]

Molecular background

Since AE is driven by the activation of the immune system, B and T lymphocytes are its main actors. Both derive from precursors in the bone marrow, but they complete their maturation in different primary lymphoid organs: B cells in the bone marrow itself, T cells in the thymus. Mature cells then colonize the secondary lymphoid organs: the lymph nodes, the spleen and the mucosa-associated lymphoid tissue.

The following overview, based on the Figure 2, summarizes the adaptive response that leads to antibody production and immune memory.

A local infection [a], for instance in the lungs, rapidly activates innate immunity, and dendritic cells (DC) travel to the draining associated lymph nodes, where they drive the activation and differentiation of naïve T cells [b]. The resulting CD4+ and CD8+ effector T cells return to the infected tissue and coordinate the clearance of the agents that cause the infection [c]. Simultaneously, in the lymph nodes, CD4+ follicular helper cells (T_{FH} cells) engage antigen-specific B cells and support their entry into the germinal centre, a specialized microenvironment where these B cells expand and differentiate into antibody-secreting cells (ASC) [e]. The antibodies released circulate in plasma and reach secretions and tissues [f], where they are important for the clearance of free virus through direct binding and neutralization or by marking virus for destruction by macrophages and serum proteins (opsonization). Immune memory is maintained by memory T cells, memory B cells and long-lived plasma cells (LLPC), which can persist in tissues as resident populations even without antigen [f]. Tissue-resident memory B cells are found mainly in lymphoid organs and mucosal sites, whereas LLPCs settle in the bone marrow, intestine, skin and other sites [g][12].

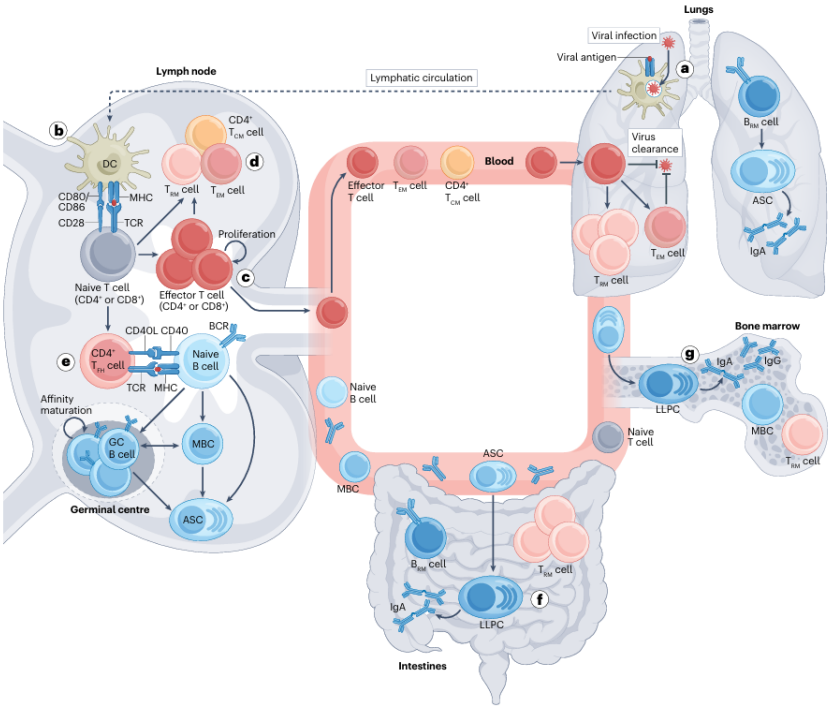


Figure 2. Antibody production and immune memory[12].

Autoimmune disease, AE included, result from a dysregulated immune system in which autoreactive immune cells become aberrantly activated and ultimately damage the tissues[13]. The underlying cause is the survival of autoreactive lymphocytes that would normally be removed by the mechanisms of immune tolerance[14]. Tolerance operates at two levels: central tolerance, enforced in the primary lymphoid organs, and peripheral tolerance, which acts in the secondary lymphoid organs and in non-lymphoid tissues throughout the body.

The checkpoints are not perfect. According to Weill and Reynaud (2015) and Kenison et al. (2023), roughly 40-70% of precursor B cells carry receptors directed against self-antigens, including cytoplasmic and nuclear components, and many of them are polyspecific, i.e. able to bind several unrelated antigenic structures. Peripheral tolerance reduces this fraction, but about 20% of the mature B cells that remain still recognise self-structure[15,16]. T cells behave similarly: an estimated 25-40% evade central tolerance[16]. Some of these self-reactive T cells escape and persist in the periphery, where they are normally held in check by anergy, deletion, and suppression

by regulatory T cells (Tregs). Autoimmunity develops only when these remaining safeguards fail.

The loss of tolerance can depend on different factors such as genetic predisposition and environmental risk factors[13] that were previously mentioned. The HLA region is the gene cluster most relevant to the immune system, and variation at some of its loci can favour the onset of autoimmune disease[17]. Environmental risk factors can be chronic microbial infections, physical agents, medications and diet[18]. Molecular mimicry is a key mechanism in microbe-induced autoimmunity. A microbe may express epitopes that are identical or structurally similar to those of the host, leading to the production of antibodies against the microorganism that may cross-react with self-antigens and potentially trigger autoimmunity[14].

About 40% of patients with Herpes Simplex virus Encephalitis develop antibodies against neuronal surface proteins, mainly the NMDA receptor, which directly links a viral infection of the CNS to brain autoimmunity[5]. A plausible interpretation is that the virus-induced inflammation and neuronal destruction release surface and synaptic proteins that are normally hidden from the immune system. These then trigger the production of autoantibodies, in line with the general model of inflammation-driven release of self-antigens[19].

Unlike classical PNS of the CNS, which are almost always tumor-associated[8], AE can occur in the presence or in the absence of a tumor. In AE with a frequent cancer association, the tumor cells express the neuronal surface or synaptic protein targeted by the antibody (molecular mimicry). The best-known example is anti-NMDAR encephalitis, where ovarian teratomas contain neural tissue expressing the receptor[20,21]. In these forms, the trigger mainly primes a B-cell response and produces antibodies directed at surface or synaptic targets. The resulting neuronal damage is therefore mostly functional and reversible, unlike the structural and irreversible damage seen in syndromes mediated by cytotoxic T cells against intracellular antigens[22]. A mechanistic explanation comes from Hughes et al. (2010), who showed that patient antibodies cross-link NMDARs and reduce their density at the synapse, and this effect is reversible once the antibodies are removed[23]. This is consistent with the generally favourable response to immunotherapy observed in these patients.

Structure and classification of antibodies

Antibodies are heterodimeric glycoproteins composed of two heavy chains and two light chains (Figure 4). Each chain contains variable regions, which form the antigen-binding site (paratope), and constant regions, which are responsible for molecular assembly and effector activity. The chains are held together by interchain disulfide bonds. Both heavy and light chains consist of an N-terminal variable domain (VH in heavy chain, VL in light chain) and constant domains, with one constant domain in light chains (CL) and three in heavy chains (CH1, CH2, CH3); the latter also contain an additional region between CH1 and CH2, known as the "hinge region". The isotype and subclass of an antibody are determined by the constant region of its heavy chain[24].

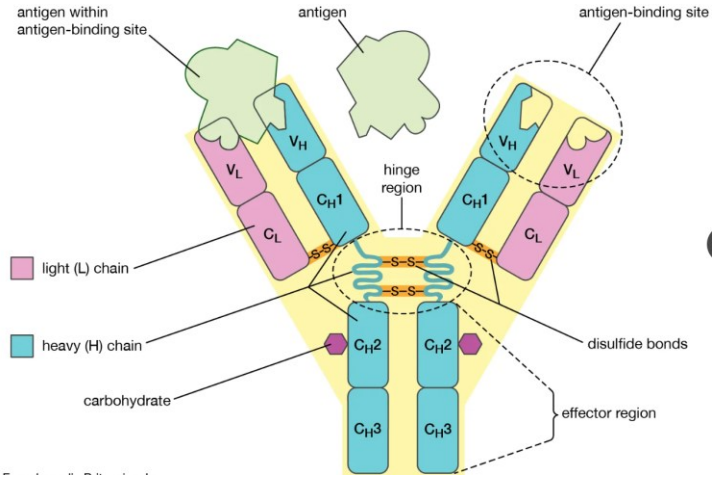


Figure 4. Illustration of antibodies structure[25].

Human immunity relies on five immunoglobulin isotypes – IgM, IgD, IgA, IgE, and IgG – each with a distinct functional profile. The latter isotype is the most abundant, accounting for 10-20% of total serum proteins[26].

Four subclasses of IgG exist – IgG1, IgG2, IgG3, and IgG4 – sharing more than 90% amino acid sequence (Figure 5). The variation is concentrated in the hinge region and the N-terminal portion of the CH2 domain.

- IgG1, the predominant subclass under normal conditions, is produced in response to soluble and membrane protein antigens.

- IgG2 is the principal responder to bacterial capsular polysaccharides.
- IgG3 is a potent driver of inflammation, and its short half-life (~7 days, versus ~21 days for the other subclasses) is thought to act as a natural brake on excessive inflammatory activity. Together with IgG1, it efficiently triggers effector mechanisms such as classical complement-mediated lysis, antibody-dependent cellular toxicity, and antigen cross-linking followed by endocytosis [26].
- IgG4 tend to arise after prolonged or intense antigenic stimulation, and it is generally considered as anti-inflammatory. A key feature of this subclass is Fab-arm exchange (Figure 6): a single hinge-region amino acid substitution gives IgG4 greater structural flexibility, permitting – under reducing conditions – intrachain rather than interchain disulfide bonding. This weakens the covalent link between the two antibody half-molecules, allowing them to dissociate and recombine at random with half-molecules from other IgG4 antibodies, producing bispecific, functionally monovalent antibodies. Because such monovalent antibodies cannot cross-link antigen, trigger antigen internalization, or form immune complexes, this mechanism is thought to be advantageous when non-pathogenic antigens must be cleared without provoking overt inflammation[26,27].

  The main mechanism of action of IgG4 is the disruption of the protein-protein interaction[1].

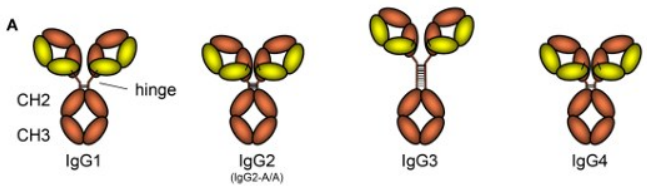


Figure 5. Illustration of IgG subclasses[26].

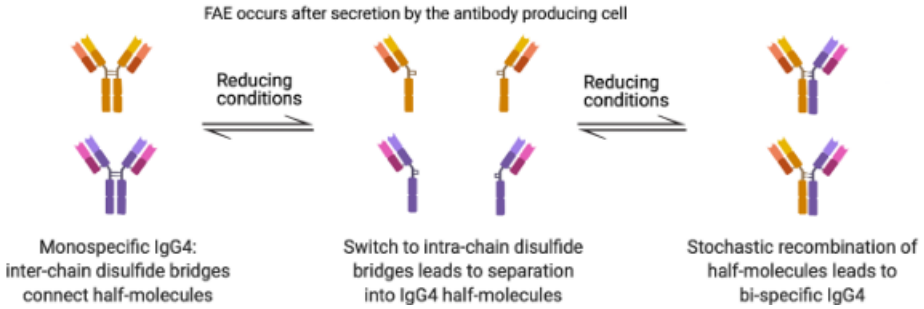


Figure 6. Fab arm exchange[27].

Regarding complement activation cited before, IgG1 and IgG3 are by far the most effective activators of the classical pathway; IgG2 can also engage complement, but only when antibody titres or antigen density are high, whereas IgG4 is essentially unable to activate it[24].

More details, related to IgG subclasses, can be found in the table below (Table 4).

	IgG1		IgG2		IgG3		IgG4	
General								
Molecular mass (kD)	146		146		170		146	
Amino acids in hinge region	15		12		62[a]		12	
Inter-heavy chain disulfide bonds	2		4[b]		11[a]		2	
Mean adult serum level (g/l)	6.98		3.8		0.51		0.56	
Relative abundance (%)	60		32		4		4	
Half-life (days)	21		21		7/~21[a]		21	
Placental transfer	++++		++		++/++++[a]		+++	
Antibody response to:								
Proteins	++		+/–		++		++[e]	
Polysaccharides	+		+++		+/–		+/–	
Allergens	+		(–)		(–)		++	
Complement activation								
C1q binding	++		+		+++		–	
Fc receptors								
FcγRI	+++[c]	65[d]	–	–	++++	61	++	34
$FcγRIIa_{H131}$	+++	5.2	++	0.45	++++	0.89	++	0.17
$FcγRIIa_{R131}$	+++	3.5	+	0.10	++++	0.91	++	0.21
FcγRIIb/c	+	0.12	–	0.02	++	0.17	+	0.20
$FcγRIIIa_{F158}$	++	1.2	–	0.03	++++	7.7	–	0.20
$FcγRIIIa_{V158}$	+++	2.0	+	0.07	++++	9.8	++	0.25
FcγRIIIb	+++	0.2	–	–	++++	1.1	–	–
FcRn (at pH < 6.5)	+++		+++		++/++++[a]		+++	

[a] *Depends on allotype.*
[b] *For A/A isomer.*
[c] *Multivalent binding to transfected cells. Adapted from Bruhns et al. (2).*
[d] *Association constant (× 10^6 M^{-1}) for monovalent binding (2).*
[e] *After repeated encounters with protein antigens, often allergens.*

Table 4. Properties of human IgG subclasses[26].

Differences between intracellular neuronal antibodies (ICN-Abs) and neuronal surface antibodies (NS-Abs)

The main difference between ICN-Abs and NS-Abs lies in the localisation of antigen target: the former are directed against intracellular antigens, whereas the latter target antigens located on the cell membrane, which are therefore accessible to circulating

antibodies. NS-Abs are thought to be directly pathogenic, although this has been proven for a limited number of NS-Abs.

Intraventricular infusion of anti-NMDAR antibodies in rat brains, leads to downregulation of receptor density[23]. In contrast, passive transfer in animal models of Yo, then classified as a high-risk antibody, did not reproduce the neurological findings or the extensive neuronal destruction seen in human disease[28]. This suggests that these antibodies are merely markers of an underlying malignancy rather than direct mediators of damage[29]. The pathogenic role is thought to be played by cytotoxic T cells, which have been identified in the brains of affected patients[30]. T cells recognise intracellular antigens presented on the cell surface by major histocompatibility complex type 1 (MHC1) molecules[31], which may be upregulated in the paraneoplastic setting[32].

Despite extensive efforts, no animal model fully recapitulates the pathogenesis of classical PNS, as the transfer of cytotoxic T cells does not produce the neurological phenotype[29]. Further studies are therefore needed to understand this pathology in depth.

The main pathogenic mechanisms associated with NS-Abs are: cross-linking and internalization, disruption of protein-protein interaction, receptor blockade, complement activation, and Fc-dependent effector mechanism that activates the microglia (Figure 7) [33].

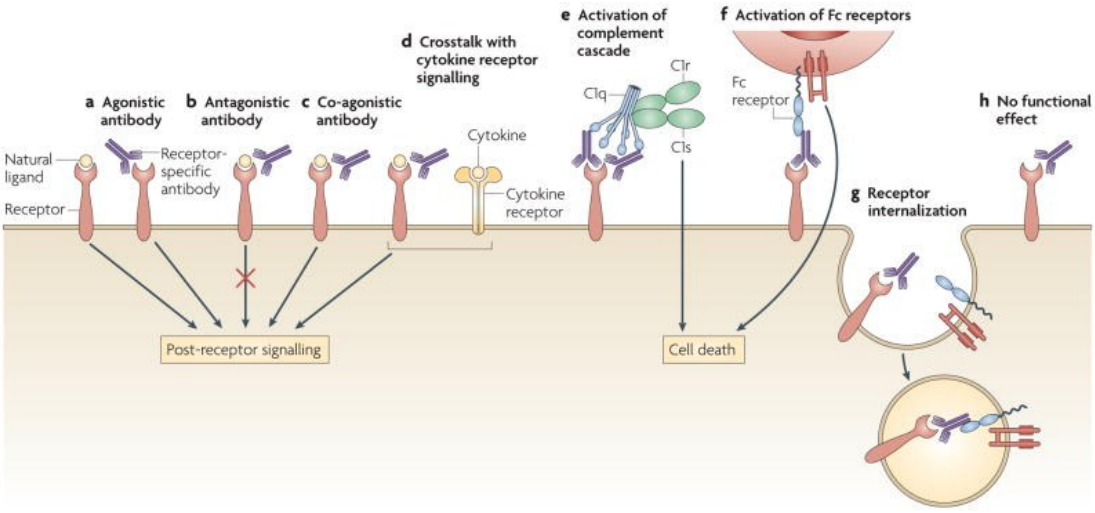


Figure 7. Pathogenic mechanisms associated with NS-Abs[33]

Sources of autoantibodies production

Two classical models, which are not mutually exclusive and often operate together, have been proposed to explain how B cells escaping tolerance checkpoints give rise to the pool of circulating autoantigen-specific antibodies (Figure 8). The first model holds that autoreactive B cells are repeatedly recruited into germinal centre reactions, where they differentiate into short-lived ASCs that release the pathogenic autoantibodies into the bloodstream[34]. This scenario requires persistent stimulation by the autoantigen. Since IgM is normally the first isotype produced during a humoral response, autoantigen-specific IgM would be expected to be generated continuously and to be detectable in the circulation[35]. The B cells feeding these germinal centres could be naïve autoreactive cells that keep slipping through defective tolerance checkpoints, or memory B cells formed during previous autoimmunizing episodes[35]. The second model proposes that an autoimmunizing event leads to the generation of autoantibody-secreting LLPCs, which settle in specialized bone marrow niches[36]. In this case, germinal centre activation produces a population of plasma cells that homes to the bone marrow and from there supplies most of the autoantibodies, largely independently of further antigenic stimulation[35].

Taken together, experimental and clinical data suggest that both active germinal centre and LLPCs contribute to the autoantibody repertoire found in the circulation of patients with neuronal surface antibody-associated diseases[35].

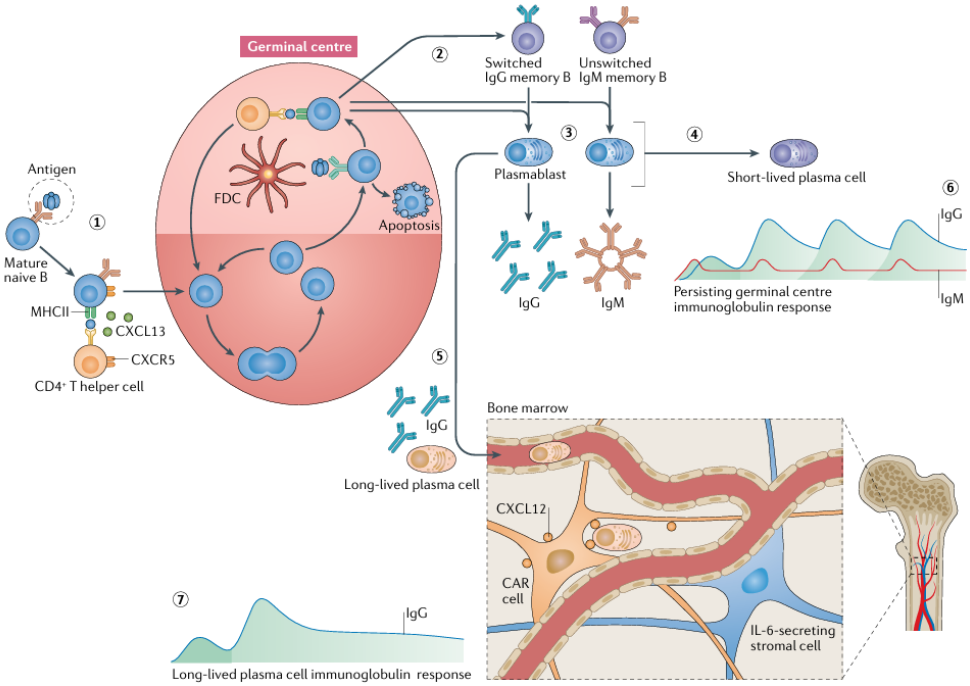


Figure 8. Representation of conventional models of autoantibodies sources[35].

Autoantibody access in the CNS

Historically, the CNS has been considered an immune-privileged organ, owing to the presence of the blood-brain barrier (BBB) and the lack of a classical lymphatic drainage. Recent discoveries, such as the ability of peripheral immune cells to cross the intact BBB and the identification of a functional lymphatic drainage from the CSF to the cervical lymph nodes, have challenged this concept. In this context, autoantibodies are known to reach the CNS, although it remains unclear whether they do so through passive diffusion of IgG or through the trafficking of peripheral B cells[35].

Figure 9 illustrates a possible mechanism underlying the entry of autoantibodies into the CNS. Infections or inflammation can damage nervous tissue and release neuronal proteins into the CSF [1]. These proteins can then be carried to the cervical lymph nodes by the lymphatic vessels of the brain [2]. In the lymph nodes, the immune system can elicit a B cell response against them, producing antigen-specific B cells, plasma cells and large amounts of circulating autoantibodies [3]. Activated B cells can then migrate into the intrathecal space, attracted by chemical signals such as the chemokine CXCL13, where they proliferate, improve their affinity for the antigen and

differentiate into ASCs that release pathogenic autoantibodies [4]. These steps may occur in ectopic tertiary lymphoid structures. Finally, the antibodies produced in the intrathecal space can directly impair neuronal function [5][35].

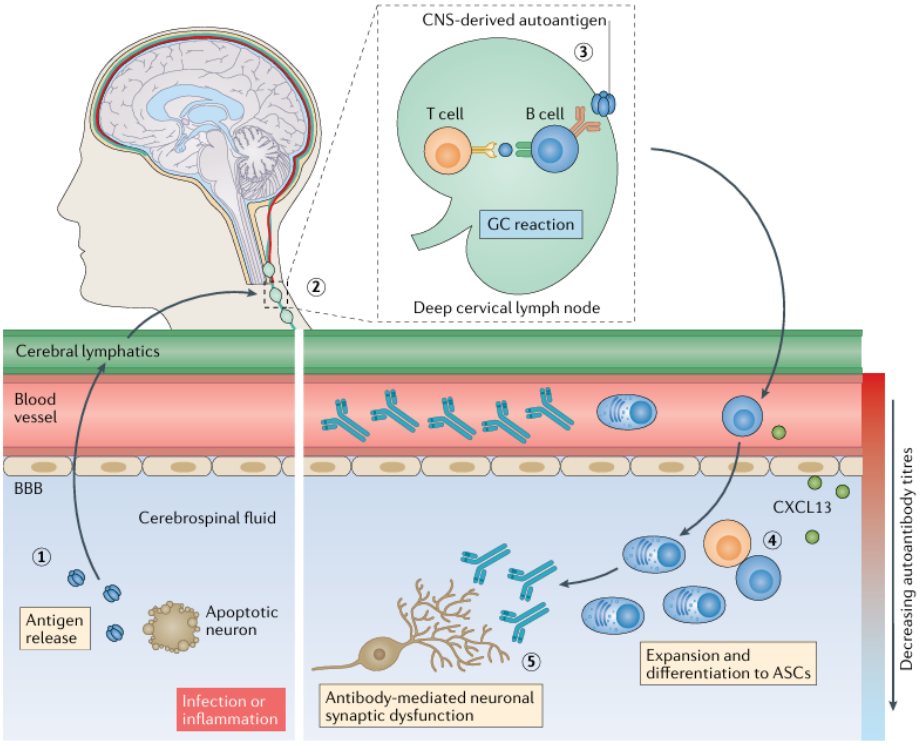


Figure 9. Autoantibody access to CNS targets[35].

Limbic encephalitis

Limbic encephalitis is an inflammatory neurological syndrome that primarily targets the structures of the limbic system, most notably the medial temporal lobes, including the hippocampus and amygdala. In a substantial subset of patients, the inflammatory process extends beyond these core structures to involve extra limbic regions such as the insula, the orbitofrontal cortex, and the basal ganglia[37,38].

The principal symptoms of limbic encephalitis include short-term memory impairment (being reported in approximately 84-92% of patients), neuropsychiatric manifestations (including confusion, temporal disorientation and behavioural disorders), epileptic seizures and sleep disturbances[37].

Classification of the principal autoantibodies against neuronal surface antigens

1. Anti-NMDA Receptor Encephalitis

The N-methyl-D-aspartate (NMDA) receptor is an ionotropic glutamate receptor permeable to calcium, found at excitatory synapses in the brain and important for brain development, memory formation and learning. This role has been demonstrated by the spatial and temporal learning impairments observed in hippocampal CA1 region-specific GluN1 knockouts[39].

The receptor consists of a tetrameric structure composed of four subunits: two GluN1 subunits and two GluN2A-D or GluN3A-B subunits. It is also composed of an extracellular amino-terminal domain (ATD), an extracellular ligand-binding domain (LBD), a transmembrane domain (TMD), and an intracellular carboxyl-terminal domain (CTD)[40] (Figure 10).

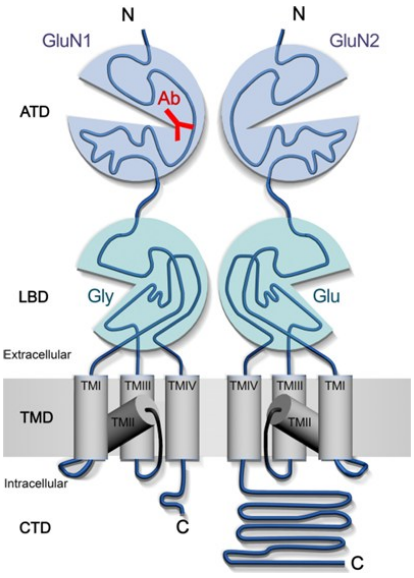


Figure 10. Schematic representation of the GluN1 and GluN2 subunits of NMDA receptor, highlighting the main antibody-binding site[39].

Antibodies against the NMDA receptor are not found only in AE but also in other disorders such as schizophrenia, Parkinson's disease, stroke and dementia. Depending on the IgG subclass involved, the specific receptor subunit targeted and whether the

antibodies are detectable in the CSF, the clinical and pathological significance is determined[39].

Once the autoantibodies bind to the target, receptor density decreases due to internalization, leading to the typical symptoms of the disease, which progress from prodromal symptoms such as fever and nausea to psychiatric symptoms, autonomic dysfunction and movement impairment in later stages[41].

Hughes et al. (2010) showed, both in vitro and in vivo, that antibodies against NMDA receptors isolated from patients with anti-NMDA receptor encephalitis reduce receptor density and synaptic localisation through antibody-mediated capping and internalization. This effect was complement-independent and did not alter other synaptic proteins. Moreover, they demonstrated that the magnitude of these changes correlates with antibody titre, and the effects are reversible upon reduction of the titre[23].

As stated by Dalmau et al. (2017) and later confirmed by Nissen et al. (2020), the disease predominantly affects young women with a median age of 22 years[39,41].

The trigger of the pathology can be a tumor, especially ovarian teratomas, seen in up to 58% of female patients, or it can develop after Herpes Simplex Encephalitis[41].



Figure 11. Sagittal and coronal sections of rat brain immunostaining with autoantibodies of patients targeting NMDA receptor[43].

2. Anti-AMPA Receptor Encephalitis

The α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid (AMPA) receptor is an ionotropic receptor activated by glutamate that mediates most of the fast excitatory transmission in the brain[44].

The receptor is a tetramer formed by combinations of four subunits, GluA1, GluA2, GluA3, and GluA4[45].

Most autoantibodies found in AE patients recognise conformational, extracellular epitopes located on the GluA1 or GluA2 subunits, as demonstrated by the decrease in synaptic AMPA receptors clusters observed in cultured rodent neurons exposed to patients' IgG against GluA1 or GluA2[39].

The classical presentation is a subacute limbic encephalitis, characterised by memory impairment, behavioural change, and seizures. However, some patients show other symptoms, such as rapidly worsening cognitive decline or psychiatric symptoms (psychosis).

According to Nissen et al. 2020, AMPAR encephalitis affects predominantly older adults, with a median age at onset of 60-62 years, with a female predominance[41].

Anti-AMPAR encephalitis is strongly associated with an underlying tumor, identified in 64%, most commonly small-cell lung carcinoma and thymoma[46].



Figure 12. Sagittal and coronal sections of rat brain immunostaining with autoantibodies of patients targeting AMPA receptor[43].

3. Anti-LGI1 Encephalitis

Leucine-rich Glioma-Inactivated 1 (LGI1) is a secreted glycoprotein involved in CNS development and physiology.

Its function is to create a bridge, in the synaptic cleft, between two membrane proteins, ADAM23 presynaptically and ADAM22 postsynaptically (Figure 13). In this way they organize a trans-synaptic protein complex that additionally incorporates presynaptic

Kv1.1 potassium channels and postsynaptic AMPA receptors, thereby coupling presynaptic excitability to postsynaptic glutamatergic signalling[47].

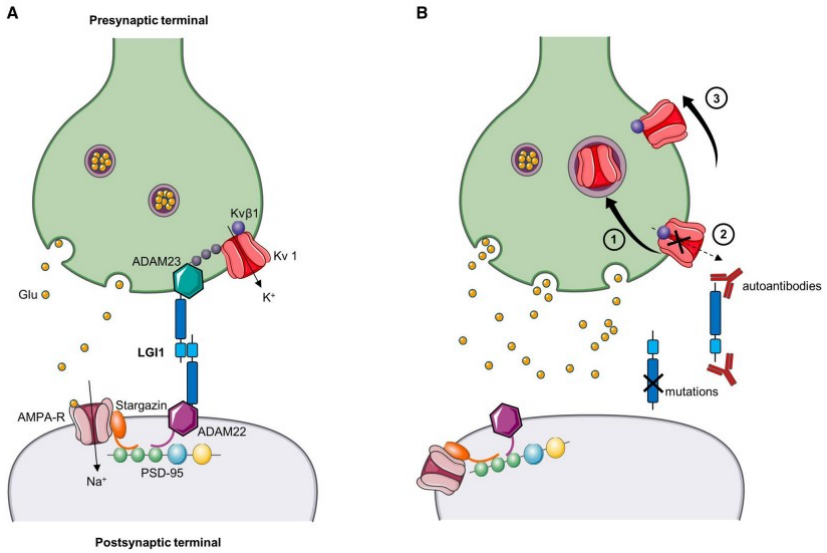


Figure 13. Synaptic organization of LGI1 (A) and disruption by mutations and autoantibodies (B)[47].

A distinctive immunological feature of LGI1 and Caspr2 antibodies, the latter of which will be described next, is their subclass. Indeed, most pathogenic antibodies in AE are IgG1, while LGI1 and Caspr2 antibodies are predominantly IgG4.

LGI1 encephalitis typically presents as limbic encephalitis, with behavioural change, memory deficits, confusion, and seizure.

It preferentially affects older patients with a median age around 60 years, with a slight-to-moderate male predominance[39].

LGI1 encephalitis carries a low overall tumor association[41].

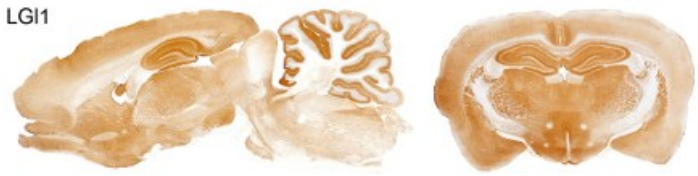


Figure 14. Sagittal and coronal sections of rat brain immunostaining with autoantibodies of patients targeting LGI1[43].

4. Anti-Caspr2 Encephalitis

Contactin-associated protein 2 (Caspr2) is a cell adhesion molecule that belongs to the neurexin IV superfamily. It is expressed at the juxtaparanodal region of myelinated axons, both in the CNS and in the peripheral nervous system (PNS), where it plays a central role in organizing the local architecture of the axolemma[39].

As the Figure 15 demonstrates, Caspr2 acts as a transmembrane bridge between Contactin-2 extracellularly and Postsynaptic Density Protein 95 (PSD95) intracellularly; this complex anchor Kv1 potassium channels at the juxtaparanode[48].

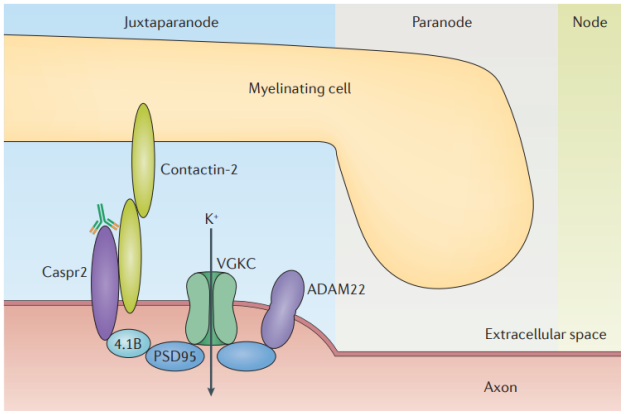


Figure 15. Schematic representation of the Caspr2 protein in the juxtaparanodal region of myelinated axons[48].

There is some overlap between LGI1 and Caspr2 clinical presentations; indeed, patients with Caspr2 antibodies also often present with limbic encephalitis.

Given that Caspr2 is also located in the PNS, peripheral symptoms are more common, such as peripheral nerve hyperexcitability and Morvan syndrome, which combines neuromyotonia and neuropathic pain with hallucinations and insomnia.

Caspr2 encephalitis carries a low overall tumor association[41].



Figure 16. Sagittal and coronal sections of rat brain immunostaining with autoantibodies of patients targeting Caspr2[43].

5. Anti-GABA$_B$ Receptor Encephalitis

γ-Aminobutyric acid (GABA) type B receptor belongs to the metabotropic class of GABA receptors. It is a G protein coupled receptor (GPCR) and mediates slower, sustained inhibitory signalling through intracellular second-messenger cascades.

Functionally, the receptor is heterodimer comprised of GABA$_{B1}$ and GABA$_{B2}$ subunits (Figure 17). The first one is the GABA binding site, while the second is essential for trafficking the heterodimer to the plasma membrane and for the activation of the downstream cascades[39].

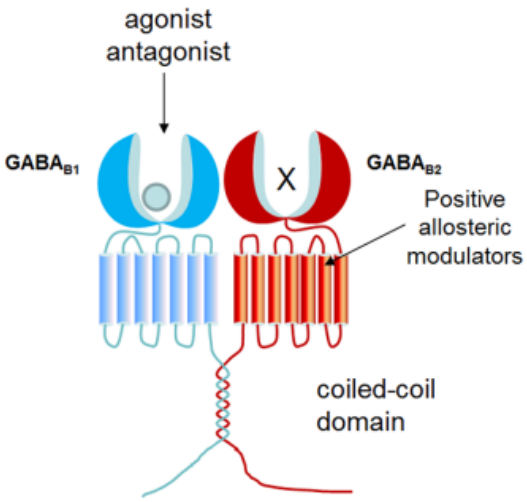


Figure 17. Structural organization of GABA$_B$ receptor[49].

The mechanism of action of autoantibodies against GABA$_B$ receptors is not the internalization of it, but the blockage of the receptor itself. This is demonstrated by

Nibber et al. (2017) in which patients' IgG modified the functionality of the receptors but not the expression of them[50].

Anti-$GABA_B$ receptor encephalitis typically manifests as limbic encephalitis with a prominent seizure phenotype.

This type of AE shows a strong association with underlying malignancy: approximately 50% of patients have an underlying small-cell lung carcinoma, while less commonly it has been associated with neuroendocrine lung tumours, malignant melanoma, and other carcinomas[41].



Figure 18. Sagittal and coronal sections of rat brain immunostaining with autoantibodies of patients targeting $GABA_B$ receptor[43].

Classification of autoantibodies identified in the study

In the study cohort, as described in the "Results" chapter, we identified three types of autoantibodies that are not considered among the "Principal autoantibodies against neuronal surface": SEZ6L2, mGluR5, and mGluR1.

1. SEZ6L2

Seizure 6-like protein 2 (SEZ6L2) is a transmembrane protein localised to the plasma membrane and endoplasmic reticulum, highly expressed in the brain, particularly in the hippocampus and cerebellum[51].

In the CNS, SEZ6L2 plays a role in intracellular protein trafficking and neuronal differentiation: it has been shown to bind the aspartic protease cathepsin D and to mediate its trafficking from the trans-Golgi network to endosomes, while proteolytic cleavage of SEZ6L2 by cathepsin D releases a soluble N-terminal fragment that

modulates neurite outgrowth and neuronal differentiation[52]. More recently, SEZ6L2 has also been shown to act as a synaptic scaffolding protein linking the AMPA receptor subunit GluA1 to adducin, thereby contributing to the regulation of excitatory synaptic connectivity[51].

SEZ6L2 autoimmunity was first described in 2014, in a patient presenting with subacute cerebellar ataxia and retinopathy[53]. Since then, it has been increasingly recognised as a rare but distinct and immunotherapy-responsive cause of subacute cerebellar ataxia[54] and is now considered a differential diagnosis of atypical parkinsonism[55]. Patients exhibit a parkinsonian phenotype; other symptoms include depression, pyramidal signs, nausea, tremor not typical of Parkinsonism, dysautonomia, sleep disturbances, and cognitive disfunction[54].

2. mGlur5

Metabotropic glutamate receptors (mGluRs) are GPCRs activated by glutamate, the main excitatory neurotransmitter of the central nervous system[56]. mGluR5 belongs to Group I metabotropic glutamate receptors, together with mGluR1, and this group has been implicated in physiological processes, including learning, memory, epilepsy, and pain processing[57].

Within the CNS mGluR5 is concentrated at postsynaptic terminals of hippocampus and amygdala neurons, and it is also expressed by microglial cells. Receptor engagement triggers Gq/G11-dependent signalling, which activates phospholipase C and, downstream, drives calcium mobilisation together with protein kinase C activity[58].

Anti-mGluR5 antibodies were first identified in 2011, in a patient with limbic encephalitis associated with Hodgkin's lymphoma (HL)[59]. Since then, anti-mGluR5 antibody-associated encephalitis has been most commonly linked to HL and frequently presents with coexisting antibodies or peripheral neuropathy[58].

Clinically, the disease is heterogeneous: no clear gender differences have been reported[58], and in the largest case series to date, all patients experienced prodromal symptoms preceding the onset of neurological manifestations, followed by behavioural and personality changes, mood disturbances, or psychiatric symptoms -

ranging from negative symptoms such as apathy and reduced verbal output to positive symptoms such as irritability, mania, and hallucination – together with cognitive deficits[58].

Although anti-mGluR5 encephalitis has traditionally been classified as a form of limbic encephalitis, extra-limbic lesions may also be present on brain MRI, either independently or in combination with limbic involvement[58]; indeed, brain abnormalities are not restricted to the mesial temporal lobes but may also involve the parieto-occipital cortex, thalamus, pons, and cerebellum[60].

Regarding pathogenicity, a recent animal model study has confirmed that mGluR5 antibodies are pathogenic, causing a reduction of mGluR5 clusters on the neuronal surface[61]. Specifically, cerebroventricular infusion of patients' IgG in mice led to memory impairment, increased anxiety, and a decrease in hippocampal mGluR5 clusters, supporting the pathogenic role of these antibodies[61].

3. mGluR1

mGluR1 is a GPCR with a particularly dense distribution across Purkinje cell dendrites in the cerebellar cortex, though its expression is not confined there: it is also detectable in the thalamus, hippocampus, globus pallidus, substantia nigra, deep cerebellar nuclei, and superior colliculus[62]. Within the cerebellar circuitry specifically, the receptor is not restricted to the postsynaptic Purkinje cell membrane but is also found along parallel and climbing fibre terminals[63], placing it at a strategic position for shaping cerebellar synaptic signalling.

Antibodies directed against mGluR1 give rise to a rare autoimmune condition affecting both the central and peripheral nervous system, in which the hallmark presentation is an acute or subacute cerebellar syndrome of variable severity[64].

Across reported cohorts, ataxia emerges overwhelmingly as the dominant clinical feature[58], consistent with the receptor's concentration in the cerebellum, although the syndrome rarely remains an isolated cerebellar disorder: behavioural changes and cognitive impairment are frequently superimposed on the motor picture[65].

Unlike many other antibody-mediated encephalitis, CSF analysis and brain imaging are frequently unremarkable, and an underlying tumour is only rarely identified[65];

when present, the oncological associations reported include testicular seminoma, cutaneous T-cell lymphoma, prostate cancer, and mantle cell non-Hodgkin lymphoma, among others[58].

Mechanistic studies have provided converging evidence for a pathogenic role of these antibodies. In non-neuronal cells engineered to express mGluR1, patient antibodies were shown to block glutamate-dependent receptor signalling[66], while in cultured rodent Purkinje cells they prevented the induction of long-term depression[67]. When infused directly into the CSF surrounding the cerebellum, these antibodies were sufficient to produce motor incoordination in mice[67], and in cultured neurons they caused a selective and significant reduction of both total and synaptic mGluR1 clusters, without affecting other synaptic markers such as PSD95[65], findings that together point to a direct, antibody-driven disruption of mGluR1-dependent cerebellar signalling as the likely basis of the clinical syndrome.

Treatment of Autoimmune Encephalitis

Treatment approaches are generally similar across all antibody-related subclasses.

First, tumor removal is performed, when present, to eliminate the trigger of antibody production.

Then, for the acute treatment aimed to ameliorating the patient's conditions, physicians can administer a combination of immunomodulators, including high-dose corticosteroids, intravenous immunoglobulin and plasma exchange, which non-specifically eliminate a portion of circulating antibodies.

However, these approaches cannot alter the underlying pathological process within the CNS.

Second-line treatment, also used as chronic treatment, may include rituximab and cyclophosphamide[68]. Rituximab is a monoclonal antibody that binds and kills B cells expressing CD20 on their surface, including precursor, mature, and memory B cells. It does not directly affect antibody-producing cells (plasma cells), since they do not

express CD20, but it reduces the generation of new plasma cells. The duration of depletion is limited, approximately six months, and depends on the individual[69].

Cyclophosphamide is a cytotoxic alkylating agent that is not cell-cycle phase-specific. Most of its antineoplastic effects are due to the phosphoramide mustard formed through hepatic metabolism of the drug by enzymes such as cytochrome P-450[70]. In this way, there is a reduction in antibody-producing cells.

Currently, two phase II clinical trials are investigating novel therapeutic approaches for AE:

- Inebilizumab (NCT04372615) is a monoclonal antibody targeting CD19, a B-cell surface antigen. It has recently demonstrated efficacy and an acceptable safety profile in neuromyelitis optica spectrum disorder (NMOSD), another antibody-mediated disorder of the CNS. Unlike rituximab, which primarily targets CD20-expressing B cells, Inebilizumab also depletes CD19-expressing plasmablast and plasma cells, potentially resulting in a broader suppression of antibody-mediated immune responses[71].
- Efgartigimod (NCT07583641) is a human IgG1 Fc fragment that binds to and inhibits the neonatal Fc receptor (FcRn), thereby preventing the recycling of IgG. This mechanism increases IgG degradation and reduces circulating IgG levels without directly affecting IgG production, resulting in a reduction of pathogenic IgG antibodies across IgG subclasses[72,73].

Diagnostic tools

The diagnosis of AE remains a challenging process, requiring the integration of clinical, neurological, radiological, and laboratory findings. AE can manifest in different ways and often resembles infectious, neoplastic, or other neurological diseases, so identifying the underlying cause is rarely straightforward. The diagnostic work-up usually combines CSF analysis, brain MRI, electroencephalography (EEG), and testing for neuronal autoantibodies in serum and CSF. Each of these tests provides different information and has its own limitations, so the results must be read together with the clinical phenotype and not considered in isolation[74,75]. Since delays in

diagnosis and treatment can worsen the outcome, the goal is to reach a reliable diagnosis quickly while ruling out alternative causes, such as infections. To facilitate this process, several diagnostic approaches have been proposed to guide the work-up of patients with suspected AE[8,75].

Focusing on the laboratory setting, Masciocchi et al. (2023) proposed an algorithm for the detection of autoantibodies in patients with suspected AE. A mainstay of this algorithm is that, since there is substantial clinical overlap in clinical symptoms between disorders associated with ICN-Abs and disorders associated with NS-Abs, most patients with suspected AE should be tested for both antibody groups[1].

As shown in the Figure 19, the algorithm is organized into three levels of testing. The first level, which can be performed in any laboratories, includes commercial fixed tissue-based assay (TBA) used in combination with line blots, and commercial fixed-CBA (discussed in more detail below). The second level, restricted to specialized referral laboratories, comprises in-house TBA and in-house CBA [see Materials and Methods]. The third level is reserved for research purposes only and includes assays on brain homogenates and neuronal cultures [see Materials and Methods][1].

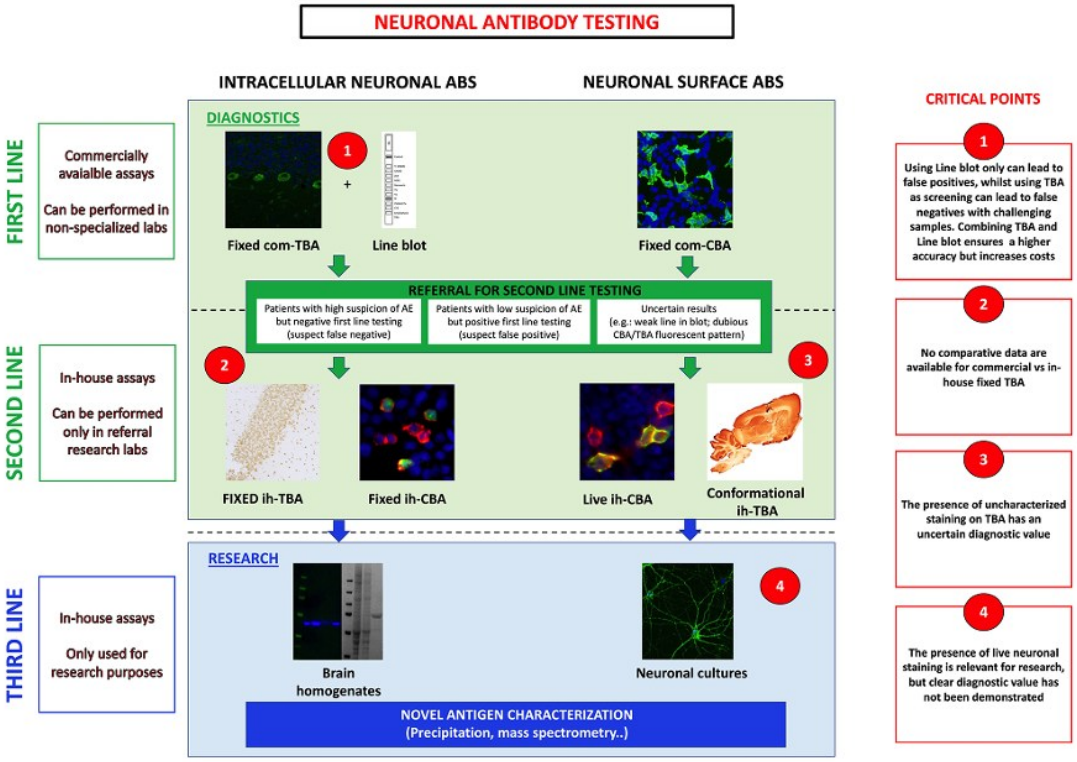


Figure 19. Proposed algorithm for detecting autoantibodies in patients with suspected AE[1].

For the detection of ICN-Abs, commercial fixed-TBA and commercial line blots are the assays most commonly used. These two techniques should be used in combination, both to compensate for inaccurate line blot results and to facilitate the detection of challenging TBA staining[1]. Thirty microliters of patient sample, diluted 1:10 in PBS-Tween, are loaded onto a single-BIOCHIP monkey cerebellum substrate (EUROIMMUN, Lübeck, Germany), followed, after 30 minutes, by 25µl of anti-human IgG antibody conjugated with fluorescein isothiocyanate, for a further 30 minutes. After mounting the slides, sample positivity can be evaluated using a fluorescence microscope (Zeiss Axioskop2). Positivity should then be confirmed using line blot. The Euroimmun immunoblot is a multiparameter line blot based on purified antigens fixed in parallel lines on nitrocellulose strips. Patient's autoantibodies bind specifically to the target antigens and are subsequently detected using an alkaline phosphatase-conjugated anti-human IgG antibody, yielding a visible dark band upon enzymatic substrate conversion. The antigens included in the kit are: amphiphysin, CV2, PNMA2 (Ma2/Ta), Ri, Yo, Hu, recoverin, SOX1, titin, zic4, GAD65 and Tr

(DNER). Following the manufacturer's protocol, strips are initially pre-treated with sample buffer on a rocking shaker and then incubated with samples diluted 1:100 in sample buffer for 30 minutes. Subsequently, the strips are incubated with an alkaline phosphatase-conjugated antibody diluted 1:10 for 30 minutes. Finally, all channels are filled with 1,5 ml of substrate solution, and when the control band is correctly coloured, the reaction is stopped with unionized water. At the end of the procedure, the strips are evaluated using EUROLineScan, which assigns a score to each band. Scores from 6 to 10 are considered borderline, scores from 11 to 50 are considered positive, and scores >50 strongly positive.

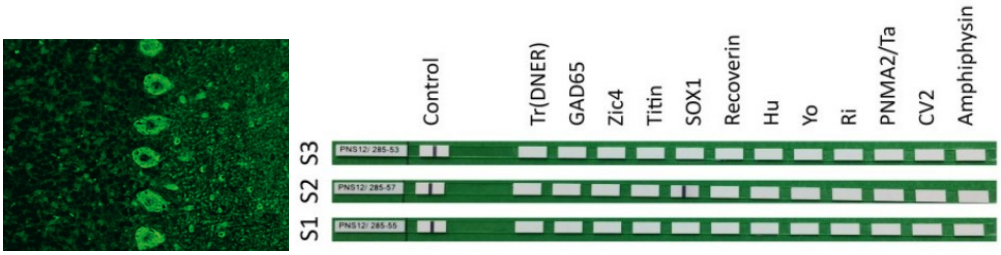


Figure 20. On the left an example of commercial fixed-TBA with positivity on Purkinje cells. On the right an example of Euroimmun strips with different antibodies detected.

Commercial fixed-CBA is used to detect NS-Abs: the Autoimmune Encephalitis Mosaic 6 by EUROIMMUN (Lübeck, Germany) consists of six BIOCHIPS per field with EU 90-transfected cells; each representing one specific antigen that can detect one specific autoantibody located in the serum or CSF of patients. This kit, for instance, allows detection of antibodies against NMDA receptor, Caspr2, AMPA receptor, LGI1, DPPX, and $GABA_B$ receptor. According to the manufacturer's instruction, patient samples are diluted 1:10 in PBS-Tween and 30µl are added to each well; after 30 minutes, 25µl of anti-human IgG antibody conjugated with fluorescein isothiocyanate are added, for a further 30 minutes. After mounting the slides, sample positivity can be evaluated using a fluorescence microscope (Zeiss Axioskop2).

Some studies have found reduced sensitivity of fixed CBA compared to live CBA; indeed, fixation may cause modifications to protein tertiary structure, preventing the binding of low-affinity autoantibodies[76]. On the other hand, fixed CBA can be considered a first-level diagnostic tool[1], as it offers relevant practical advantages,

being less technically demanding and time-consuming than live CBA, as well as more affordable[76].

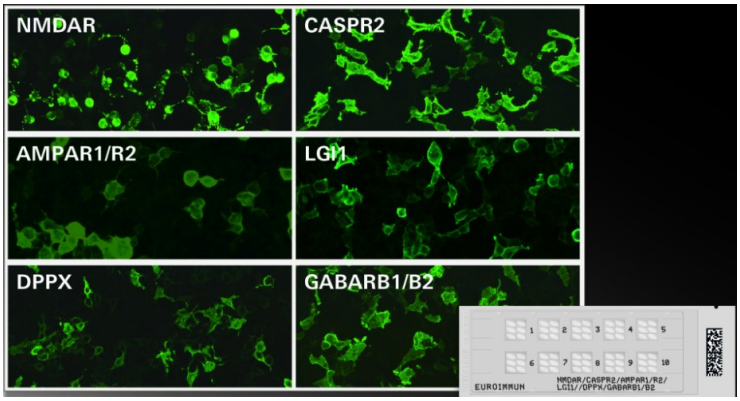


Figure 21. Euroimmun IFA: encephalitis autoimmune mosaic 6[77].

In Materials and methods section there is the discussion of the other techniques used for the detection of autoantibodies and the diagnosis of AE.

Aim of the study

This study aimed to characterise the previously uncharacterised immunohistochemical staining pattern observed in the patient cohort, with the ultimate goal of identifying novel NS-Abs through CBA analysis.

A key component of this work also involved the development and implementation of new experimental assays for the detection of novel antibodies linked to the staining pattern observed on immunohistochemistry (IHC).

This methodological approach was intended not only to support the identification of the specific antigen underlying the observed reactivity in the present cohort, but also to provide a reproducible framework that could be applied to future studies investigating novel or atypical patterns of neuronal autoimmunity.

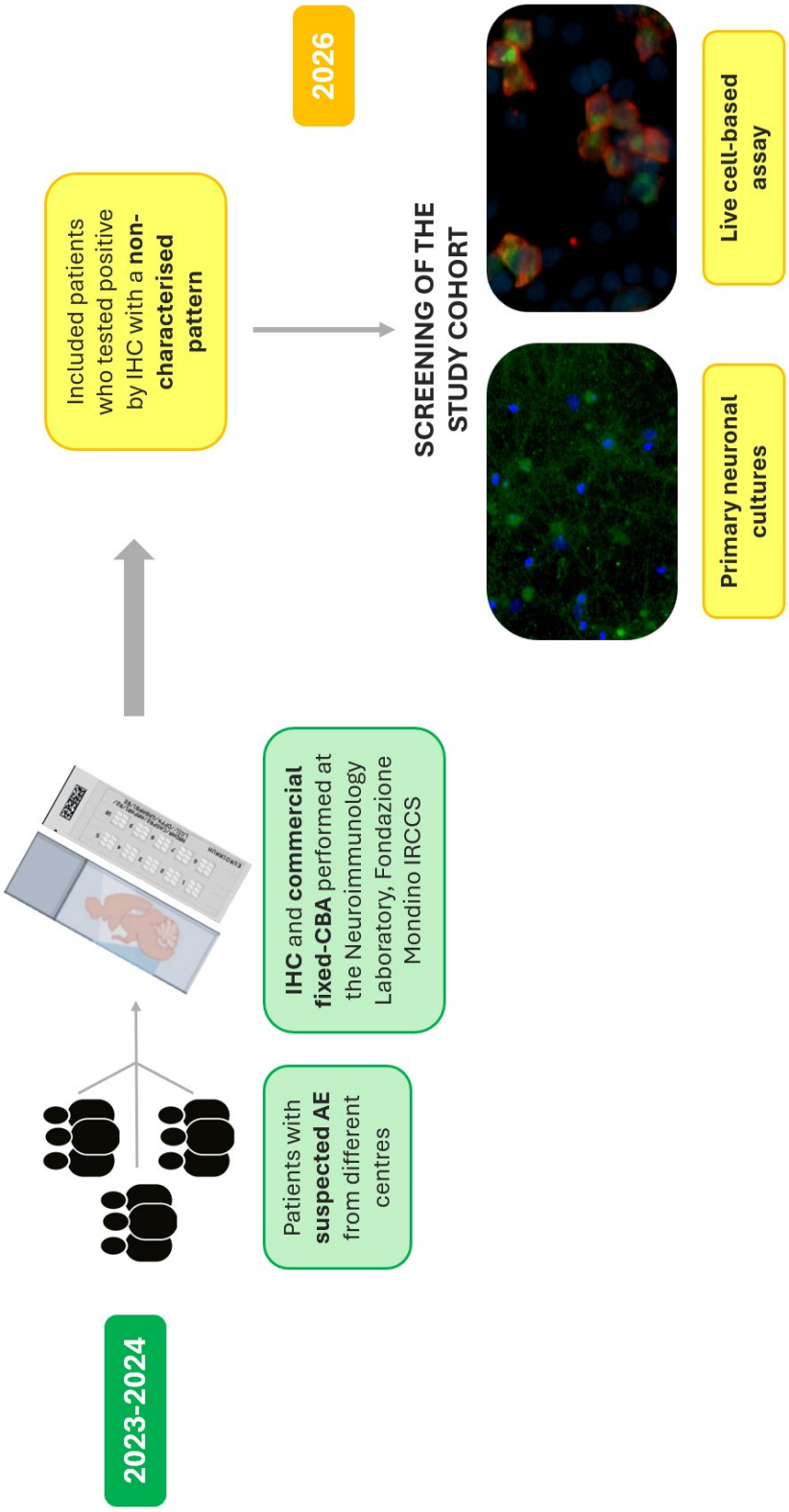


Figure 22. Study workflow illustrating the main steps of the study.

Materials and methods

STUDY COHORT								
UNC-01	UNC-02	UNC-03	UNC-04	UNC-05	UNC-06	UNC-07	UNC-08	UNC-09
UNC-10	UNC-11	UNC-12	UNC-13	UNC-14	UNC-15	UNC-16	UNC-17	UNC-18
UNC-19	UNC-20	UNC-21	UNC-22	UNC-23	UNC-24	UNC-25	UNC-26	UNC-27
UNC-28	UNC-29	UNC-30	UNC-31	UNC-32	UNC-33	UNC-34	UNC-35	UNC-36
UNC-37	UNC-38	UNC-39	UNC-40	UNC-41	UNC-42	UNC-43	UNC-44	UNC-45
UNC-46	UNC-47	UNC-48	UNC-49	UNC-50	UNC-51	UNC-52	UNC-53	UNC-54

MALE

FEMALE

Figure 23. Illustration of the study cohort, divided by sex.

This is a retrospective study in which 54 patients were recruited from multiple referring hospitals. Patients were included in the cohort based on CSF and/or serum positivity on IHC, displaying a non-characterised pattern.

Of the 54 patients enrolled, CSF samples were available for 19 patients (35%).

The cohort comprised 24 females (~44%) and 30 males (~56%), with a mean age of approximately 52 years (median age, 57 years) at the time of sample collection.

Inclusion criteria

Consecutive samples with clinical data, sent to the Laboratory of Neuroimmunology of “Fondazione IRCCS Istituto Neurologico Nazionale Casimiro Mondino” with a suspected autoimmune encephalitis, resulted positive to IHC with a non-characterised pattern (neuropilar pattern) but negative for conventional neuroimmunology panel (commercial fixed-CBA); they were collected between December 2022 and December 2024, except for five samples (three from 2020, one from 2019, and one from 2018) that were retrospectively tested by IHC in 2023-2024.

Exclusion criteria

Patients with CSF as the only available sample.

Tissue-based assay (TBA)

In-house TBA, also known as IHC, was used in the clinical setting to screen patients suspected of having AE or other autoimmune-related disorders. TBA is based on rat brain sections onto which the patient sample is loaded.

Adult Wistar rats were sacrificed with CO_2 inhalation. The brains were extracted and immersed in 4% paraformaldehyde (PFA) at 4°C for one hour, followed by immersion in 40% sucrose for 72 hours. Brains were then stored at -80°C in cooled isopentane, to prevent ice crystal formation and consequent tissue cracking. Sagittal brain slices of seven to eight micrometres in thickness were obtained using a cryostat and stored at -80°C until later use.

For the IHC protocol, slides were first thawed at room temperature and rehydrated with PBS. They were then treated with hydrogen peroxide to block endogenous peroxidase activity, which would otherwise generate false-positive background staining. Slides were subsequently incubated with 5% goat serum for one hour at room temperature, to prevent non-specific binding. Patient samples (serum diluted 1:200 in 5% goat serum, or CSF used undiluted) were then applied and incubated overnight at 4°C.

Slices were subsequently incubated with a biotinylated goat anti-human IgG antibody (BA-3000, Vector Labs), used as the secondary antibody. Immunoreactivity was detected using the Avidin-Biotin Complex (ABC; Vectastain PK-6100, Vector Labs), which binds to the secondary antibody: avidin is a tetrameric protein with high affinity for biotin, and since the secondary antibody is biotinylated, each avidin molecule can bind multiple biotin molecules, thereby amplifying the signal (Figure 24). Immunoreactivity was visualised using the chromogen 3,3'-Diaminobenzidine (DAB), which forms an insoluble brown polymer deposit at the site of antibody-antigen interaction[78]. Slices were then dehydrated by immersion in increasing alcohol concentrations (70% to 100%) and in xylene. Finally, slices were mounted with dibutylphthalate polystyrene xylene (DPX).

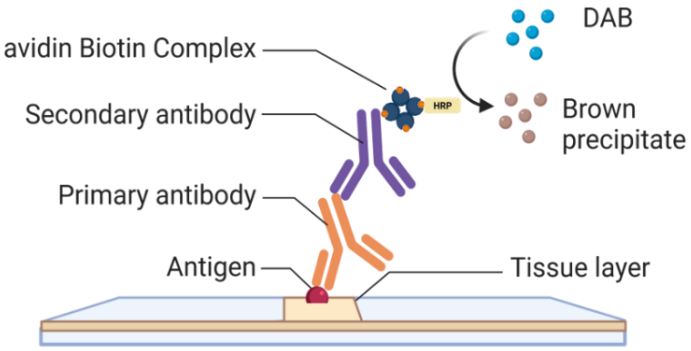


Figure 24. Principle of indirect immunohistochemistry using the avidin-biotin-peroxidase complex (ABC) method[79].

Staining patterns were evaluated with bright-field microscopy, focusing the analysis on three principal brain regions:

- Cerebellum (CB),
- Hippocampus (HIP),
- Striatum (STR).

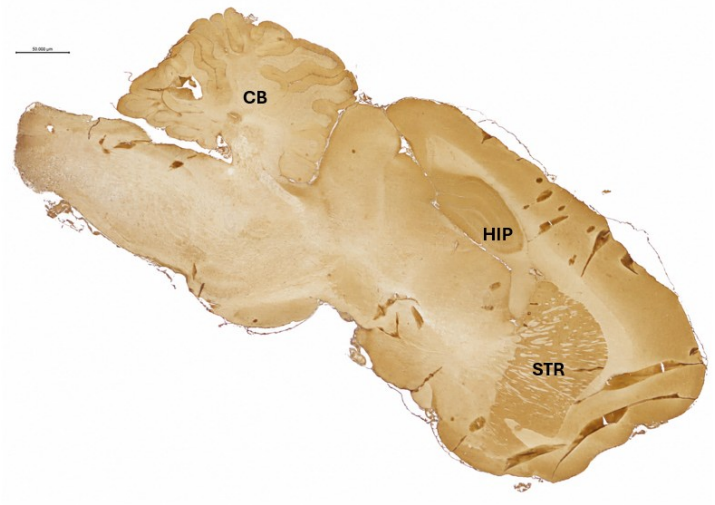


Figure 25. Immunohistochemical staining of a rat brain section.

By observing where the antibodies bind within the brain section (the resulting staining pattern), we can hypothesise about the antigenic target of the antibodies themselves.

However, it is important to emphasize that the staining pattern alone does not allow the antigenic target to be identified unequivocally. Different antibodies recognising distinct antigens may produce partially overlapping staining patterns; moreover, the distribution observed in the tissue may also be influenced by factors such as antigen accessibility, antibody affinity, and tissue processing. Consequently, the observations obtained through TBA should be considered as suggestive rather than conclusive evidence of antibody specificity.

For this reason, the hypotheses generated from TBA staining should be validated using CBA, allowing the identification of the specific antibodies present in the sample[1].

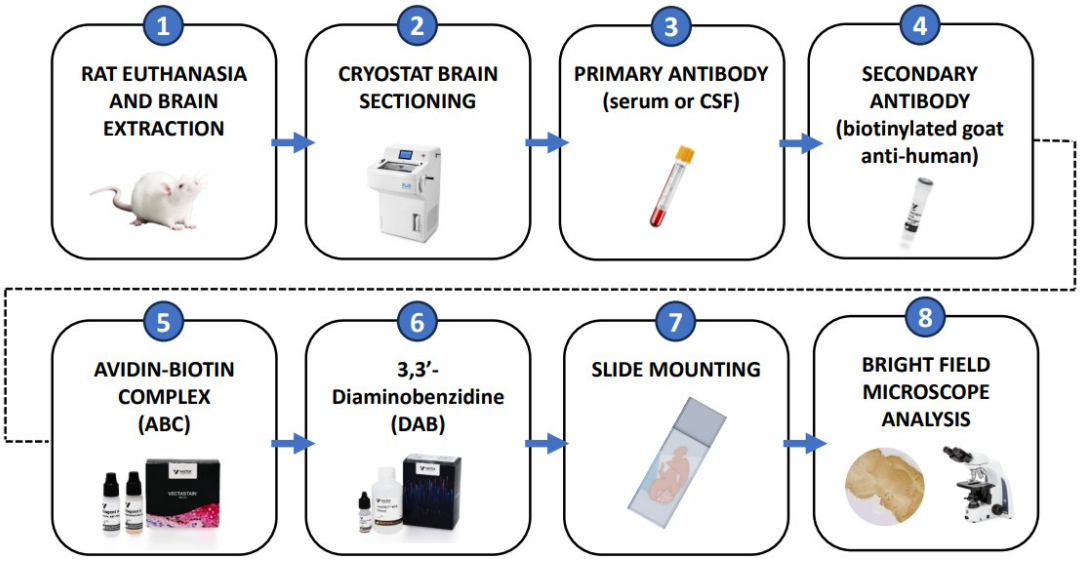


Figure 26. IHC workflow.

Live Cell-Based Assay (L-CBA)

In the current study, we used L-CBA to confirm and identify the antibody positivity resulting from IHC.

Immortalized cells, human embryonic kidney cells (HEK293T; IST Cell Factory, Genoa, Italy), were induced to express the antigen of interest through plasmid transfection, preserving its native conformation. Cells were seeded on twelve mm glass

coverslip (VWR, 631-1577) treated with poly-L-lysine (PLL, Sigma P1399), in a 6-well plate at a density of approximately $2.5*10^5$ cells per well and grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, D6429), enriched with 10% Fetal Bovine Serum (FBS; Sigma, F7524) and 1% penicillin-streptomycin (Sigma, P4333). Incubation was carried out at 37°C in a humidified atmosphere containing 5% CO_2, until cells reached 75-80% confluence.

For the transfection, plasmids were added at a concentration of 3 ug/well using Lipofectamine 2000 (concentration 6µL/well; Invitrogen, 11668027).

One day after the transfection, the test can be performed. Coverslip with transfected cells were transferred to 24-well plates containing patients' serum diluted 1:20 or 1:40, depending on the antibody, while CSF was diluted 1:5, in DMEM supplemented with 1% Bovine Serum Albumin (BSA; Sigma A7906) and 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma H337). In this study, given the limited availability of CSF, it was tested only when serum was positive.

After 45 minutes, cells are fixed with 4% PFA, and then the secondary antibody against the crystallizable fragment (Fc) segment of human immunoglobulin (Alexa Fluor 594 AffiniPure Goat Anti-Human IgG, Fcγ fragment specific, 109-585-008 or Alexa Fluor 488 AffiniPure Goat Anti-Human IgG, Fcγ fragment specific, 109-545-098) can be loaded at a concentration of 1:750 in DMEM + 1% BSA + 0.1M HEPES for 45 minutes.

At the end coverslip were mounted on slides (Epredia AA00008032E01MNZ10) with mounting fluid (Dako, S302380-2) containing 4',6-diamidino-2-phenylindole (DAPI at 1:1000 concentration, Sigma D9542) for the visualization of nuclei.

IgG subclasses were also assessed using subclass-specific antibodies against IgG1 (MH1015, Invitrogen), IgG2 (05-3500, Invitrogen), IgG3 (MH1031, Invitrogen), and IgG4 (A-10651, Invitrogen), all used at a 1:500 dilution.

Samples positivity can be observed using a fluorescence microscope (Zeiss Axioskop2), which allows visualization of the interaction between the antigen and the antibody.

Using a score from 0 to 4, the positivity can be evaluated, and endpoint titres were obtained by performing 1:2 serial dilutions[1].

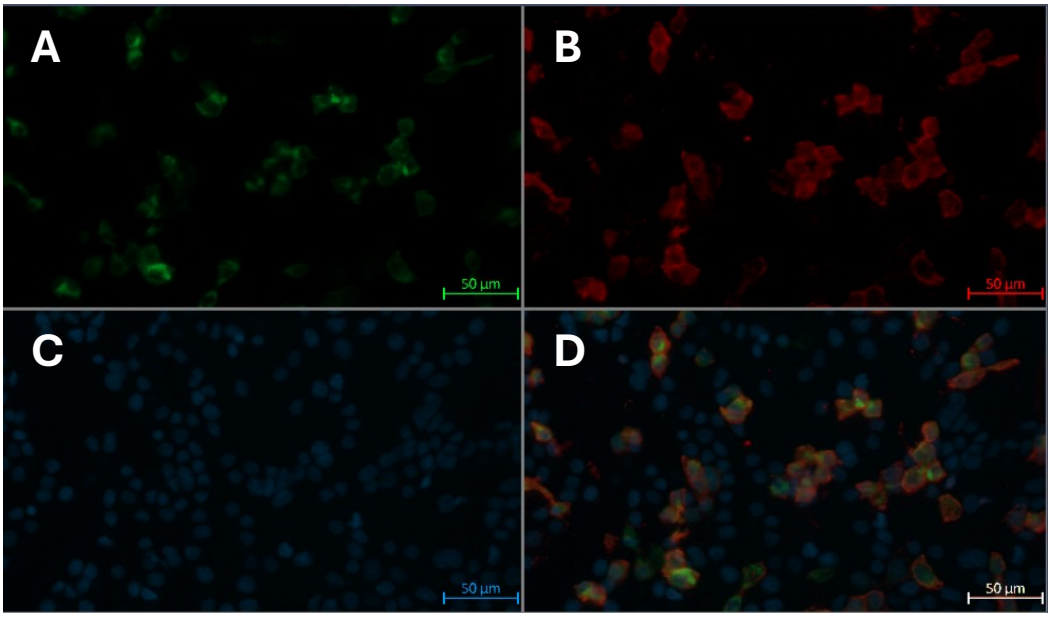


Figure 27. Example of a live-CBA. In this case, using a fluorescence microscope, positivity against mGluR5 is evaluated. Panel A shows transfection (GFP-tagged), panel B shows positivity (Fc red anti-human), panel C shows nuclei (DAPI), and panel D shows the merge of all three channels.

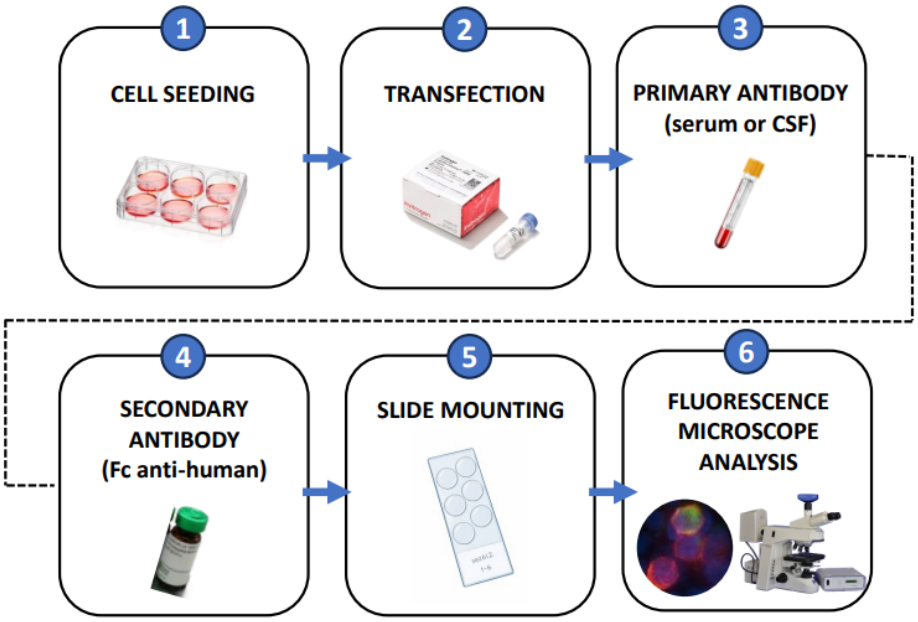


Figure 28. L-CBA workflow.

Primary neuronal cultures

The cohort was also screened on primary neuronal cultures to further confirm the presence of NS-Abs in the samples.

Neurons were isolated from embryonic hippocampus of E17-E18 rat embryos, a method that minimizes the presence of non-neuronal cells and placed in a Petri dish containing cold Hibernate medium supplemented with B27. Tissues was transferred with a glass pipette into a trypsin solution prepared at a 1:10 dilution in Hank's Balanced Salt Solution and kept for 15 minutes at room temperature for enzymatic digestion. Tissues were then placed in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, D6429) and dissociated by repeated trituration with a glass pipette. The resulting cell suspension was then resuspended in DMEM and seeded onto 10 mm coverslip, previously coated with poly-L-lysine (PLL, Sigma P1399), at a density of 10,000 neurons per coverslip in Neurobasal medium (Life technologies A21103049) and maintained under standard incubator conditions (37°C containing 5% CO_2).

Neuronal cultures were then allowed to differentiate for three weeks, with medium supplementation performed on days 7, 14, and 21. During this period, neurons developed extensive axonal and dendritic branching[80].

After 21 days in culture, the coverslips were transferred to a 48-well plate and incubated with patient samples for 45 minutes. The cells were then fixed with 4% PFA for 5 minutes and permeabilized with 0.3% Triton X-100 in PBS for 5 minutes. Samples were subsequently incubated with the secondary antibody (Alexa Fluor 488 AffiniPure Goat Anti-Human IgG, Fcγ fragment specific, 109-545-098), diluted 1:1000 in PBS containing 5% NGS, for 45 minutes. Finally, the coverslips were mounted using mounting fluid (Dako, S302380-2) containing 4',6-diamidino-2-phenylindole (DAPI at 1:1000 concentration, Sigma D9542). Positivity was assessed using a fluorescence microscope (Zeiss Axioskop2).

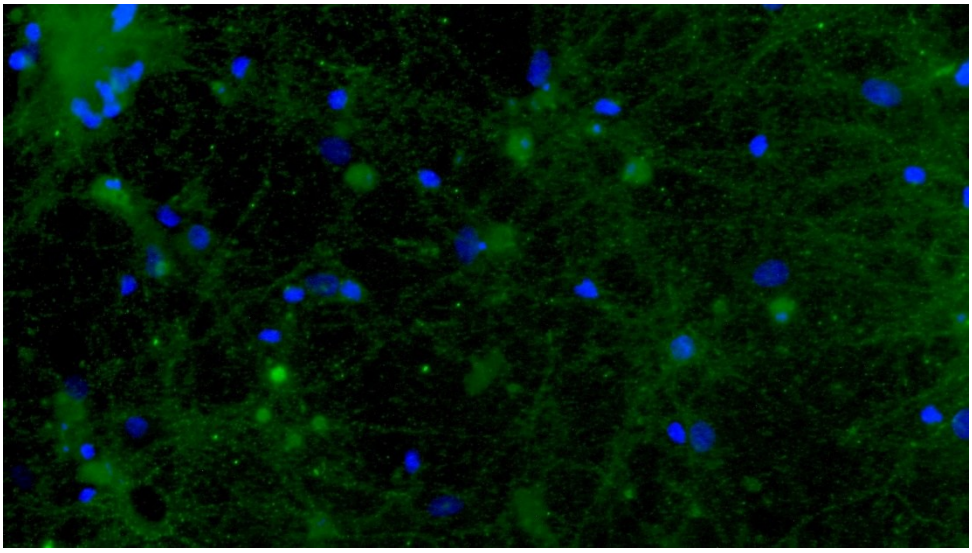


Figure 29. Example of a neuronal culture; axonal and dendritic branch positivity is shown in green, while nuclei are shown in blue.

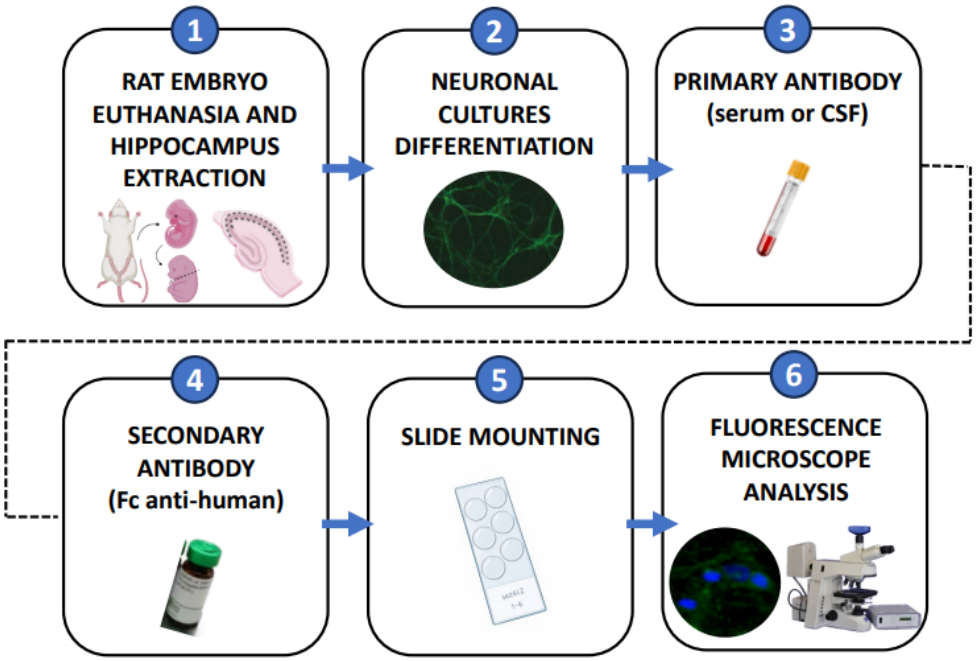


Figure 30. Primary neuronal culture workflow.

Results

- L-CBA

STUDY COHORT								
UNC-01	UNC-02	UNC-03	UNC-04	UNC-05	UNC-06	UNC-07	UNC-08	UNC-09
UNC-10	UNC-11	UNC-12	UNC-13	UNC-14	UNC-15	UNC-16	UNC-17	UNC-18
UNC-19	UNC-20	UNC-21	UNC-22	UNC-23	UNC-24	UNC-25	UNC-26	UNC-27
UNC-28	UNC-29	UNC-30	UNC-31	UNC-32	UNC-33	UNC-34	UNC-35	UNC-36
UNC-37	UNC-38	UNC-39	UNC-40	UNC-41	UNC-42	UNC-43	UNC-44	UNC-45
UNC-46	UNC-47	UNC-48	UNC-49	UNC-50	UNC-51	UNC-52	UNC-53	UNC-54

SEZ6L2
MGLUR5
MGLUR1

Figure 31. Summary of the study cohort results obtained in L-CBA. Positive patients are highlighted according to the colour coding indicated in the legend.

We screened our cohort, previously resulted positive to IHC, for twelve different antibodies with L-CBA: GABA A, GABA B, IgLON5, DPPX, mGluR1, mGluR2, mGluR5, SEZ6L2, Caspr2, NMDAR, AMPAR and GluK2.

As the figure illustrates, among the 54 patients, 4 resulted positive for at least one of the following antibodies: SEZ6L2, mGluR1, and mGluR5. Here a briefly description of positive samples: UNC-07 (female, 13 years), UNC-23 (male, 38 years), UNC-02 (male, 49 years), and UNC-33 (female, 37 years).

50% of positive patients are female/male, and the mean age is about 34 years.

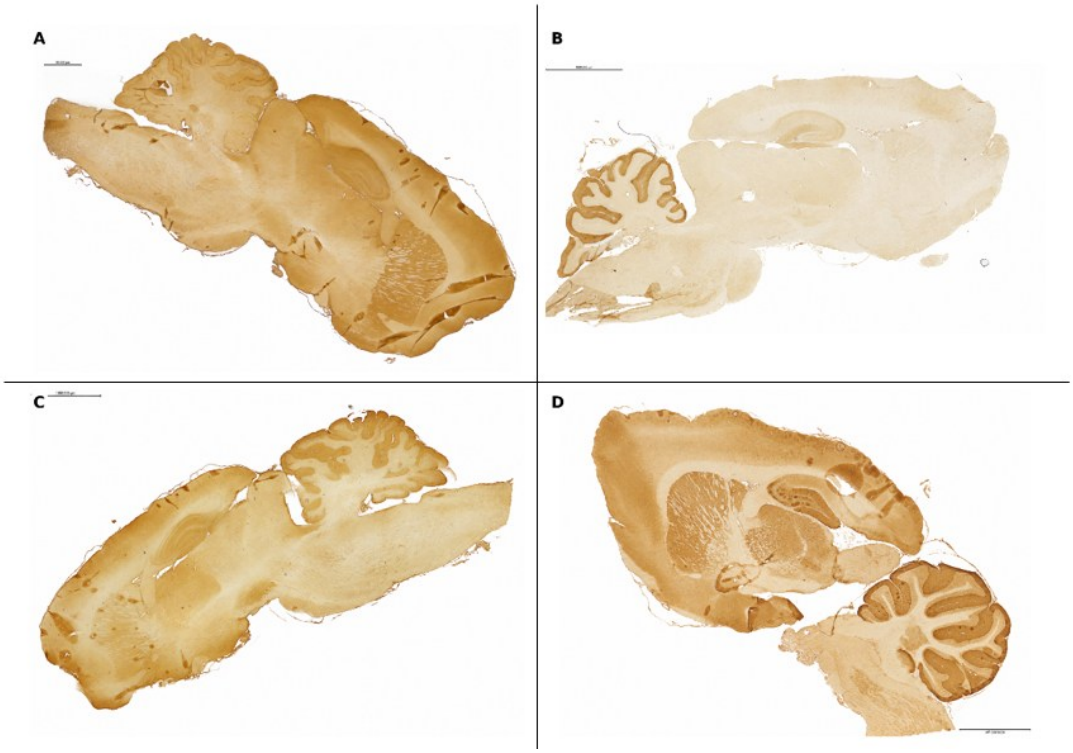


Figure 32. Representative immunohistochemical staining patterns observed in brain tissue sections from samples subsequently confirmed as positive by CBA. Panels A, B, C, and D correspond to UNC-07, UNC-02, UNC-23, and UNC-33, respectively.

- Anti-SEZ6L2 positive patients

Three patients (UNC-07, UNC-23, UNC-02) tested positive for anti-SEZ6L2 antibodies. UNC-07 revealed an endpoint titre of 1:2560, also confirmed in the CSF; UNC-23 and UNC-02 revealed an endpoint titre of 1:80, and in both cases the reactivity could not be confirmed in the CSF.

With regard to the IgG subclasses, the anti-SEZ6L2 antibodies of UNC-07 and UNC-02 were of the IgG1 subclass, whereas those of UNC-23 belonged to the IgG2 subclass. This difference in subclass distribution indicates that the antibody response against SEZ6L2 is not homogeneous across the cohort.

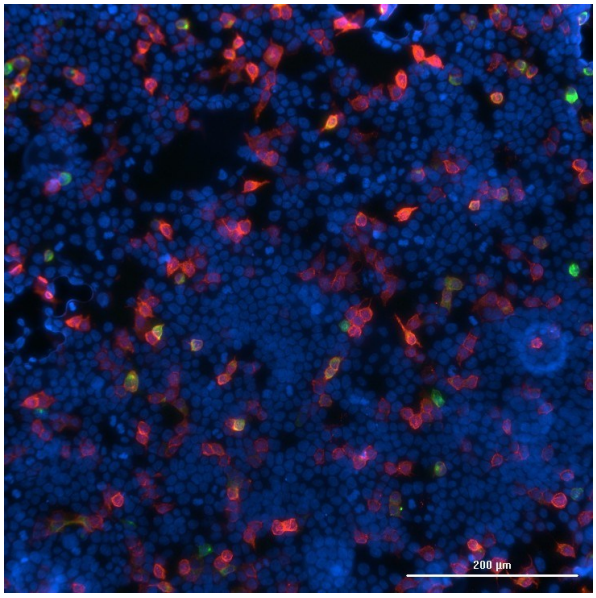


Figure 33. UNC-07 SEZ6L2 positivity: red indicates the positivity, green indicates the cell transfection with the plasmid, and blue indicates the nuclei. Image acquired using Agilent BioTek Cytation 5 Cell Imaging Multi-Mode Reader.

- Anti-mGluR1 positive patients

One patient (UNC-33) resulted positive for anti-mGluR1 antibodies, with an endpoint titre of 1:2560, also confirmed in CSF. In this specific case, UNC-33 resulted positive to both IgG1 and IgG2 subclasses, suggesting a heterogeneous IgG response against mGluR1.

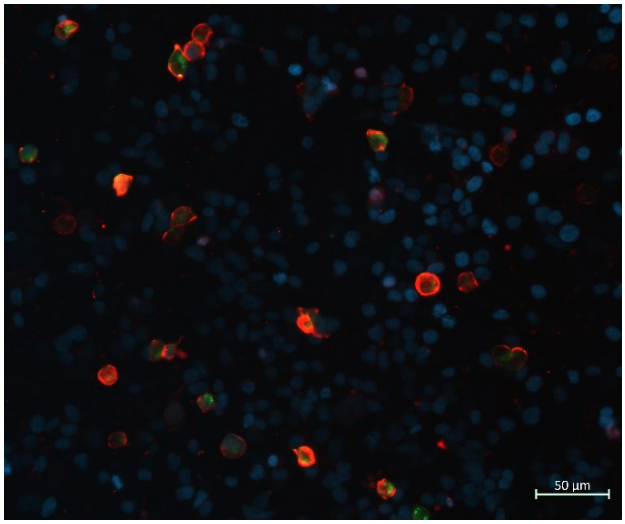


Figure 34. UNC-33 mGluR1 positivity: red indicates the positivity, green indicates the cell transfection with the plasmid, and blue indicates the nuclei. Image acquired using Zeiss Axioskop2.

- Anti-mGluR5 positive patients

One patient (UNC-07) resulted positive for anti-mGluR5 antibodies, with an endpoint titre of 1:20480, also confirmed in CSF. In this specific case, UNC-07 resulted positive to IgG1 subclass.

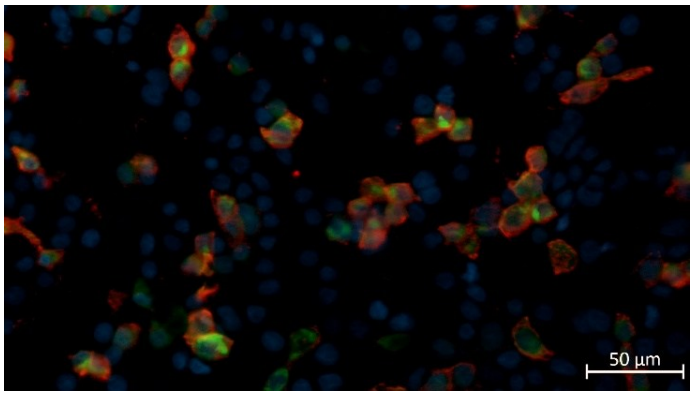


Figure 35. UNC-07 mGluR5 positivity: red indicates the positivity, green indicates the cell transfection with the plasmid, and blue indicates the nuclei. Image acquired using Zeiss Axioskop2.

- Primary neuronal cultures

STUDY COHORT								
UNC-01	UNC-02	UNC-03	UNC-04	UNC-05	UNC-06	UNC-07	UNC-08	UNC-09
UNC-10	UNC-11	UNC-12	UNC-13	UNC-14	UNC-15	UNC-16	UNC-17	UNC-18
UNC-19	UNC-20	UNC-21	UNC-22	UNC-23	UNC-24	UNC-25	UNC-26	UNC-27
UNC-28	UNC-29	UNC-30	UNC-31	UNC-32	UNC-33	UNC-34	UNC-35	UNC-36
UNC-37	UNC-38	UNC-39	UNC-40	UNC-41	UNC-42	UNC-43	UNC-44	UNC-45
UNC-46	UNC-47	UNC-48	UNC-49	UNC-50	UNC-51	UNC-52	UNC-53	UNC-54

ONLY SERUM

SERUM + CSF

Figure 36. Summary of the study cohort results obtained in primary neuronal cultures. Positive patients are highlighted according to the colour coding indicated in the legend.

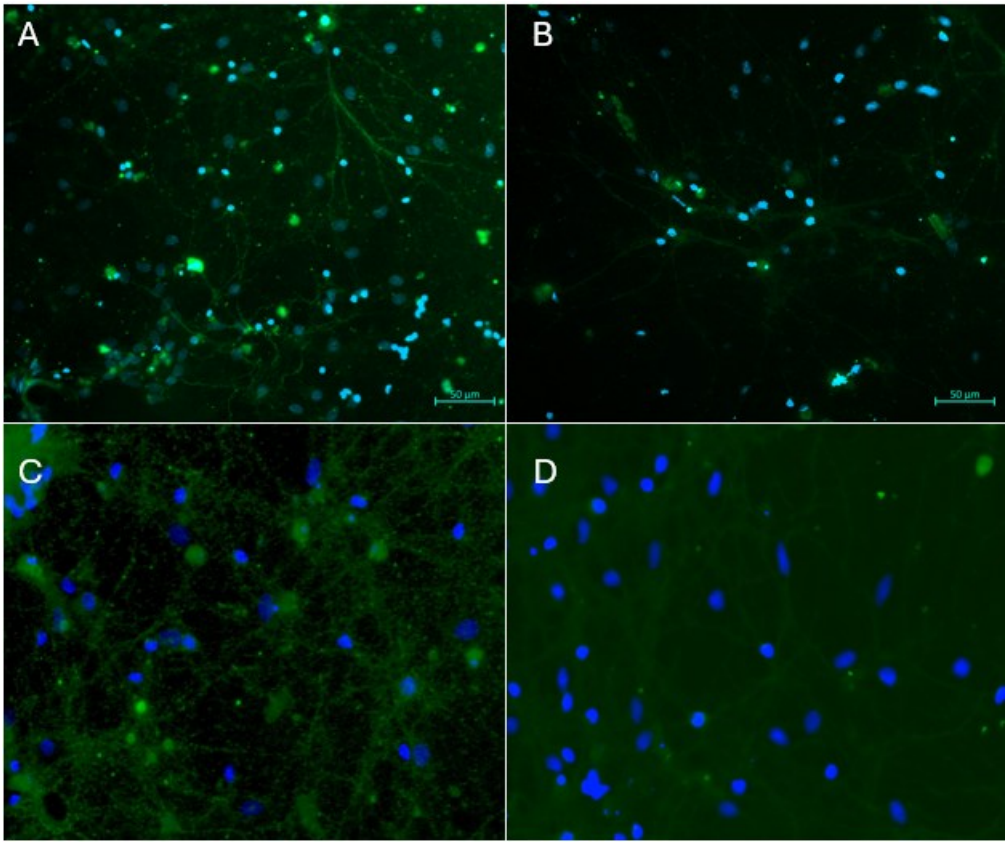


Figure 37. Example of positivity to neuronal cultures. Panel A shows UNC-37, panel B shows UNC-23, panel C shows UNC-07, and panel D shows UNC-05. Image acquired using Zeiss Axioskop2.

Among the 54 patients, 8 tested positive for primary neuronal cultures, suggesting in this way the presence of NS-Abs.

As the image illustrates some patients resulted positive for both serum and CSF: UNC-07 (female, 13 years), UNC-14 (male, 72 years), and UNC-33 (female, 37 years); while UNC-05 (female, 62 years), UNC-23 (male, 38 years), UNC-34 (male, 16 years), UNC-37 (female, 10 years), and UNC-50 (female, 78 years) were positive only in serum (due to the absence of it (UNC-34, UNC-37 and UNC-50) or for its negativity (UNC-05 and UNC-23)).

62,5% of positive patients are female, and the mean age is about 40 years.

Discussion

The screening of the study cohort with L-CBA against twelve neuronal surface antigens identified SEZ6L2, mGluR1, and mGluR5 as the targets underlying reactivity in 4 out of 54 patients, extending the antigenic characterisation of this cohort beyond antibodies already well established in clinical practice.

The three SEZ6L2-positive patients (UNC-07, UNC-23, UNC-02) are of particular interest, since anti-SEZ6L2 antibody-associated syndrome has been described in only a small number of reports, starting with the first patient reported by Yaguchi et al. (2014)[53] and followed by the case series by Landa et al. (2021)[51] and Kather et al. (2022)[81]. The marked difference in endpoint titre between UNC-07 (1:2560) and UNC-23/UNC02 (1:80), together with the fact that only UNC-07 showed CSF-confirmed reactivity, suggests that these three cases may not represent a homogeneous group. UNC-07 appears to display a more robust and clinically relevant antibody response, whereas the low-titre, serum-restricted reactivity observed in UNC-23 and UNC-02 raises the possibility of a less specific or even inaccurate finding.

Regarding IgG subclass distribution, UNC-07 and UNC-02 were IgG1-positive, while UNC-23 showed an IgG2 response. This partially overlap with Landa et al. (2021), who found IgG4 SEZ6L2-antibodies in all four of their patients, with IgG1 co-occurring in three of them[51]. Since IgG4 is generally poor activators of the classical complement pathway, whereas IgG1 efficiently fixes C1q and activates complement[82], the IgG1 pattern observed in the study cohort raises the possibility of a distinct, and potentially more complement-dependent pathogenic mechanism than in the previously described cases. This discrepancy could reflect true biological heterogeneity of the anti-SEZ6L2 humoral response across patients, methodological differences between subclass assays, or the limited sample size of both studies, and warrants further investigation in larger cohorts.

UNC-07 testes positive to mGluR5 antibodies, at a very high titre (1:20480) confirmed in the CSF, and IgG1 positivity, consistent with the findings of Spatola et al. (2018)[60]. The coexistence of SEZ6L2 and mGluR5 antibodies in the same patient raises the question of whether it reflects true antigenic co-reactivity, a broader breakdown of immune tolerance, or a technical cross-reactivity, and deserves further investigation.

Finally, UNC-33 tested positive for mGluR1 antibodies, with a high endpoint titre (1:2560) confirmed in the CSF, and reactivity for both IgG1 and IgG2, showing that the humoral response can be heterogeneous even within a single serum. The subclass positivity was, in part, in line with the previous publication, who founded IgG1 as the main subclass founded in their cohort[65].

Taken together, these L-CBA findings explain only a fraction of the reactivity detected by IHC and, more strikingly, by primary neuronal culture assays, where 8 of 54 patients tested positive, indicating the presence of NS-Abs not captured by the twelve antibodies screened. This is consistent with the complementarity of the two techniques: unlike CBA on HEK293 cells, which is limited by the specific repertoire of transfected antigens, primary neuronal cultures preserve the native conformation, density and membrane context of surface proteins, and can therefore reveal reactivity against antigens that are absent, underrepresented, or conformationally altered in heterologous expression systems[83,39]. However, since these cultures are derived from mouse embryos, antibodies directed against epitopes not conserved between species may go undetected.

The discrepancy observed in UNC-02, who tested positive for SEZ6L2 by L-CBA at a low titre but negative on primary neuronal culture, is particularly relevant to the aims of this study. It shows that neither assay can by itself definitively confirm or exclude the presence of neuronal surface antibodies. A negative result on neurons does not rule out antibodies, which may be missed because of differences in antigen expression, conformation or assay sensitivity. Further studies are therefore needed to clarify this discrepancy and establish whether the SEZ6L2 reactivity in UNC-02 is genuine. Conversely, patients who tested positive on primary neuronal cultures but negative for the antigens screened by L-CBA (4 of 8 patients) may have autoantibodies against targets not included in the study, whether already known or yet to be identified. In these cases, expanding the antigen panel will be necessary to determine the specific target.

Overall, these results suggest that a substantial fraction of NS-Abs reactivity in this IHC-positive cohort remains unexplained by the currently available antigen panel.

Patients positive on primary neuronal culture but negative for all twelve antigens screened by L-CBA are the most promising subset for the discovery of novel, still uncharacterised autoantigens.

This study has some limitations. First, the cohort is limited (54 patients), so the results should be considered preliminary, and the frequency of each antibody cannot be generalized. Second, the screening was performed on serum, while CSF was tested only in patients whose serum was already positive on L-CBA, to confirm their reactivity. As a result, reactivities present only, or predominantly, in the CSF may have been missed in the remaining patients. Finally, the low-titre reactivities, such as those of UNC-23 and UNC-02, need further confirmation.

Conclusions

In conclusion, screening the study cohort with L-CBA identified four patients positive for SEZ6L2, mGluR5 or mGluR1. However, many patients who were positive on IHC, and especially those positive on primary neuronal cultures, still have no identified target. For this reason, the next steps should be to look for new candidate antigens and test them with L-CBA, to enlarge the study cohort, and to test the CSF of all patients alongside serum.

Bibliography

1. Masciocchi, S. *et al.* General features, pathogenesis, and laboratory diagnostics of autoimmune encephalitis. *Critical Reviews in Clinical Laboratory Sciences* **61**, 45–69 (2024).
2. Abboud, H. *et al.* Autoimmune encephalitis: proposed best practice recommendations for diagnosis and acute management. *J Neurol Neurosurg Psychiatry* **92**, 757–768 (2021).
3. Kim, T. *et al.* Anti-LGI1 encephalitis is associated with unique HLA subtypes. *Annals of Neurology* **81**, 183–192 (2017).
4. Van Sonderen, A. *et al.* Anti- LGI1 encephalitis is strongly associated with HLA-DR7 and HLA-DRB4. *Annals of Neurology* **81**, 193–198 (2017).
5. Armangue, T. *et al.* Herpes simplex virus encephalitis is a trigger of brain autoimmunity. *Annals of Neurology* **75**, 317–323 (2014).
6. Yucel, Y., Sidow, N. O. & Yilmaz, A. Approach and overview of autoimmune encephalitis: A review. *Medicine* **104**, e42472 (2025).
7. Yang, M. & Lian, Y. Clinical Features and Early Recognition of 242 Cases of Autoimmune Encephalitis. *Front. Neurol.* **12**, 803752 (2022).
8. Graus, F. *et al.* A clinical approach to diagnosis of autoimmune encephalitis. *The Lancet Neurology* **15**, 391–404 (2016).
9. Autoimmune encephalitis. *Nat Rev Dis Primers* **11**, 66 (2025).
10. Devine, M. F., Kothapalli, N., Elkhooly, M. & Dubey, D. Paraneoplastic neurological syndromes: clinical presentations and management. *Ther Adv Neurol Disord* **14**, 1756286420985323 (2021).
11. Graus, F. *et al.* Updated Diagnostic Criteria for Paraneoplastic Neurologic Syndromes. *Neurol Neuroimmunol Neuroinflamm* **8**, e1014 (2021).
12. Lam, N., Lee, Y. & Farber, D. L. A guide to adaptive immune memory. *Nat Rev Immunol* **24**, 810–829 (2024).

13. Song, Y., Li, J. & Wu, Y. Evolving understanding of autoimmune mechanisms and new therapeutic strategies of autoimmune disorders. *Sig Transduct Target Ther* **9**, 263 (2024).

14. Yan, L. *et al.* A Unique Perspective on Auto-reactive Antibody Production in Autoimmune Disease Induced by Microbiome. *Front. Biosci. (Landmark Ed)* **31**, 45424 (2026).

15. Weill, J.-C. & Reynaud, C.-A. The ups and downs of negative (and positive) selection of B cells. *Journal of Clinical Investigation* **125**, 3748–3750 (2015).

16. Kenison, J. E., Stevens, N. A. & Quintana, F. J. Therapeutic induction of antigen-specific immune tolerance. *Nat Rev Immunol* **24**, 338–357 (2024).

17. Dendrou, C. A., Petersen, J., Rossjohn, J. & Fugger, L. HLA variation and disease. *Nat Rev Immunol* **18**, 325–339 (2018).

18. Pisetsky, D. S. Pathogenesis of autoimmune disease. *Nat Rev Nephrol* **19**, 509–524 (2023).

19. Suurmond, J. & Diamond, B. Autoantibodies in systemic autoimmune diseases: specificity and pathogenicity. *J. Clin. Invest.* **125**, 2194–2202 (2015).

20. Dalmau, J. *et al.* Paraneoplastic anti– *N* -methyl- D -aspartate receptor encephalitis associated with ovarian teratoma. *Annals of Neurology* **61**, 25–36 (2007).

21. Day, G. S., Laiq, S., Tang-Wai, D. F. & Munoz, D. G. Abnormal Neurons in Teratomas in NMDAR Encephalitis. *JAMA Neurol* **71**, 717 (2014).

22. Bien, C. G. *et al.* Immunopathology of autoantibody-associated encephalitides: clues for pathogenesis. *Brain* **135**, 1622–1638 (2012).

23. Hughes, E. G. *et al.* Cellular and Synaptic Mechanisms of Anti-NMDA Receptor Encephalitis. *J. Neurosci.* **30**, 5866–5875 (2010).

24. Valenzuela, N. M. & Schaub, S. The Biology of IgG Subclasses and Their Clinical Relevance to Transplantation. *Transplantation* **102**, S7–S13 (2018).

25. Britannica Editors. Antibody. (2026).

26. Vidarsson, G., Dekkers, G. & Rispens, T. IgG Subclasses and Allotypes: From Structure to Effector Functions. *Front. Immunol.* **5**, (2014).

27. Koneczny, I. *et al.* IgG4 Autoantibodies in Organ-Specific Autoimmunopathies: Reviewing Class Switching, Antibody-Producing Cells, and Specific Immunotherapies. *Front. Immunol.* **13**, 834342 (2022).

28. Tanaka, K. *et al.* Passive transfer and active immunization with the recombinant leucine-zipper (Yo) protein as an attempt to establish an animal model of paraneoplastic cerebellar degeneration. *Journal of the Neurological Sciences* **127**, 153–158 (1994).

29. Greenlee, J. E. *et al.* Paraneoplastic and Other Autoimmune Encephalitides: Antineuronal Antibodies, T Lymphocytes, and Questions of Pathogenesis. *Front. Neurol.* **12**, 744653 (2022).

30. Marsili, L. *et al.* Paraneoplastic Neurological Syndromes of the Central Nervous System: Pathophysiology, Diagnosis, and Treatment. *Biomedicines* **11**, 1406 (2023).

31. Zekeridou, A. & Lennon, V. A. Neurologic Autoimmunity in the Era of Checkpoint Inhibitor Cancer Immunotherapy. *Mayo Clinic Proceedings* **94**, 1865–1878 (2019).

32. Darnell, R. B. Onconeural antigens and the paraneoplastic neurologic disorders: at the intersection of cancer, immunity, and the brain. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 4529–4536 (1996).

33. Diamond, B., Huerta, P. T., Mina-Osorio, P., Kowal, C. & Volpe, B. T. Losing your nerves? Maybe it's the antibodies. *Nat Rev Immunol* **9**, 449–456 (2009).

34. Boes, M. Role of natural and immune IgM antibodies in immune responses. *Molecular Immunology* **37**, 1141–1149 (2000).

35. Sun, B., Ramberger, M., O'Connor, K. C., Bashford-Rogers, R. J. M. & Irani, S. R. The B cell immunobiology that underlies CNS autoantibody-mediated diseases. *Nat Rev Neurol* **16**, 481–492 (2020).

36. Leyendeckers, H. *et al.* Correlation analysis between frequencies of circulating antigen-specific IgG-bearing memory B cells and serum titers of antigen-specific IgG. *Eur. J. Immunol.* **29**, 1406–1417 (1999).

37. Asztely, F. & Kumlien, E. The diagnosis and treatment of limbic encephalitis. *Acta Neurol Scand* **126**, 365–375 (2012).

38. Graus, F. *et al.* Syndrome and outcome of antibody-negative limbic encephalitis. *Euro J of Neurology* **25**, 1011–1016 (2018).

39. Dalmau, J., Geis, C. & Graus, F. Autoantibodies to Synaptic Receptors and Neuronal Cell Surface Proteins in Autoimmune Diseases of the Central Nervous System. *Physiological Reviews* **97**, 839–887 (2017).

40. Michalski, K. *et al.* Structural and functional mechanisms of anti-NMDAR autoimmune encephalitis. *Nat Struct Mol Biol* **31**, 1975–1986 (2024).

41. Nissen, M. S., Ryding, M., Meyer, M. & Blaabjerg, M. Autoimmune Encephalitis: Current Knowledge on Subtypes, Disease Mechanisms and Treatment. *CNSNDDT* **19**, 584–598 (2020).

42. Mohn, A. R., Gainetdinov, R. R., Caron, M. G. & Koller, B. H. Mice with Reduced NMDA Receptor Expression Display Behaviors Related to Schizophrenia. *Cell* **98**, 427–436 (1999).

43. Dalmau, J. NMDA receptor encephalitis and other antibody-mediated disorders of the synapse: The 2016 Cotzias Lecture. *Neurology* **87**, 2471–2482 (2016).

44. Shepherd, J. D. & Huganir, R. L. The Cell Biology of Synaptic Plasticity: AMPA Receptor Trafficking. *Annu. Rev. Cell Dev. Biol.* **23**, 613–643 (2007).

45. Palmer, C. L., Cotton, L. & Henley, J. M. The Molecular Pharmacology and Cell Biology of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptors. *Pharmacological Reviews* **57**, 253–277 (2005).

46. Höftberger, R. *et al.* Encephalitis and AMPA receptor antibodies: Novel findings in a case series of 22 patients. *Neurology* **84**, 2403–2412 (2015).

47. Baudin, P., Cousyn, L. & Navarro, V. The LGI1 protein: molecular structure, physiological functions and disruption-related seizures. *Cell. Mol. Life Sci.* **79**, 16 (2022).

48. Van Sonderen, A., Petit-Pedrol, M., Dalmau, J. & Titulaer, M. J. The value of LGI1, Caspr2 and voltage-gated potassium channel antibodies in encephalitis. *Nat Rev Neurol* **13**, 290–301 (2017).

49. Xu, C., Zhang, W., Rondard, P., Pin, J.-P. & Liu, J. Complex GABAB receptor complexes: how to generate multiple functionally distinct units from a single receptor. *Front. Pharmacol.* **5**, (2014).

50. Nibber, A. *et al.* Pathogenic potential of antibodies to the $GABA_B$ receptor. *Epilepsia Open* **2**, 355–359 (2017).

51. Landa, J. *et al.* Seizure-related 6 homolog like 2 autoimmunity: Neurologic syndrome and antibody effects. *Neurol Neuroimmunol Neuroinflamm* **8**, e916 (2021).

52. Boonen, M. *et al.* Cathepsin D and its newly identified transport receptor SEZ6L2 can modulate neurite outgrowth. *Journal of Cell Science* **129**, 557–568 (2016).

53. Yaguchi, H. *et al.* Identification of anti-Sez6l2 antibody in a patient with cerebellar ataxia and retinopathy. *J Neurol* **261**, 224–226 (2014).

54. Hoshina, Y., Kobayashi, G. & Reda, H. Clinical Spectrum of SEZ6L2 Autoimmunity: A Case Report and Systematic Review. *Cerebellum* **25**, 51 (2026).

55. Borsche, M. *et al.* Sez6l2-antibody-associated progressive cerebellar ataxia: a differential diagnosis of atypical parkinsonism. *J Neurol* **266**, 522–524 (2019).

56. Mazzitelli, M., Presto, P., Antenucci, N., Meltan, S. & Neugebauer, V. Recent Advances in the Modulation of Pain by the Metabotropic Glutamate Receptors. *Cells* **11**, 2608 (2022).

57. Chemin, J. *et al.* Mechanisms underlying excitatory effects of group I metabotropic glutamate receptors via inhibition of 2P domain K+ channels. *EMBO J* **22**, 5403–5411 (2003).

58. Liu, Y. *et al.* Anti-mGluR encephalitis: case series and literature review. *BMC Neurol* **26**, 150 (2026).

59. Lancaster, E. *et al.* Antibodies to metabotropic glutamate receptor 5 in the Ophelia syndrome. *Neurology* **77**, 1698–1701 (2011).

60. Spatola, M. *et al.* Encephalitis with mGluR5 antibodies: Symptoms and antibody effects. *Neurology* **90**, (2018).

61. Maudes, E. *et al.* Human Metabotropic Glutamate Receptor 5 Antibodies Alter Receptor Levels and Behavior in Mice. *Annals of Neurology* **92**, 81–86 (2022).

62. Martin, L. J., Blackstone, C. D., Huganir, R. L. & Price, D. L. Cellular localization of a metabotropic glutamate receptor in rat brain. *Neuron* **9**, 259–270 (1992).

63. Yoshikura, N. *et al.* Long-term clinical follow-up of a patient with non-paraneoplastic cerebellar ataxia associated with anti-mGluR1 autoantibodies. *Journal of Neuroimmunology* **319**, 63–67 (2018).

64. Khojah, O., Makkawi, S. & Alghamdi, S. Anti-mGluR1 encephalitis: Case illustration and systematic review. *Front. Neurol.* **14**, 1142160 (2023).

65. Spatola, M. *et al.* Clinical features, prognostic factors, and antibody effects in anti-mGluR1 encephalitis. *Neurology* **95**, (2020).

66. Smitt, P. S. *et al.* Paraneoplastic Cerebellar Ataxia Due to Autoantibodies against a Glutamate Receptor. *N Engl J Med* **342**, 21–27 (2000).

67. Coesmans, M. *et al.* Mechanisms underlying cerebellar motor deficits due to mGluR1-autoantibodies. *Annals of Neurology* **53**, 325–336 (2003).

68. Bartolini, L. & Muscal, E. Differences in treatment of anti-NMDA receptor encephalitis: results of a worldwide survey. *J Neurol* **264**, 647–653 (2017).

69. Randall, K. L. Rituximab in autoimmune diseases. *Aust Prescr* **39**, 131–134 (2016).

70. Ogino, M. H. & Tadi, P. Cyclophosphamide. in *StatPearls* (StatPearls Publishing, Treasure Island (FL), 2023).

71. Wong, K.-H. *et al.* A Phase-2B Double-Blind Randomized International Prospective Trial of Inebilizumab in NMDAR Encephalitis: The ExTINGUISH Trial. *Neurotherapeutics* **23**, e00980 (2026).

72. Argenx. A Study to Learn About How Well the Medicine Efgartigimod Works to Treat Autoimmune Encephalitis In Children 12 Years or Older and Adults. *All Clinical Trials* https://www.allclinicaltrials.com/study/NCT07583641?utm_source=chatgpt.com (2026).

73. Howard, J. F. *et al.* Subcutaneous efgartigimod PH20 in generalized myasthenia gravis: A phase 3 randomized noninferiority study (ADAPT-SC) and interim analyses of a long-term open-label extension study (ADAPT-SC+). *Neurotherapeutics* **21**, e00378 (2024).

74. Lee, S. K. & Lee, S.-T. The Laboratory Diagnosis of Autoimmune Encephalitis. *J Epilepsy Res* **6**, 45–50 (2016).

75. Abboud, H. *et al.* Autoimmune encephalitis: proposed best practice recommendations for diagnosis and acute management. *J Neurol Neurosurg Psychiatry* **92**, 757–768 (2021).

76. Spagni, G. *et al.* Comparison of Fixed and Live Cell-Based Assay for the Detection of AChR and MuSK Antibodies in Myasthenia Gravis. *Neurol Neuroimmunol Neuroinflamm* **10**, e200038 (2023).

77. Clinical Laboratory International. Together at the pulse of neurobiology. *Clinical Laboratory International* https://clinlabint.com/together-at-the-pulse-of-neurobiology/?utm_source=chatgpt.com (2024).

78. Ramos-Vara, J. A. Technical Aspects of Immunohistochemistry. *Vet Pathol* **42**, 405–426 (2005).

79. OriGene Technologies, Inc. Signal Amplification Using Chromogenic Detection. *OriGene Technologies* https://www.origene.com/products/assay-kits/ihc-kits-reagents/chromogenic-detection.

80. Sahu, M. P., Nikkilä, O., Lågas, S., Kolehmainen, S. & Castrén, E. Culturing primary neurons from rat hippocampus and cortex. *Neuronal Signaling* **3**, NS20180207 (2019).

81. Kather, A. *et al.* Anti-SEZ6L2 antibodies in paraneoplastic cerebellar syndrome: case report and review of the literature. *Neurol. Res. Pract.* **4**, 54 (2022).

82. Ramanathan, S., Al-Diwani, A., Waters, P. & Irani, S. R. The autoantibody-mediated encephalitides: from clinical observations to molecular pathogenesis. *J Neurol* **268**, 1689–1707 (2021).

83. Cunquero, M., Aguilar, E., Loza-Alvarez, P. & Planagumà, J. Hippocampal Neuronal Cultures to Detect and Study New Pathogenic Antibodies Involved in Autoimmune Encephalitis. *JoVE* 63829 (2022) doi:10.3791/63829.