Neurological Laboratory

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New serological markers for the differential diagnosis of autoimmune limbic encephalitis

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Abstract

Recently, several novel autoantibodies have been identified which are closely associated with different subtypes of autoimmune encephalitis. These antibodies are directed against structures located on the neuronal cell surface: glutamate receptors (types NMDA and AMPA), GABA_B receptors, as well as the voltage-gated potassium channel-associated proteins LGI1 and CASPR2. They are much more common than the classical paraneoplastic antibodies (anti-Hu, -Yo, -Ri, -Ma, -CV2, -amphiphysin), less frequently associated with a tumor, and the corresponding clinical syndromes respond significantly better to immunotherapy. Monospecific detection of these autoantibodies in the serum or cerebrospinal fluid of patients is primarily performed by indirect immunofluorescence using transfected HEK293 cell lines recombinantly expressing the membrane-associated target antigens. Owing to the symptom overlap of the respective disorders, it is highly appropriate to determine these parameters in parallel for each patient (autoantibody profiles). Early diagnosis (substantially supported by the serological laboratory), the immediate initiation of immunotherapeutic intervention and, in cases of paraneoplastic etiology, tumor resection are crucial for prognosis. In our own investigations, antibodies against glutamate receptors (type NMDA) are most frequently found among the newly identified forms of autoimmune encephalitis, accounting for 42% of cases. In laboratory practice, one-third of positive reactions were caused by an autoantibody whose determination was not requested by the clinician. Considering the urgency for therapeutic measures in positive cases, these findings substantiate the need to implement multiparametric serological test systems in this diagnostic area.

Keywords: AMPA receptor; autoimmune encephalitis; BIOCHIP mosaic; CASPR2; differential diagnosis; GABA_B receptor; indirect immunofluorescence; LGI1; neuropil antibodies; NMDA receptor; voltage-gated potassium channel (VGKC).

Introduction

Autoimmune limbic encephalitis is characterized clinically by memory deficits, neuropsychiatric symptoms and epileptic seizures accompanied by MRI and EEG abnormalities. A few years ago, this disease was primarily associated with paraneoplastic autoantibodies against intracellular neuronal antigens (Hu, Ma, CV2, amphiphysin), which are induced by underlying tumors with ectopic expression of neuronal antigens. As highly specific pathognomonic markers, these antibodies often allow for the early diagnosis of a previously unknown tumor. If the reactivities anti-Hu, anti-amphiphysin, anti-CV2 or anti-Ma are detected, the probability of finding a tumor at the time of the assessment or within the following 5 years is >95% [1]. Tumor removal may contribute to the stabilization or clinical improvement of the affected patients. The efficacy of immunotherapeutic interventions is limited, however, as with these paraneoplastic syndromes, cytotoxic T-cell responses with the resulting destruction of neurons must be regarded as decisive for the pathogenesis [2–10].

With growing awareness of the symptoms of autoimmune limbic encephalitis, an increasing number of patients have been diagnosed in the past decade. But despite the typical symptoms, none of the classic paraneoplastic autoantibodies was detected in those patients. Instead, they often presented with antibodies against antigens of the cell surface of neurons, particularly in the hippocampal neuropil and the cerebellum.
Meanwhile, glutamate receptors (types NMDA and AMPA), GABA<sub>B</sub> receptors and the potassium channel (VGKC)-associated proteins LGI1 and CASPR2 have been identified as specific target antigens [14–19]. Antibodies against these neuronal surface antigens are in some cases also associated with malignancies; in the majority of patients, however, no tumor is detected (facultative paraneoplastic antibodies) (Table 1). In contrast to the classic paraneoplastic antibodies, autoantibodies against neuronal surface antigens are ascribed a direct pathogenic role. This is based on their direct interaction with pre- or postsynaptic target structures (Table 2), which causes severe neuropsychiatric deficits due to an impairment of the synaptic signal transduction [59]. Since immunotherapy and, in paraneoplastic cases, tumor resection often lead to a significant improvement of the potentially reversible symptoms, an early diagnosis is prognostically important. This is helped not only by the continuous advancement in diagnostic methods, but also by the increasing differential diagnostic consideration and the ever more extensive clinical characterization of associated encephalitis subtypes [2–4, 60–62].

**Autoimmune encephalitis with autoantibodies against neuronal surface antigens**

**Anti-glutamate receptor (type NMDA) encephalitis**

The anti-glutamate receptor (type NMDA) encephalitis, first described in 2007, is associated with autoantibodies against extracellular epitopes of the NR1 subunit of glutamate receptors (type NMDA) [13, 14]. These are not identical to autoantibodies against the NR2 subunit, which have occasionally been discussed as an indication of neurological involvement in SLE (neuropsychiatric lupus) [63]. The anti-glutamate receptor (type NMDA) encephalitis was initially observed in young women with ovarian teratomas, but is now increasingly diagnosed in women without cancer, men, adolescents and children [20–22, 62, 64–67]. This relatively common form of severe but potentially reversible encephalitis is characterized by an almost stereotypical multistage clinical course. A flu-like prodrome (low-grade fever, headache, fatigue) is followed in 100% of patients by a psychotic stage of severe behavioral and personality changes, delusions, paranoid thoughts and hallucinations. As a result of these symptoms, many patients first undergo psychiatric treatment. Subsequently, they develop impaired consciousness, hypoventilation, epileptic seizures, autonomic instability, dyskinesia and involuntary repetitive movement patterns. Due to the severity of the disease (coma, status epilepticus, etc.), those affected often have to be treated in intensive care for a long time [13, 14, 20–22, 67–79]. About half of patients show abnormalities in the cerebral MRI, while the EEG is pathologically changed in over 90% of patients. The examination of cerebrospinal fluid (CSF) reveals in 90% of cases a mild lymphocytic pleocytosis, in 33% of cases an intrathecal increase in protein, and in 25% of cases oligoclonal bands [13, 14, 20, 74, 78]. If the anti-glutamate receptor (type NMDA) encephalitis is associated with a tumor, it is a paraneoplastic syndrome.

<table>
<thead>
<tr>
<th>Autoantibodies</th>
<th>Clinical syndrome</th>
<th>Common symptoms</th>
<th>Paraneoplastic cases</th>
<th>Associated tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-glutamate receptor (type NMDA)</td>
<td>Limbic encephalitis</td>
<td>Psychosis, memory/language deficits, seizures, impaired consciousness, dysequilibrium, dyssynergia, dyshydrosis</td>
<td>35%–40% (depending on gender, age, ethnicity)</td>
<td>Ovarian teratoma; less commonly: testicular teratoma, breast cancer, neuroendocrine ovarian cancer, ovarian sex cord-stromal tumor, pseudopapillary neoplasm of the pancreas, SCLC, neuroblastoma, Hodgkin’s lymphoma</td>
</tr>
<tr>
<td>Anti-glutamate receptor (type AMPA)</td>
<td>Limbic encephalitis</td>
<td>Memory deficits, confusion, disorientation, seizures, dysautonomia, myokymia</td>
<td>70%–75%</td>
<td>SCLC, and non-SCLC, thymoma, breast cancer</td>
</tr>
<tr>
<td>Anti-GABA&lt;sub&gt;B&lt;/sub&gt; receptor</td>
<td>Limbic encephalitis</td>
<td>Seizures, confusion, memory deficits, disorientation, memory loss, hyponatremia, myoclonus, dysautonomia</td>
<td>50%–80%</td>
<td>SCLC, carcinoid of the thymus, SCLC, renal cell carcinoma, ovarian teratoma</td>
</tr>
<tr>
<td>Anti-LGI1</td>
<td>Limbic encephalitis</td>
<td>Epileptic seizures, memory deficits, confusion, disorientation, seizures, dysautonomia, myokymia</td>
<td>0%–10%</td>
<td>Thyroid carcinoma, thymoma, SCLC, renal cell carcinoma, ovarian teratoma</td>
</tr>
<tr>
<td>Anti-CASPR2</td>
<td>Limbic encephalitis</td>
<td>Peripheral neural hyperexcitability, muscle cramps/fasciculations, sensory symptoms, memory deficits, confusion, disorientation, neuropathic pain, insomnia, dysautonomia, weight loss</td>
<td>0%–35%</td>
<td>Thymoma, endometrial adenocarcinoma</td>
</tr>
</tbody>
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SCLC: small-cell lung carcinoma.
Table 2  Characterization of neuronal surface antigens.

**NMDA receptors** belong to the ionotropic glutamate receptors and were named according to their activation by the synthetic amino acid N-methyl-D-aspartate (NMDA). Localized in the postsynaptic membrane, they form cation channels with central importance for synaptic transmission and plasticity [30, 31]. The receptors are tetrameric complexes consisting of 2 glycine-binding NR1 subunits and 2 glutamate-binding NR2 subunits (NR2A-NR2D), whose heteromeric assembly generates multiple receptor subtypes with different synaptic localization as well as various physiological and pharmacological properties [32]. Their activity is regulated by the binding of ligands such as glutamate, the key excitatory neurotransmitter. Localization of diagnostically relevant epitopes: extracellular region of NR1 [14].

**AMPA receptors** belong to the ionotropic glutamate receptors and have a high affinity for glutamate and for the synthetic glutamate agonist AMPA (α-amino-3-hydroxy-5-methyl-4-isozazole-propionic acid). They are mainly localized in the postsynaptic membrane of glutamatergic synapses and are expressed in particular in the hippocampus, cerebellum, cerebral cortex and in the basal ganglia. As ligand-gated cation channels, AMPA receptors facilitate the fast excitatory neurotransmission in the CNS, are important for the modulation of synaptic plasticity and thus for neurophysiological mechanisms underlying learning and memory processes. The receptors are composed of 4 subunits (GluR1 to GluR4) whose combination can vary locally and influence ion specificity and kinetic properties of the receptors [33–36]. Localization of diagnostically relevant epitopes: GluR1 and GluR2 [15].

**GABA<sub>B</sub> receptors** are metabotropic receptors that specifically bind the inhibitory neurotransmitter γ-amino butyric acid (GABA). Throughout the CNS, particularly in the hippocampus, thalamus, and cerebellum, they are enriched in pre- and postsynaptic membranes forming heterotetrameric complexes composed of 2 subunits each of GABA<sub>B1</sub> and GABA<sub>B2</sub>. Each receptor is associated with 2 tetrmers of KCTD proteins (potassium channel tetramerization domain-containing proteins), whose respective subtypes determine the kinetic and pharmacological properties of the receptor [37, 38]. The binding of GABA to the GABA<sub>B1</sub> subunits leads to receptor activation. This is followed by a G-protein-mediated transduction cascade for the regulation of synaptic cation channels and the inhibition of presynaptic transmitter release [37, 39]. GABA<sub>B</sub> receptors also play a role in modulating synaptic plasticity [40, 41]. Localization of diagnostically relevant epitopes: GABA<sub>B1</sub> [16].

**Voltage-gated potassium channels** (VGKC) are found in neuronal cell membranes, especially in paranodal and terminal regions of myelinated nerves. Among other things, they are responsible for membrane repolarization after action potentials.

**Leucine-rich glioma-inactivated protein 1** (LGI1) is expressed primarily by neurons in specific areas of the neocortex and the limbic system (e.g., hippocampus, amygdala, putamen, medulla, frontal lobe, temporal lobe) [42, 43]. It owes its name to its controversial role as a tumor suppressor in glioma progression [43–46], and to an N-terminal LRR (leucine-rich repeat) domain with relevance for the interaction with other proteins [43, 47]. Together with the pre-/postsynaptic ADAM23/ADAM22 receptors, presynaptic potassium channels (VGKC), scaffold proteins (e.g., PSD-95) and other factors, secreted LGI1 forms a transsynaptic protein complex and probably regulates the VGKC inactivation and the AMPA receptor-mediated synaptic signal transduction [48–51]. Because of so-called “EPTG repeats” in the C-terminus, LGI1 is allocated to a superfamily of proteins in a causal relationship with epilepsy and other neurological disorders [52].

**Contactin-associated protein-2** (CASPR2), a transmembrane protein with expression in the CNS and peripheral nerves, belongs to the neurexin superfamily of synaptic cell adhesion molecules, whose mutations are associated with autism and schizophrenia [53, 54]. CASPR2 is part of adhesion complexes of the neuronal cell surface that interact with the subunits of voltage-gated potassium channels (VGKC) and are essential to the VGKC localization in the juxtaparanodal regions of myelinated axons of the central and peripheral nervous system [55–58].

The probability of an underlying neoplasm is 35%–40% and is dependent on age, gender and ethnicity (Table 1). Ovarian teratomas are found most commonly in patients between 13 and 42 years old [13, 14, 20, 21, 68, 70, 78]. Some of the tumor cells express glutamate receptors (type NMDA) and thus induce the synthesis of receptor-specific autoantibodies [14, 80]. In men, patients of advanced age and children, tumors occur less often [14, 20, 21, 66, 67]. Interestingly, teratomas are overall more commonly found in women of African-American origin [20]. It is to be assumed that the anti-glutamate receptor (type NMDA) encephalitis is currently heavily underdiagnosed. The disease constitutes an important differential diagnosis, especially in patients with encephalitis of unknown cause (no infectious etiology) and in young women with initial epileptic seizures (de novo epilepsy) [73, 77].

The prognosis improves with adequate immunomodulatory therapy and, in the case of paraneoplastic etiology, early tumor detection and resection [13, 14, 22, 70–72, 81]. An early start of immunotherapy is associated with a significantly better long-term outcome than a later start [14, 62]. Even in severe cases of anti-glutamate receptor (type NMDA) encephalitis during pregnancy, immediate therapeutic measures can effect a good outcome for the mother and newborn [82]. Since the disease process is primarily maintained by intrathetically formed autoantibodies, there is a good correlation between the improvement of clinical symptoms and a decrease in specific antibody titers in the CSF. By contrast, serum antibodies can persist over a longer period, even during remission [14, 20, 22, 73, 81, 83]. The recovery process may take some time (up to years), but even then may result in a regression of fronto-temporal atrophy and hypoperfusion [84]. Particularly severe and protracted disease is presumably caused by autoantibody-producing plasma cells that infiltrate the brain’s parenchyma and are hard to reach therapeutically [20, 65, 85]. In general, a substantial regression of the symptoms can be achieved in about 75% of all patients. But 25% of patients suffer severe neurological deficits or die. Among the survivors, memory loss (amnesia) persists for the duration of the disease and there is a risk of relapse of the encephalitic syndrome, the latter especially if the tumor was removed late or not at all or if no tumor was found [13, 14, 20, 22, 68, 74, 78].

The occurrence of receptor-specific autoantibodies, their correlation with the disease course, immuno-pathological
findings, and the therapeutic reversibility of the disease suggest an immune-mediated pathogenesis of anti-glutamate receptor (type NMDA) encephalitis. Cytotoxic T-cell mechanisms and complement reactions, however, are of little relevance [20, 59, 69, 80, 85]. The pathogenic role of antibodies is supported by the antibody-mediated dysfunction of glutamatergic synapses [86]. In cell culture experiments on hippocampal neurons, it was also demonstrated that antibody binding induces a reversible, titer-dependent reduction in glutamate receptors (type NMDA) on the neuronal cell surface [14, 87]. Furthermore, in the case of a pharmacological blockade of the receptors with NMDA antagonists, clinical symptoms have been observed that are similar to anti-glutamate receptor (type NMDA) encephalitis, especially psychoses [88, 89].

**Anti-glutamate receptor (type AMPA) encephalitis**

Antibodies against glutamate receptors (type AMPA) are markers for a specific form of limbic encephalitis that so far has been diagnosed only in few patients (>90% women) with an average age of 60 years (age range: 38–87). Commonly associated symptoms include progressive memory deficits, confusion, disorientation, seizures, agitation, aggressive behavior, and (occasionally) hallucinations, nystagmus or lethargy [15, 25, 26]. In some cases, the clinical picture is limited to acute psychotic symptoms with rapidly progressive behavioral abnormalities [25]. In about 70%–75% of those affected, lung cancer, breast cancer or thymoma (with expression of the receptor subunits GluR1/GluR2) is detected, suggesting a paraneoplastic etiology of the encephalitic syndrome [15, 25, 26] (Table 1). In about 50% of the cases, there is an overlap with other systemic autoimmune diseases (e.g., stiff-person syndrome, diabetes mellitus, Raynaud’s syndrome, hypothyroidism), and additional autoantibodies against GAD, CV2, VGCC, SOX1 or other antigens are detectable. The cerebral MRI shows signal intensities of the medial temporal area in 89% of patients, while EEG abnormalities occur in 75%. Moreover, pathological changes in the CSF can be found almost always (90% lymphocytic pleocytosis, 70% intrathecal protein increase) [15].

Immunotherapy and, in case of paraneoplastic syndromes, tumor resection/chemotherapy usually lead to an improvement of symptoms [15, 25]. Strikingly, however, there is a tendency (56%) toward relapsing-remitting courses, with neurological relapses occurring once or several times even in the absence of neoplasms or after their removal. Without adequate treatment, or when treatment is not successful, this form of limbic encephalitis can lead to death of the patient.

Both the success of immunomodulatory interventions and the observation that, in cultured hippocampal neurons, the application of the antibodies leads to a reversible reduction of synaptic receptor density point to an antibody-mediated pathogenesis [15, 59]. Individual case studies suggest a correlation between receptor-specific antibody titers and disease activity [15]. The majority of patients studied to date exhibit an isolated reactivity against the receptor subunit GluR1 (30%) or GluR2 (60%); less frequently (10%) there are antibodies against both subunits [15].

**Anti-GABA<sub>α</sub> receptor encephalitis**

Limbic encephalitis with autoantibodies against GABA<sub>α</sub> receptors has so far been diagnosed in patients (68% men) with a mean age of 60 years (age range: 24–75 years) [16, 27]. All patients suffer from epileptic seizures, confusion and disruption of short-term memory, with the seizures representing the primary clinical symptom (complex focal seizures with frequent origin in temporal lobes and secondary generalization, status epilepticus, non-convulsive seizures). In addition, there may be behavioral problems, psychoses, paranoia, hallucinations, disturbances of sleep and consciousness, coma, and fatal outcomes. The occurrence of a tumor (usually small cell lung cancer) in 50%–80% of patients suggests a common paraneoplastic etiology of the neurological syndrome (Table 1). After the anti-Hu antibodies, the anti-GABA<sub>α</sub> receptor antibodies represent the second most frequent immunoreactivity in patients with limbic encephalitis and small cell lung cancer [27]. Almost half of the patients exhibit additional autoantibodies, for example, against GAD, TPO, VGCC, VGKC and/or SOX1, suggesting a generalized autoimmune disorder. In about 70% of all cases, the cerebral MRI shows abnormalities (usually increased FLAIR/T2 signals of the medial temporal lobes), and in more than 90% of cases, epilepsy-typical potentials occur in the EEG. Often the CSF findings are pathological in terms of a lymphocytic pleocytosis, intrathecal protein increase or (rarely) oligoclonal bands. Partial to full recovery can be achieved by early detection of the syndrome, immunotherapy and, if necessary, tumor resection/chemotherapy [16].

The current assumption of an antibody-mediated pathogenesis through the inhibition or destruction of GABA<sub>α</sub> receptors corresponds to the specific binding of patient antibodies to GABA<sub>α</sub> receptors of hippocampal neurons [16], the success of immunomodulatory interventions [16], the observation of symptoms similar to those of limbic encephalitis in mouse GABA<sub>α</sub><sub>1</sub>- and GABA<sub>α</sub><sub>2</sub>-null mutants (e.g., spontaneous epileptic seizures, memory deficits, anxiety, hyperalgesia, excessive motor activity) [90–93], as well as an increased risk of temporal lobe epilepsy in patients with GABA<sub>α</sub><sub>1</sub> receptor polymorphism [94]. A tumor-induced immune response against GABA<sub>α</sub> receptors with the concomitant appearance of the described symptoms is likely considering the frequent association of this encephalitis form with small cell lung cancer and the ability of cancer cells to express synaptic proteins. In the serum or CSF of all patients identified so far, it was possible to detect antibodies to the GABA<sub>α</sub><sub>1</sub> subunit, while additional anti-GABA<sub>α</sub><sub>2</sub> antibodies were present only in isolated cases [16]. Accordingly, it can be assumed that the relevant epitopes are primarily localized in the GABA<sub>α</sub><sub>1</sub> subunits, which are essential for substrate binding and receptor function [37].

**Encephalitis with autoantibodies against voltage-gated potassium channels (VGKC) and VGKC-associated proteins: LGI1 and CASPR2**

Autoantibodies against voltage-gated potassium channels (VGKC) constitute the entirety of all antibodies that precipitate <sup>125</sup>I-γ-dendrotoxin-labeled VGKC complexes from brain homogenates. <sup>125</sup>I-α-
dendrotoxin is a snake venom capable of binding specifically to the \( \alpha \)-subunits \( \kappa_{1.1} \), \( \kappa_{1.2} \) and \( \kappa_{1.6} \) of potassium channels. Only in 2010 did it emerge that the autoantibodies detected in patients with “VGKC antibody-associated” syndromes are directed against these potassium channel subunits in only approx. 3% of cases [95–101]. In fact, about 80% of relevant epitopes are localized in VGKC-associated subunits in only approx. 3% of cases [95–101]. In fact, about 80% of relevant epitopes are localized in VGKC-associated subunits in only approx. 3% of cases [95–101].

Autoantibodies against LGI1 are found almost exclusively in patients with limbic encephalitis (approx. 65% men, mean age 60 years). The main symptoms are seizures, memory deficits, confusion and disorientation. Additional symptoms are hyponatremia, myoclonus, sleep disturbances, and (rarely) dysautonomia, neuropathic pain, weight loss or neuromyotonia [17, 19, 103]. Patients with LGI1 antibodies may develop brief, short-lasting myoclonic-like movements that can affect the face, arm and leg and are often preceded by electroencephalographic responses in video EEG, similar to tonic seizures [28, 104]. These episodes may indicate anti-LGI1-associated encephalitis at an early stage, so that (along with the serological verification) the progression of the syndrome can be countered by an immediate start of therapy. Compared with forms of encephalitis with other antibody specificities, the incidence of tumors (thyroid carcinoma, small cell lung cancer, renal cell carcinoma, ovarian teratoma and thymoma) is low with 0%–10% in the case of anti-LGI1 positivity (Table 1). More than half of those affected exhibit T2 signal intensities of the medial temporal lobe in the MRI (56%–84%), and EEG abnormalities (76%). The CSF is usually normal; only rarely does a lymphocytic pleocytosis or protein elevation occur. In about 80% of cases, immunotherapy causes a substantial or complete regression of the symptoms, whereby the antibody titers correlate with the decline in disease activity. The likelihood of recurrence is ≈20% and the mortality rate is approximately 2%–6% [17, 19, 28].

Pathophysiologically, the antibody-mediated impairment of the LGI1 function could lead to increased excitability and, according to the primarily hippocampal localization of the antigen, result in the symptoms of limbic encephalitis. Correspondingly, \( LGI1 \) gene mutations that cause a malfunction or secretion dysfunction of LGI1 are associated with a hereditary epilepsy syndrome (ADLTE or ADPEAF) [42, 105–107]. Moreover, the inactivation of the \( LGI1 \) gene is associated with lethal epilepsy and a hypomyelination in the peripheral and central nervous system [48, 108].

Autoantibodies against CASPR2 are also found in patients with limbic encephalitis, but mostly occur in connection with acquired neuromyotonia or Morvan’s syndrome, affecting mainly men (approx. 85%) aged on average 60 years. The core symptom of neuromyotonia is peripheral neuronal hyperexcitability, which in Morvan’s syndrome is also associated with central nervous system disorders such as memory deficits, seizures, confusion, disorientation, hallucinations, insomnia, weight loss and hyponatremia [18, 19, 29]. Information on the frequency of associated tumors (mostly thymomas) diverges in different study cohorts (0%–35%) (Table 1). In patients without tumors, immunomodulatory interventions and symptomatic treatment usually (80%) lead to a significant improvement of symptoms and prognosis [18, 19, 62]. Paraneoplastic cases, however, appear to be linked with little or no therapeutic success and poor prognosis, with a possible fatal outcome [19].

Regarding the pathophysiology, it is thought that CASPR2 autoantibodies lead to a quantitative decrease in CASPR2-VGKC-complexes on the axons of peripheral nerves and thus to neuromuscular hyperexcitability typical of neuromyotonia [19, 109]. Corresponding to this, mutations or polymorphisms of the gene encoding CASPR2 (\( CNTNAP2 \)) are closely related to symptoms similar to those of anti-CASPR2-positive patients, for example, focal epilepsy or neuropsychiatric changes [110, 111].

### Diagnosis

The diagnosis of limbic encephalitis with autoantibodies against neuronal surface antigens is initially based on the combination of the characteristic clinical picture and any supporting evidence from MRI (mesial temporal FLAIR/T2 signal increase), EEG (temporal focus or generalized slowing) and CSF analysis (lymphocytic pleocytosis, protein increase, oligoclonal bands). Due to the overlap of clinical symptoms, the final diagnosis is made on the basis of the monospecific detection of serum or CSF antibodies against glutamate receptors (types NMDA and AMPA), GABA\(_{\alpha}\) receptors, LGI1 or CASPR2. A positive serological finding should always be followed by an intensive tumor search (Table 1). If initial oncological findings are negative, a tumor may not be identified until months after the first neurological symptoms, or along with a relapse. Therefore, follow-up should include periodic repetitions of MRI and ultrasound examinations over a period of at least 2 years [14, 21, 68]. An early diagnosis including adequate laboratory analysis is of high prognostic relevance, as most patients respond well to early immunotherapeutic measures (corticosteroids, plasmapheresis, intravenous immunoglobulin, rituximab, cyclophosphamide and others) and, in the case of neoplasms, respond to the removal of the tumor [14–20, 62, 73].

As differential diagnoses, primarily infectious encephalitis (e.g., HSV encephalitis) [112, 113] as well as various autoimmune etiologies with neuropsychiatric manifestations must be taken into consideration. The latter include encephalitis with classical paraneoplastic autoantibodies (against intracellular onconeural antigens: Hu, Ma, CV2, amphiphysin), neuropsychiatric lupus, Sjögren’s syndrome, Hashimoto’s encephalopathy, antiphospholipid syndrome, metabolic encephalopathy, Korsakoff’s syndrome, primary angiitis of the CNS and others [2, 3, 61, 62, 114]. Occasionally, the symptoms give rise to a diagnosis of a suspected drug-induced psychosis (e.g., neuroleptic malignant syndrome). In cases of anti-glutamate
receptor (type NMDA) encephalitis, the disease was initially classified as “idiopathic encephalitis with psychiatric manifestations and dyskinesias,” or as “encephalitis lethargica” [20, 115, 116].

Serological laboratory diagnosis

Fluorescent or light microscopic immunohistochemistry and immunocytochemistry are the primary methods used for the determination of autoantibodies against antigens of the neuronal cell surface. The presence of these antibodies in serum or CSF produces (i) a characteristic staining on tissue sections of the rat brain (cerebellum, hippocampus, “anti-neuropil antibodies”), (ii) surface staining of cultured hippocampal neurons (anti-CASPR2 antibodies: juxtaparanodal staining of peripheral nerves), and (iii) reactivity with specifically transfected human embryonic kidney cells (HEK) (Figure 1) [14–19, 74].

The tissue and cellular substrates of neuronal origin (i, ii) contain the target molecules expressed in native form with in-situ localization on the neuronal surface, resulting in maximum authentic antigen presentation (conformational epitopes), characteristic binding patterns and high diagnostic sensitivity. A definite conclusion about the respective target antigen or a reliable differentiation of the various autoantibodies by means of these substrates is not fully possible, since some of the staining patterns differ only slightly from each other and the pattern interpretation may be rendered more difficult by possible parallel autoreactivities against other neuronal or nuclear targets (e.g., ANA). However, the knowledge of the target antigen is of great clinical importance because the therapeutic approach and the tumor association vary between the different forms of encephalitis (Table 1).

Recombinant human cell lines (iii) expressing the relevant antigen on the cell surface are optimally suitable for the highly sensitive, monospecific antibody detection. As the target molecules are membrane-associated proteins (receptors/ion channels and associated factors), an expression and detection system is required for their synthesis and presentation that is as authentic as possible. For maximum sensitivity and specificity, the transfected cells selectively express those antigenic components that contain the immunologically relevant epitopes: the NR1 subunit of glutamate receptors (type NMDA) [14, 22], GluR1 and GluR2 subunits of glutamate receptors (type AMPA) [15], the B1 subunit of GABA_A receptors [16], and the VGKC-associated proteins LGI1 and CASPR2 [17–19]. The recombinant cell-based assays were previously reserved for only a few specialized research laboratories, because this method is associated with complex molecular biological and immunological techniques. In addition, the long-term culturing of cell lines and their transfection prior to each test series are not only laborious and time consuming, but can also affect the reproducibility of the analysis. Through the development of standardized test systems based on BIOCHIPs, the detection method can now also be applied in any laboratory in which indirect immunofluorescence has been established [74, 117–120]. A comprehensive serodiagnosis with transfected cells and CNS tissues as target substrates allows not only the differentiation of symptomatically overlapping encephalitis subtypes, but also the distinction from infectious encephalitis and syndromes with classic paraneoplastic autoantibodies or other autoimmune etiologies.

A diagnostic particularity relates to the detection of antibodies against antigens of the VGKC complex. Since approximately 80% of them are directed against the VGKC-associated proteins LGI1 or CASPR2, it is advisable to examine the patient samples first monospecifically by means of recombinant cell substrates for their reactivity against LGI1 and/or CASPR2. If this analysis is negative, a radioimmunoassay must be performed additionally, i.e., an immunoprecipitation of 125I-α-dendrotoxin-labeled VGKC complexes from brain homogenates. This approach enables detection of antibodies directed against the potassium channel subunits K_1,1, K_1,2 and K_1,6 or against other, as yet unidentified, VGKC complex proteins [19].

A quantification of the antibody titers or a titer comparison between longitudinal samples can be done using the specific fluorescence intensities in the indirect immunofluorescence test (IIFT). Often a reduction in antibody titers is associated with clinical improvement [14, 15, 20, 22, 28, 73]. Since intrathecal antibody synthesis can be combined with low or negative serum titers, serum and CSF samples should always be analyzed in parallel. The inclusion of the CSF is of particular importance in the search for antibodies against glutamate receptors (type NMDA), and in general in patients who are already undergoing plasmapheresis [14, 16, 65].

Prevalence of anti-neuronal autoantibodies

To investigate the relative frequency of antibodies against neuronal surface antigens, we analyzed all findings resulting from the determination of anti-neuronal autoantibodies in 2716 samples sent in to the clinical immunology laboratory of the last author between October and December 2010. The detection of autoantibodies (IgG) in serum and CSF was carried out using multiparametric IIFT (Figure 2). For the production of monospecific cell substrates, cDNA fragments were generated that encode for the respective target antigens: glutamate receptor (type NMDA; subunit NR1), glutamate receptor (type AMPA; subunits GluR1/ GluR2), GABA_A receptor (subunit B1), LGI1 (membrane-bound fusion protein), CASPR2, GAD65, aquaporin-4 and glycine receptor. After insertion of the cDNA into eukaryotic expression vectors, HEK293 cells were transfected with these plasmids, seeded on coverslips and fixed after 48 h. Subsequently, the coverslips were cut into millimeter-sized fragments (BIOCHIPs) that were assembled into mosaic rows on the ridges of special microscope slides. The substrate spectrum of the mosaics included, in addition to the different recombinant cell lines, frozen sections of neuronal tissue (hippocampus, cerebellum, nerve and intestine)
Figure 1  Detection of autoantibodies against neuronal surface antigens by indirect immunofluorescence. Antigen substrates: hippocampus (frozen section; rat), cerebellum (frozen section; rat), transfected HEK293 cells with surface expression of recombinant target antigens. (A) Autoantibodies against glutamate receptors (type NMDA): fluorescence of the molecular layer of the hippocampus (neuropil staining) and neuronal nuclei of the cerebellar stratum granulosum; monospecific detection with NR1-transfected HEK293 cells. (B) Autoantibodies against glutamate receptors (type AMPA): fluorescence of the molecular layer of the hippocampus (neuropil staining), the strata moleculare and granulosum of the cerebellum and the Purkinje cells; monospecific detection with GluR1/GluR2-transfected HEK293 cells. (C) Autoantibodies against GABA\(_B\) receptors: coarse granular fluorescence of the molecular layer of the hippocampus (neuropil staining) and of the cerebellum; additional patchy fluorescence of the granular layer of the cerebellum; monospecific detection with GABA\(_B1\)-transfected HEK293 cells. (D) Autoantibodies against LGI1: fine granular fluorescence of the molecular layer of the hippocampus (neuropil staining) and the cerebellum; outer molecular layer of the hippocampus is more sensitive than the inner one; additional patchy fluorescence of the stratum granulosum of the cerebellum; monospecific detection with LGI1-transfected HEK293 cells. (E) Autoantibodies against CASPR2: fluorescence of the molecular layer of the hippocampus (neuropil staining) and the cerebellum; additional patchy fluorescence of the granular layer of the cerebellum; monospecific detection with CASPR2-transfected HEK293 cells.
Optimized assay conditions were achieved by applying the EUROTIDE incubation technology, in which reactions are accelerated by forced convection (Figure 2).

The identification of classic paraneoplastic autoantibodies (anti-Hu, -Yo, -Ri, -Ma, -CV2, -amphiphysin) was based on the presence of the respective characteristic fluorescence pattern on different diagnostically relevant tissues (IIFT) parallel to the reactivity of the monospecific line blot assay. The study included not only the requested parameters according to the analysis order (main findings), but also anti-neural reactivities that were detected instead or in addition (secondary findings).

Among the 2716 serum/CSF samples, 108 cases were found with anti-neuronal IgG, including 68 with antibodies against one of the neuronal surface antigens (group A, 63%), 33 with antibodies against an intracellular onconeural antigen (group B, 31%) and 7 with antibodies against multiple target antigens (group C, 6%) (Table 3 and Figure 3). Based on these positive samples, the incidence of reactivities to antigens of the neuron surface (groups A and C) was 67% (72/108) and thus corresponded to almost twice the prevalence of classic paraneoplastic autoantibodies (groups B and C), which accounted for only 35% (38/108) of positive samples. A possible bias due to the laboratory specialization and novel character of the group A parameters cannot be excluded, however.

Table 3 Prevalence of anti-neuronal IgG autoantibodies. The data (%) refer to 108 positive findings in a study period of 3 months.

<table>
<thead>
<tr>
<th>Autoantibodies</th>
<th>Positive findings (n=108)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Autoantibodies against neuronal surface antigens</td>
<td></td>
</tr>
<tr>
<td>NMDAR</td>
<td>41 (38.0%)</td>
</tr>
<tr>
<td>AMPAR</td>
<td>–</td>
</tr>
<tr>
<td>GABAβ2R</td>
<td>3 (2.8%)</td>
</tr>
<tr>
<td>LGI1</td>
<td>12 (11.1%)</td>
</tr>
<tr>
<td>CASPR2</td>
<td>12 (11.1%)</td>
</tr>
<tr>
<td>Total</td>
<td>68 (63.0%)</td>
</tr>
<tr>
<td>B. Classic paraneoplastic autoantibodies</td>
<td></td>
</tr>
<tr>
<td>Hu</td>
<td>6 (5.6%)</td>
</tr>
<tr>
<td>Yo</td>
<td>9 (8.4%)</td>
</tr>
<tr>
<td>Ri</td>
<td>10 (9.3%)</td>
</tr>
<tr>
<td>Ma</td>
<td>2 (1.8%)</td>
</tr>
<tr>
<td>PCA-2</td>
<td>1 (0.9%)</td>
</tr>
<tr>
<td>CV2</td>
<td>4 (3.7%)</td>
</tr>
<tr>
<td>Amphiphysin</td>
<td>1 (0.9%)</td>
</tr>
<tr>
<td>Total</td>
<td>33 (30.6%)</td>
</tr>
<tr>
<td>C. Simultaneous detection of 2 anti-neuronal autoantibodies</td>
<td></td>
</tr>
<tr>
<td>NMDAR/GABAβ2R</td>
<td>1 (0.9%)</td>
</tr>
<tr>
<td>NMDAR/CASPR2</td>
<td>1 (0.9%)</td>
</tr>
<tr>
<td>NMDAR/Hu</td>
<td>2 (1.8%)</td>
</tr>
<tr>
<td>Hu/CV2</td>
<td>3 (2.8%)</td>
</tr>
<tr>
<td>Total</td>
<td>7 (6.4%)</td>
</tr>
</tbody>
</table>
Autoantibodies with binding specificity for glutamate receptors (type NMDA) were detected most frequently (45/108, 42%). With decreasing prevalence reactions occurred against CASPR2 (13/108, 12%), LGI1 (12/108, 11%), Hu (11/108, 10%), Ri (10/108, 9%), Yo (9/108, 8%), CV2 (7/108, 6%), GABA<sub>R</sub> receptor (4/108, 4%), Ma (2/108, 2%), PCA-2 (1/108, 1%) and amphiphysin (1/108, 1%). Autoantibodies to glutamate receptors (type AMPA) were not detected in any of the samples (Table 3, Figure 3).

In 33% (24/72) of the findings with autoantibodies against neuronal surface antigens and 26% (10/38) of the findings with classic paraneoplastic autoantibodies, the positive reactions were caused by an autoantibody other than that expected according to the analysis order. In total, 31% (33/108) of all positive findings were such secondary results (Table 4). The large proportion of relevant secondary findings underscores the importance of multiparametric antibody diagnostics and the analysis of antibody profiles for diagnosing autoimmune encephalitis: without serology going beyond the expected parameters (analysis order), the reactivities identified as secondary findings would not have been detected. Since antibodies against neuronal surface antigens accounted for the majority of secondary findings (71%, 24/34), a limited awareness and insufficient consideration of these new parameters has to be assumed, resulting in underdiagnosis of the associated autoimmune diseases.

The relatively high prevalence of autoantibodies against receptors/ion channels of the neuronal surface, as presented herein, reflects the high relevance of their detection in the case of a suspected autoimmune encephalitis. With recombinant cell-based BIOCHIP mosaics, the reactivity of a patient sample can be examined on different substrates in the same test run, and various diagnostically relevant autoantibodies can be detected or ruled out immediately.

**Conclusions and outlook**

Anti-glutamate receptor (type NMDA) encephalitis and the receptor-specific antibody detection required to diagnose it

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**Table 4** Discrepancies between requested (analysis order) and detected anti-neuronal autoantibodies. In 33 of 108 positive samples (31%), the multiparametric testing revealed the presence of an autoantibody other than the one requested.

<table>
<thead>
<tr>
<th>Requested</th>
<th>Detected</th>
<th>Autoantibodies against neuronal surface antigens</th>
<th>Classic paraneoplastic autoantibodies</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoantibodies against (another) neuronal surface antigen</td>
<td></td>
<td>NMDAR  GABA&lt;sub&gt;R&lt;/sub&gt;  LGI1  CASPR2</td>
<td>Hu  Yo  Ri  PCA-2  CV2</td>
<td>NMDAR/Hu</td>
</tr>
<tr>
<td>(Other) classic paraneoplastic autoantibodies</td>
<td>7</td>
<td>– 1 1 1</td>
<td>– 2 1 1</td>
<td>–</td>
</tr>
<tr>
<td>Other</td>
<td>6</td>
<td>– – 2</td>
<td>1 1 1</td>
<td>–</td>
</tr>
</tbody>
</table>

*Anti-aquaporin-4; anti-myelin-associated glycoprotein.
were first described 4 years ago. Since then, autoantibodies against other neuronal surface antigens have also been incorporated into the diagnosis of autoimmune limbic encephalitis. The identification of target antigens has been associated with a rapid development of standardized test systems, in particular assays containing specifically transfected cell substrates, which allow for a highly sensitive and specific antibody determination in serum and CSF. As a result, the associated, potentially fatal diseases are diagnosed and treated early in more and more patients – frequently resulting in partial to full recovery and thus lower mortality.

In the future, it will be important to increase awareness of these parameters, to enforce their inclusion in the neurological differential diagnosis, and to make better use of the diagnostic potential of multiparametric test systems for analyzing antibody profiles. Given the continuous efforts to identify target structures on the surface of neurons and to characterize them more precisely, the establishment of further serological markers in the diagnosis of autoimmune encephalitis is very likely in the foreseeable future.

Conflict of interest statement

The corresponding author points to the following relationship: K.P. Wandinger, C. Klingbell, S. Saschenbrecker, K. Borowski, C. Probst and W. Stöcker are employees of Euroimmun AG. Euroimmun develops and sells test systems for the detection of autoantibodies, including antibodies against neuronal antigens. Current research projects by J. Dalmau and A. Vincent are supported by Euroimmun. Despite the potential conflict of interest, the content of this paper is independent and product neutral.

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