Autoantibodies in the nervous system

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**Autoantibodies in the nervous system:
pathophysiology and new therapeutic
strategies**

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Maastricht University on the authority of the Rector Magnificus

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in accordance with the decision of the Board of Deans.

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by

Marina Mané Damas

Supervisor:

Prof. Dr. Pilar Martinez

Co-supervisors:

Dr. Mario Losen

Dr. Rob Rouhl

Assessment Committee:

Prof. Dr. B.P.F. Rutten (Chair)

Prof. Dr. G.M.J. Bos

Dr. J.G.J. Hoeijmakers

Prof Dr. E. Tuzun (University of Istanbul, Turkey)

Dr. E. Hofman (Argenx, Gent, Belgium)

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The background of the slide is a light gray with a complex, abstract pattern. It features a grid of squares in various shades of gray, overlaid with thin, dark gray line art. These lines form various shapes, including a spiral, a gear, a hand, and other organic, flowing patterns. The overall effect is a textured, artistic backdrop.

01.

General introduction

Abstract

The immune system is a host defence system, which has two major pillars, innate and adaptive immunity. Even though it is designed to protect us against external harmful agents, it can also play detrimental roles. When this happens, the adaptive immune system can cause diseases of the central and peripheral nervous system, involving the activation of an immune response against self-antigens leading to highly specific cellular reactions, inflammation and antibody secretion, known as antibody-mediated autoimmune disorders.

Impairment of nervous conduction and synaptic transmission by autoantibodies can manifest itself in many ways, depending on the targeted antigen, its location and on the action mechanisms involved in the pathophysiology, leading to a large number of different autoimmune disorders [1, 2]. Demyelinating disorders such as multiple sclerosis, neuromyelitis optica and Guillain-Barré syndrome, encephalomyelitis including NMDAR encephalitis, progressive encephalomyelitis with rigidity and myoclonus (PERM) and neuromuscular disorders like myasthenia gravis and neuromyotonia are important antibody-mediated disorders of the central and peripheral nervous system [3].

Symptomatic and immunosuppressive drugs are used as treatment for antibody-mediated autoimmune disorders. However, treatments can fail or have a poor response in some patients, and may cause side effects resulting from lack of specificity.

The adaptive immune system and its players

The immune system protects the body against pathogens by eliminating external harmful agents such as viruses, bacteria or fungi thanks to two different responses that differ in time and specificity: innate and adaptive immunity. The adaptive immune response is a very precise and elaborate reaction resulting from a previous exposition to the pathogen.

B lymphocytes and their progeny plasma cells are specialized white blood cells. They are, as part of the adaptive immune response, responsible for the production of the antibodies. The secretion of antibodies is maintained by the activation of reactive B cells that will differentiate into short-lived plasma cells and finally into specialized long-lived plasma cells. Additionally, they serve as antigen-presenting cells, resulting in the secretion of pro-inflammatory cytokines and recruitment of other cells involved in the immune response.

Antibodies, also referred to as immunoglobulins, are heterodimeric glycoproteins in a “Y” shape consisting of four chains, two heavy (H) and two light (L), each of them with constant (C) and variable (V) regions. The constant regions together form the Fc fragment, responsible of the recruitment of several cells involved in the immune response, while the variable regions compose the Fab fragment, which exclusively identifies a specific region, known as epitope, of the target antigen. Immunoglobulin G (IgG) is the major class of the five classes of immunoglobulins present in humans and one of the most abundant proteins in human serum (10–20% of plasma protein) [4]. IgGs can be further classified into four different subclasses [5]. Each subclass differs in heavy chain structure and has a unique effector function profile with respect to antigen binding, immune complex formation, complement activation, triggering of effector cells, half-life and placental transport. IgG1 and IgG3 bind strongly to soluble and cell membrane proteins; they stimulate other effector cells via Fcγ receptors [6] and activate complement via the “classical pathway” [7], resulting in inflammation, tissue damage and loss of antigen function. IgG2, however, is mostly secreted as a response to bacterial polysaccharide antigens and has a reduced capacity to activate complement. Finally, IgG4 is directed against proteins and allergens and lacks the capacity to fix complement.

Tolerance and autoimmunity

The high specificity of antibodies to foreign particles results from a sophisticated and highly regulated mechanism which prevents recognition of self-antigens by a process known as tolerance which includes multiple selection steps that deplete or functionally silence autoreactive T cells. However, when this orchestrated mechanism to discriminate between non-self and self-antigens fails, antibodies become autoantibodies that attack self-antigens inducing autoimmunity with pathological consequences [8].

Currently, a growing 2.5 % of the general population suffer from autoimmune diseases caused by autoantibodies [9].

Autoantibodies were first discovered in serum from patients with systemic lupus erythematosus (anti-nuclear antibodies) and rheumatoid arthritis (rheumatoid factors). These autoantibodies bound to nuclear antigens and immunoglobulins respectively [10]. Autoantibodies can be directed against nucleic acids, lipids and proteins, which can be located in the nucleus or cytoplasm, on the cell surface or in the extracellular space. The pathogenicity of autoantibodies against extracellular and circulating molecules has been extensively confirmed and autoantibodies play a pivotal role in the pathogenesis of many diseases by inducing cellular damage and inflammation.

Autoimmunity to cell surface antigens

In the mid-50s, autoantibodies against the thyroid stimulating hormone (TSH) receptor were described in autoimmune hyperthyroidism as the first autoantibodies targeting a receptor [11, 12]. Autoimmunity against cell surface receptors are T cell dependent, B cell-mediated autoimmune-disorders [13]. In these diseases, it was of paramount importance to identify the presence of circulating antibodies and locate the targeted antigen.

Neuronal receptors, ion channels and associated proteins are essential for the correct neurotransmission signalling involved in processes including learning and memory as well as muscle contraction. Nevertheless, these proteins are sometimes affected by the presence of autoantibodies and have been described in many autoimmune disorders. Autoantibodies influence their effector mechanisms for example by stimulating or blocking the targeted receptor, resulting in the disruption of synaptic transmission or the deterioration of the synaptic structures [14]. Obviously, the resulting phenotype depends on the properties of the receptor under attack, the characteristics of the autoantibodies involved and their effector mechanisms. Complement mediated lysis, antibody-mediated cell cytotoxicity, immune complex mediated damage, receptor signalling and cell architecture modulation, receptor internalization, opsonisation, and inhibition of repair mechanisms are some antibody pathogenic mechanisms observed in the nervous system [3].

Autoantibodies against the muscle nicotinic acetylcholine receptor (AChR) in myasthenia gravis (MG) or against the N-methyl-D-aspartate-(NMDA)-receptor in NMDA encephalitis share some pathogenic mechanisms [15, 16], nevertheless with very different clinical manifestations.

Myasthenia gravis

MG is an autoimmune disease caused by autoantibodies against the AChR and associated proteins at the neuromuscular junction [17, 18]. It has an annual incidence of 8 - 10 cases per 1 million persons and a prevalence of 150 - 250 cases per million, affecting individuals of all ages and both genders [19]. Weakness and fatigue of the skeletal muscles that aggravate after exercise are the marked clinical signs of MG. Thymic abnormalities are often associated with it. Specifically, follicular hyperplasia is observed in 50-60% patients and around 15% of patients with MG present with a thymoma. Additionally, 50% of thymoma patients develop MG [20-22].

Autoantibodies against the AChR are present in 85% of MG patients. The AChR is a pentameric molecule with different subunits: α , β , γ , δ and ϵ in the adult muscle, of which two are α -subunits. Interestingly, the α region keeps the main epitope targeted by autoantibodies, known as the main immunogenic region (MIR) [23-25]. The high concentration of AChRs at the top of the folds of the neuromuscular junction is crucial for an effective signal transmission from the nerve to the muscle. The muscle-specific kinase (MuSK) and lipoprotein receptor-related protein 4 (Lrp4) are elemental proteins involved in the development and maintenance of the structure of the neuromuscular junction [26, 27]. Autoantibodies against these two proteins have also been detected in patients with MG, however, in lower percentages.

In AChR-MG, IgG1 and IgG3 are the principal isotypes which induce pathogenicity by three main mechanisms [18, 28]: 1) activation of the classical complement cascade with in the formation of the membrane attack complex (MAC) [29, 30], 2) antigenic modulation [31], which both result in loss of AChR density and destruction of the postsynaptic folds, and 3) direct inhibition of ACh-AChR binding or blocking the AChR channel [32]. In MuSK-MG, IgG4 causes the disease [33]. With a reduced capacity to bind complement, these autoantibodies disrupt the association between MuSK-Lrp4 and prevents the communication with agrin, required for developing stable synapses [34]. Additionally, IgG4s can exchange arms between them, having the capability to recognize two different antigens. This property is known as Fab arm exchange and occurs spontaneously *in vivo* [35]. IgG1 is the main isotype identified for Lrp4-MG [36]. Its pathogenic mechanisms are not yet well understood.

Autoimmune encephalopathies

Autoimmune encephalopathies mostly present as a subacute encephalitis with limbic features (cognitive disturbance, psychiatric symptoms and epilepsy), and are associated with autoantibodies against several neuronal surface proteins (receptors, ion channels and associated proteins). Currently, up to 16 autoimmune encephalopathies targeting neuronal antigens have been described [37], of which the N-

methyl-D-aspartate receptor (NMDAR) is the most commonly targeted antigen [38]. NMDAR binds glutamate, the main excitatory neurotransmitter in the brain. Their annual incidence has been estimated at 3–5 cases per million, young women and children being predominantly affected [39]. Malignant diseases are highly prevalent in autoimmune encephalitis, as the antibodies might be a paraneoplastic phenomenon [15].

Autoantibodies against the NMDAR recognize a highly conserved epitope present in the GluN1 subunit of this heterotetrameric molecule [15, 40]. Even though other isotypes have been reported, IgG is the only isotype with a proven pathogenic role [41]. With a predominance of IgG1, NMDAR autoantibodies cause antigenic modulation and selective internalization of the NMDAR in neurons [42]. Even though antibodies are predominantly IgG1 [43], the few studies available showed no complement deposition in brain biopsies from patients with NMDAR encephalitis. This could be due to the low concentration of complement components in the CSF [44–46].

Clinical manifestations of NMDA-receptor encephalitis mostly start with a prodromal phase of headache or fever for hours to days, followed by clinical symptoms of abnormal behaviour and psychiatric manifestations (e.g. anxiety, hallucinations, and psychosis) and cognitive dysfunction during the following weeks. Later, patients develop seizures, abnormal movements, and eventually coma and severe autonomic dysregulation [47]. However, approximately 4% of NMDAR encephalitis patients present isolated psychotic episodes without clear neurological manifestations [48]. NMDAR autoantibodies have been described with a higher prevalence in a cohort of children with a first psychosis episode [49] and women with post-partum psychosis [50].

Besides the presence of psychiatric manifestations in antibody-mediated autoimmune forms of encephalitis, psychotic disorders are associated with inflammation and autoimmunity [51, 52]. An unbalance in the homeostasis of different neurotransmitter systems has been observed in these disorders [53, 54]. Altogether, this raises the question whether autoantibodies targeting neuronal surface antigens and altering the neuronal synapses could be the underlying cause of some forms of neuropsychiatric disorders. Despite the controversy in the field, probably as a result of the heterogeneity in the patient population and the detection methods [41, 55], no evidence for this association has been clearly found so far [56, 57]. However, it is of interest to investigate the presence of known but also novel neuronal surface autoantibodies in patients with psychotic disorders, especially if they have an atypical disease course, to describe the role of these autoantibodies and their target antigen(s).

Treatment approaches for antibody-mediated autoimmunity

Treatment strategies for antibody-mediated disorders include a combination of symptomatic treatment and non-specific immunosuppressants. High dose corticosteroids and other drugs (e.g., azathioprine, methotrexate, MMF) aim to decrease the immune response by suppressing the activation and proliferation of lymphocytes, including B cells. This approach, however, has little effect on long-lived plasma cells [58]. Plasmapheresis and high-dose intravenous IgG (IVIg) consist on the removal or neutralization of autoantibodies which is especially indicated for treating acute exacerbations thanks to the fast effect of these procedures. The beneficial effect, however, is not long-lasting due to the production of new antibodies. Cell therapies targeting B cells (e.g. rituximab), show good results mainly in MuSK-MG [59], suggesting different pathogenic mechanisms among different disease types. Proteasome inhibitors targeting long-lived plasma cells have shown to be beneficial in preclinical experimental settings [60, 61] and in some refractory cases [62-64].

Repurposed and novel preclinical treatments should be investigated in order to reduce the significant side effects resulting from the currently available treatment options and to efficiently treat a still substantial percentage of patients that do not present an optimal treatment response or do not respond at all [58, 60, 65-70].

Take home message

There is a need to elucidate the pathophysiology underlying antibody-mediated disorders. The identification of these autoantibodies and their targets will result in an early diagnosis, which is usually associated with a prompt treatment and better prognosis. Additionally, the disentanglement of the pathogenic mechanisms will help to understand these disorders and result in the development of more specific therapeutic strategies.

Overview of the thesis

Autoimmunity against neural surface receptors, ion channels and associated proteins represents a field of noteworthy interest because of its far-reaching implications in the field of neuropsychiatry including the identification of the target, the understanding of the pathophysiological mechanisms and the development of novel therapeutic strategies. The research lines of this thesis largely merge towards profiling the role of autoantibodies against neural surface proteins in antibody-mediated autoimmune disorders in the central as well as in the peripheral nervous system. The first three chapters focus on the potential role of neuronal surface autoantibodies in psychotic disorders, while the following chapters focus on novel therapeutic strategies for antibody-mediated disorders in the peripheral nervous system, using MG as a generic model.

Figure 1. Association network of the different topics discussed in this thesis including the disorders (blue), treatments (purple), the action mechanisms (yellow), the immunoglobulin isotypes (orange) and the antibody producing cells (green).

After a brief introduction on the role of the immune system in receptor antibody-mediated autoimmune disorders and a brief overview of the main disorders addressed in this thesis namely autoimmune encephalitis and MG, **CHAPTER 2** reviews the current *status quo* of autoimmunity in psychotic disorders, the relevance of the identification of the pathogenic antigens for a prompt and differential diagnosis. More specifically, **CHAPTER 3** describes a case report of a patient with mild cognitive impairment and thyroid peroxidase (TPO) autoantibodies, co-occurring with undefined neuronal surface autoantibodies. A clinical study with *de novo* recruited psychotic patients to assess the presence of autoantibodies against neuronal receptors in blood and cerebrospinal fluid (CSF) is described in **CHAPTER 4**. In this study, cognitive and psychiatric manifestations as well as neurological signs are addressed in order to common and distinct signatures of reactive patients.

In search for alternative therapeutic strategies for autoimmunity, **CHAPTER 5** gives an overview of the classic immunomodulatory therapies currently used in MG with antibodies targeting the AChR as a classic antibody-mediated autoimmune disorder. The lack of specificity and the presence of unwanted effects, takes us to the second part of the chapter, focused on innovative therapeutic strategies for symptomatic and immunomodulatory treatment approaches. Thanks to the extensive knowledge on the pathogenic mechanisms of AChR-MG, **CHAPTER 6** studies the effect of silencing of Dok-7, essential for the correct clustering of the AChRs at the neuromuscular junction and its potential therapeutic role. The final study presented in **CHAPTER 7** describes the effect of a second-generation proteasome inhibitor, ixazomib in AChR-MG. Using patient derived *in vitro* assays and the standardized model for MG, the demonstration of an efficient and safe dose dependent effect takes a step further for the application of this treatment in patients.

The key outcomes and discoveries of this thesis are summarized in CHAPTER 8. Overall strengths and limitations along with future perspectives of this thesis are described with criticism concerning the research field of antibody-mediated autoimmune disorders.

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Autoimmunity in psychotic disorders. Where we stand, challenges and opportunities

Marina Mané-Damas, Carolin Hoffmann, Shenghua Zong, Amanda Tan, Peter C. Molenaar, Mario Losen, Pilar Martinez-Martínez

Department of Psychiatry and Neuropsychology, School of Mental Health and Neuroscience, Maastricht University, Maastricht, the Netherlands

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Abstract

Psychotic disorders are debilitating mental illnesses associated with abnormalities in various neurotransmitter systems. The development of disease-modifying therapies has been hampered by the mostly unknown etiologies and pathophysiologies.

Autoantibodies against several neuronal antigens are responsible for autoimmune encephalitis. These autoantibodies disrupt neurotransmission within the brain, resulting in a wide range of psychiatric and neurologic manifestations, including psychosis. The overlap of symptoms of autoimmune encephalitis with psychotic disorders raised the question as to whether autoantibodies against a number of receptors, ion channel and associated proteins could ultimately be responsible for some forms of psychosis. Here we review our current knowledge, on antibody mediated autoimmunity in psychotic disorders, the different diagnostic methods and their limitations, as well as on varying therapeutic approaches targeting the immune system.

Keywords

Channelopathies, psychosis, neuropsychiatric, autoantibodies, immunotherapy

Highlights

- Antibodies can alter receptor and channel functions leading to psychotic symptoms.
- Neurological signs can be subtle or misinterpreted in patients with prominent psychotic symptoms.
- The use of standardized assays and CSF is essential to define autoimmunity in psychotic disorders.
- Specificity and sensitivity variations create controversial results in the field.
- Larger, homogeneous and systematically described cohorts need to be studied.

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Abbreviations

AChR	acetylcholine receptor
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
BBB	blood brain barrier
CASPR2	contactin-associated protein 2
CBA	cell-based assay
CNS	central nervous system
CSF	cerebrospinal fluid
D2DR	dopamine receptor D2
DPPX	dipeptidyl aminopeptidase-like protein 6
GABA _A R	<i>gamma</i> -aminobutyric acid A receptor
GABA _B R	<i>gamma</i> -aminobutyric acid B receptor
GAD	glutamic acid decarboxylase
GlyR	glycine receptor
Ig	immunoglobulin
IHC	immunohistochemistry
IVIG	intravenous immunoglobulin
LG11	leucine-rich glioma inactivated protein I
MAC	membrane attack complex
NMDAR	N-methyl-d-aspartate receptor
RIA	radioimmunoassay
VGKC/C	voltage-gated potassium channel/complex

1. Introduction

Psychotic disorders like schizophrenia and bipolar disorder have been well documented in terms of prevalence, demographics and co-morbidity. The pathophysiology of these disorders, however, remains poorly understood. Robust evidence suggests that synaptic transmission within dopaminergic and glutamatergic pathways is altered, which may result in a disequilibrium between inhibitory-excitatory mechanisms. Diminished function of neurotransmitter receptors like dopamine- and N-methyl-D-aspartate receptors (NMDAR) and dysfunction of ion channels such as voltage-gated potassium channels (VGKC), have all been shown to play an important role in the development and progression of schizophrenia [1-3]. Interestingly, antibodies and venom peptides targeting purinoceptors (P2X7), transient receptor potential cation channels (TRPC4 and TRPC5), cannabinoid receptor, nicotinic alpha-7 receptor, gamma-aminobutyric acid A receptor (GABA_AR) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) are currently approved or under clinical trial indicated for cognitive impairment, anxiety, depression and schizophrenia [4,5]. Furthermore, there are genetic risk factors: studies in large, pooled, cohorts have indicated that there are variations in the genetic coding for ion channels (common and rare gene variants), which thereby could increase the risk for psychotic disorders. Single nucleotide polymorphisms in genes encoding for the L-type calcium channel, affecting the gene activity of two subunits (CACNA1C and CACNB2), have been identified as risk loci, with shared effects in five major neuropsychiatric disorders, including schizophrenia and bipolar disorder [6]. Alterations in neurexin genes, coding for proteins involved in synaptic differentiation and transmission, have been associated with higher risk of developing schizophrenia [7,8] and autism [7,9-11]. Moreover, there are also genetic factors that affect the immune system. Gene variants encoding for human leukocyte antigen (HLA) regions (major histocompatibility complex (MHC) molecules), involved in building an immune response through antigen presentation, have also firmly been associated with an increased risk of schizophrenia [12,13].

The idea that autoimmunity may play a role in neuropsychiatric disorders began to take root in the last decade. Dr. Dalmau was the first to describe a group of patients with encephalitis associated with ovarian teratoma. After developing prodromal symptoms such as fever, headache and nausea, patients presented with a variety of severe psychotic and neurological manifestations, including anxiety, delusions, mania, short-term memory loss and seizures. Extensive analysis showed that serum and cerebrospinal fluid (CSF) contained antibody reactivity towards neuronal tissue, which was also present in the tumor. Autoantibodies reacted specifically against the NR1 subunits of the NMDAR. After immunosuppressive treatment and tumor resection, most of the patients recovered completely, regaining their normal social behavior and cognitive abilities in the absence of any remaining

neuropsychiatric manifestations. This previously unassociated collection of symptoms was termed autoimmune encephalitis [14].

It is not entirely unexpected, of course, that antibodies targeting neuronal receptors can cause psychotic symptoms. Neurotransmitter receptor antagonists such as phencyclidine, quinuclidinyl benzilate and lysergic acid diethylamide, that block NMDAR, muscarinic acetylcholine receptor and 5-HT receptor, respectively, are well-known to be potent hallucinogens. Agonists of the cannabinoid receptors also have the capacity to induce psychosis [15-20]. The different mechanisms by which autoantibodies alter synaptic transmission likely impacts the widespread function of neurons and neural networks, culminating in serious mental (and neurological) disturbances (Fig. 1).

Fig. 1. Venn diagram of the relationship between neurological, psychotic and autoimmune disorders. The overlap between the different diseases is being described in this review. Autoimmune encephalitis is a case in point, where autoantibodies (blue) and neurological symptoms (yellow) have been found with or without psychotic manifestations (green). However, the balance between the neurological symptoms and the psychotic manifestations can vary largely among the different disorders where autoantibodies are the organic cause of the pathology. These could lead in some cases to misdiagnoses and the use of anti-psychotic drugs instead of immunotherapy. This review focuses on the findings regarding the area shared by the 3 disciplines, where psychiatric and neurological manifestations are caused by the presence of autoantibodies. However, the scales represent the different expression of psychotic and neurological manifestations in different cases, raising the possibility of having a new subgroup of patients with autoimmune psychotic disorders with subtle or undetectable neurological symptoms.

This article reviews what is known about the occurrence of autoantibodies against neuronal neurotransmitter receptors, voltage-gated ion channels and associated proteins. We also summarize the effect of the autoantibodies in the pathophysiology of neuropsychiatric disorders and the corresponding treatment options.

2. Autoimmunity

Autoimmune diseases occur in 7-9% of the general population [21]. Specialized cells are generated in the adaptative response, namely T and B lymphocytes. This requires a maturation process with several built-in check points to detect and delete defective cells. One of these mechanisms is the acquisition of tolerance, which is generated centrally and peripherally in the lymphoid organs, where the lymphocytes are screened for their ability to distinguish between self and non-self-antigens [22]. Failure of this machinery can lead to autoimmunity where different cell mechanisms are involved. Autoimmune disorders can show a wide expression of phenotypes ranging from endocrine, musculoskeletal to neurological syndromes. Diseases where ion channels, on the surface of nerve or muscle membranes, are affected by autoantibodies, or altered by point mutations in the genome, have been named channelopathies [23,24].

2.1. Autoimmune mechanisms in channelopathies

Immunoglobulins (Igs) in the soluble form, commonly known as antibodies, are synthesized and released by B cells and plasma cells; differentiated B cells, developed in the lymphoid follicles, spleen and lymph nodes, are used as defence weapons in the adaptative immune response. IgM, IgD, IgG (1-4), IgA (1-2) and IgE are different Ig isotypes with a shared general structure (Fig. 2), but differ in characteristics such as size, capacity to activate complement, FcR binding and response to antigen, which is based on the heavy chain of the constant region. IgG is the most abundant and has the longest half-life; its isoforms are the most studied in autoimmune diseases [25]. IgG1 and IgG3 subclasses have the capacity to bind to protein antigens and induce endocytosis through cross-linking and can damage tissue as a result of complement activation, leading to loss of antigen function [26-28]. In contrast, the capacity to fix complement is lower or non-existent for the subclasses IgG2 and IgG4, which have higher affinity to polysaccharide antigens [29,30].

Fig. 2. General structure of an immunoglobulin. Immunoglobulins, commonly known as antibodies, are heterodimeric glycoproteins consisting of four chains, two heavy (H) and two light (L), each of them with constant (C) and variable (V) regions. Each H chain is composed by 3 C (CH1-3) and 1 V (VH) regions, linked with a disulfide bond at the CH2. The L chains are formed by a C (CL) and a V (VL) region and they are linked with the H chains one to one through the CH1, in a “Y” shape molecule. The constant regions together form the Fc fragment, while the variable regions compose the Fab fragment.

IgG-mediated autoimmune disease mechanisms against membrane receptors ion channels and their associated proteins (Fig. 3) are summarized as follows:

- a) Stimulation, inhibition or blocking of receptor or channel function. This mechanism has been observed in: i) infantile-onset neuropsychiatric disorders with psychomotor retardation, cerebellar ataxia, dyskinesia and in some cases, seizures due to the blocking of the folate receptor [31]; ii) autoimmune basal ganglia disorders with altered function of the dopamine receptor D2 (D2DR), comparable to the inhibitory effect of dopamine [32]; iii) cases of autoimmune encephalitis with hyper excitability in neurons in contact with antibodies targeting dipeptidyl aminopeptidase-like protein 6 (DPPX) [33] and iv) GABABR derived antibodies from limbic encephalitis patients which have been proposed to cause functional blockage of the receptors, seizures and memory dysfunction [34,35].
- b) Antigenic modulation. The binding of IgG1 leads to cross-linking of antigens, inducing internalization or endocytosis of the antigen-antibody complex, commonly followed by the degradation of the antigen. Examples are the cross-linking and selective internalization of NMDAR [14] as shown in cultured neurons [36-38] and also in an animal model of NMDAR

encephalitis where reduction of membrane-associated NMDAR is observed [39]. Reduction in the number of receptors in the synapse has also been observed *in vitro* by autoantibodies to AMPAR [40,41], GABA_AR [42], IgLON5 [43], neurexin-3 [44] and glycine receptor (GlyR) [45].

- c) Complement activation and inflammation. Antigen-antibody complexes (IgG1 and IgG3 isotypes) lead to the activation of the complement system [46,47]. The activation of the complement system leads to i) chemo-attraction and activation of phagocytes; ii) opsonization and engulfment by phagocytes and iii) formation of membrane attack complex (MAC) in cell membranes leading to lysis, extensive tissue damage and loss of tissue architecture. Activation of the classical complement pathway is an important mechanism in Rasmussen's encephalitis, where antibodies against the subunit 3 of the AMPAR have been detected [48]. Autoantibodies to GlyR, associated to progressive encephalomyelitis with rigidity and myoclonus (PERM), were shown to induce deposition of C3b in an *in vitro* assay with colocalization of the patient autoantibodies, which are predominantly of the IgG1 subclass [49]. Complement mediated pathology has been reported in the voltage-gated potassium channel complex (VGKCC) encephalitis with autoantibodies against contactin-associated protein 2 (CASPR2). In this case the patient presented brain atrophy detected by MRI and deposition of the final complement complex was observed in a hippocampus biopsy [50]. Complement activation in brain biopsies was studied in different subjects, including patients with anti-NMDAR and anti-VGKCC antibodies. In this study, 3 out of 4 patients with VGKCC autoantibodies, independently of the antigen specificity [leucine-rich glioma inactivated protein I (LGI1) or CASPR2], showed reactivity to neurons and complement deposition on neurons in brain tissue. However, none of the 3 patients with NMDAR autoantibodies presented with complement deposition [51]. Another two studies presented similar results, with no evidence of complement deposition in brain biopsies from NMDAR encephalitis patients [52,53]. Based on the Ig isotype, complement mediated pathology could be hypothesized in GABA_BR, GABA_AR, mGluR5 and neurexin-3, where IgG1 is the most prevalent isotype described [54].
- d) Loss or block of antigen-associated proteins. An important example of this is the VGKCC, where the autoantibodies are often directed against associated proteins, LGI1 and CASPR2 [55,56]. Antibodies to LGI1 can disrupt the interaction with scaffolding proteins like ADAM22 or ADAM23 [56,57]. Autoantibodies from patients with NMDAR encephalitis are found to interrupt the contact between NMDAR and Ephrin B2/EphB2 receptors, stabilizing and helping the receptor clustering in the postsynaptic membrane [58-61]. As a compensatory mechanism, increased EphB2 levels have been observed *in vitro* and *in vivo* [37]. A decrease of DPPX and Kv4.2 expression has been detected in presence of DPPX antibodies when incubated with hippocampal neurons [33,54].

Fig. 3. IgG antibody-mediated disease mechanisms. A) The binding of an antibody to a cell surface antigen can cause either stimulation, inhibition or blocking to the action of agonists on the antigen and its intracellular signaling pathways. B) Cross-linking or clustering of antigens can occur when one antibody binds to 2 antigens. This triggers the removal of the antibody-antigen complexes via endocytosis, after which the complexes are degraded. C) Formation of an antibody-antigen complex can activate the complement system leading to

chemoattraction of phagocytes, which can bind to complement via specific receptors, initiating opsonization and engulfment. Various other complement proteins are activated as well and assembled into a membrane attack complex (MAC). This creates a pore in the cell membrane through which extracellular fluids enter the cell, eventually inducing cell lysis. D) Antibodies can interfere with the contact between the antigen and its associated membrane proteins, as is the case for NMDA and EphB2 receptors. EphB2 receptors contribute to proper clustering of NMDAR in the postsynaptic membrane, which is crucial for synaptic plasticity. Antibodies can obstruct the contact between EphB2 and NMDAR, causing impaired synaptic retention of both receptors and loss of synaptic plasticity. As compensatory mechanism, EphB2R is upregulated.

3. Blood-brain-barrier

After the activation of the immune system, antibody producing cells travel in the blood to reach the organ with the targeted antigen. However, how antibodies or antibody producing cells reach the central nervous system (CNS) is still not well characterized. The blood brain barrier (BBB) unselectively prevents passage of the vast majority of molecules to the brain. The BBB is impermeable to hydrophilic substances and mostly also to large molecules such as proteins, so as to protect the CNS against the potential deleterious actions of these substances. In fact, antibodies can cross the human BBB to a limited extent [62] and reach sites in the CNS to exert pathogenic effects [63]. Diseases in which this is known to occur include paraneoplastic neurological syndromes [64], neuromyelitis optica [65], Stiff-person' s syndrome [66], epilepsy with glutamic acid decarboxylase (GAD) antibodies [66,67], narcolepsy [68], dyslexia [69], Morvan' s syndrome and limbic encephalitis [55].

Antibodies can be generated on both sides of the BBB, intrathecally [70] or peripherally from where they access the brain by crossing the BBB through different mechanisms. With an intact BBB, antibodies gain access to the CSF with a rate of 0.018 mg/min and have a turnover rate of five times per day, comparable to the CSF turnover itself of four times per day [62]. This very dynamic equilibrium results in brain IgG levels of around 1% of the plasma levels. Nonetheless IgG still composes 9.8% of the total protein in the CSF [62,71], whereas IgA and IgM are found only in trace amounts [72]. Following local inflammatory reactions, it is known that the BBB becomes much more permeable. For instance, in multiple sclerosis, the permeability is increased six fold [71]. Moreover, neuroinflammation may lead to local production of antibodies by B-cells or plasma cells in the brain itself, so that the concentration of antibodies there is strongly increased. A high level of antibodies in the CSF indicates an intrathecal synthesis of antibodies by migrated antibody producing cells in the brain, which is in line with the observation that the level of antibodies in CSF is more closely related with relapses in NMDAR encephalitis than in serum [73]. In psychotic disorders, the CSF analysis presented elevated white cell count as well as total protein levels and albumin quotient in 3.4, 42.2 and 21.8% respectively, concluding that Igs have been intrathecally produced in 7.2% of the cases [74].

Recent studies have shown that the deep cervical lymph nodes have an important role in the processing and presentation of antigens of the CNS for T-cell mediated responses [75] but not for antibody-mediated disorders. However, in NMDAR encephalitis, antibodies seem to occur earlier in CSF than in peripheral blood [76,77]. Moreover NMDAR antibodies have been cloned from CSF-derived cells [78], which indicates an intrathecal synthesis of autoantibodies [70]. This is usually accompanied with inflammatory changes such as pleocytosis and increased protein levels [73,74,79]. Furthermore, levels of the B cell attracting chemokine C-X-C motif chemokine 13 -CXCL13-, associated with plasma cells in the CNS, have been correlated with the intrathecal synthesis of NMDAR antibodies and with limited response to treatments and relapses [80].

It is important to note that in spite of the dynamic turnover of antibodies between the periphery and the brain, well-defined autoantibodies of peripheral diseases, like the Lambert-Eaton myasthenic syndrome and neuromyotonia, do not have central effects. This is remarkable because calcium Cav2.1 (P/Q-type) channels and VGKCC proteins are also present in the brain and could therefore be targeted by these antibodies. Thus, in some cases, CNS autoimmune channelopathies occur only when the integrity of the BBB is disrupted and antibodies gain access to the brain at a higher rate. From this point of view, it might be argued that CASPR2 limbic encephalitis, Morvan' s syndrome and acquired neuromyotonia harbor the same or similar disease aspects of pathophysiology and immunological pathogenesis, but that there is a relative difference in permeability of the BBB to the antibodies in these disorders [55].

In line with this, mice immunized with a peptide producing both antibodies to DNA and NMDAR did not show brain pathology unless the integrity of the BBB was challenged [63]. It is unlikely that the BBB is equally permeable to antibodies or antibody-producing cells throughout the brain. Factors affecting the BBB integrity with respect to the transport of antibodies may show regional specificity. This would explain why the “phenotype” of the antibody effect may well depend on the location where the antibody enters the brain and why the same autoantibody might have substantially different pathological effects between different groups of patients. After lipopolysaccharide treatment, NMDAR antibodies derived from systemic lupus erythematosus patients attack channels in hippocampal neurons [81], while the same antibodies damage channels in other regions of the brain, after treatment with adrenaline -which is known to breach BBB integrity [82] (see ref. [63] for a review on BBB and antibodies).

4. Antibody-mediated channelopathies in neuropsychiatric disorders

Over two-thirds of patients with autoimmune encephalitis have marked psychotic symptoms that can prevail in periods without obvious neurological manifestations; before onset of encephalitis or as a relapse. Around 45% of pediatric [83] and 16.5% of adults with limbic encephalitis [84] and antibodies to

VGKCC presented with psychotic manifestations [85]. Many patients have their initial presentation at psychiatric services with more than 60% being first admitted to a psychiatric unit [86-88]. The psychotic manifestations are similar to those seen in schizophrenia including delusional and delirious thinking, anxiety, insomnia, paranoid thoughts, manic and aggressive behaviour, hallucinations and catatonic movement disorder [70,83, 89-92]. In VGKCC antibody-associated encephalopathy, cognitive impairment is an important feature but other neuropsychiatric symptoms occur in various degrees, mainly characterized by personality changes, depression and anxiety (see ref. [85] for review including single case studies). These findings indicate that some cases with underlying encephalitis could be mistaken for a primary psychotic disorder.

4.1. Incidence of autoantibodies in psychotic disorders

In the past 10 years, numerous cohorts of patients with mental disorders have been tested for the presence of specific neuronal autoantibodies, with those against the NMDAR and the VGKCC being the most well-described. Outcomes of these studies are variable, as are the detection methods applied. This section summarizes these findings. Details of relevant studies are shown in Table 1.

Table 1. Studies on the prevalence of neuronal autoantibodies in psychotic disorders

Targeted antigen	Disorder	Ref.	Antibody source	Methods	N (patients/controls)	Isotypes studied	IgG prevalence (patients/controls)	Targeted antigen
VGKC complex	First episode psychosis	[94]	Serum	RIA	228/105	IgG	5	3
				Live CBA (LG11)	228/105	IgG	1	0
				Live CBA (CASPR2)	228/105	IgG	1	3
	First episode psychosis	[93]	Serum	RIA	46/n.a.	IgG	2.2	n.a.
	Psychotic disorders ₂	[74]	CSF	Fixed CBA (LG11/CASPR2)	125/n.a.	IgG	2.4	n.a.
Schizophrenia/schizoaffective disorder		[95]	Serum	Fixed CBA (LG11) + IHC	1378/1703	IgG, IgA, IgM	0.1	0.1
				Fixed CBA (CASPR2) + IHC	1378/1703	IgG, IgA, IgM	0.4	0.3
	Affective disorders	[95]	Serum	Fixed CBA (LG11) + IHC	310/1703	IgG, IgA, IgM	0	0.1
First episode psychosis				Fixed CBA (CASPR2) + IHC	1378/1703	IgG, IgA, IgM	0.3	0.3
		[96]	Serum	Fixed CBA (CASPR2) + IHC	70/34	IgG	0	0
	Schizophrenia spectrum disorder	[109]	CSF	Fixed CBA (LG11)	110/n.a.	IgG	0	n.a.
			Serum	Fixed CBA (LG11)	81/n.a.	IgG	0	n.a.
			CSF	Fixed CBA (CASPR2)	111/n.a.	IgG	0	n.a.
Schizophrenia			Serum	Fixed CBA (CASPR2)	81/n.a.	IgG	2.5	n.a.
		[108]	Plasma	Fixed CBA (LG11)	78/n.a.	IgG	0	n.a.

Targeted antigen	Disorder	Ref.	Antibody source	Methods	N (patients/controls)	Isotypes studied	IgG prevalence (patients/controls)	Targeted antigen
				Fixed CBA (CASPR2)	78/n.a.	IgG	0	n.a.
	Acute schizophrenia spectrum disorder	[103]	Serum	Fixed CBA (CASPR2) + IHC _{***}	144/n.a.	IgG, IgA, IgM	1.4	n.a.
	Acute bipolar disorder	[103]	Serum	Fixed CBA (CASPR2) + IHC _{***}	125/n.a.	IgG, IgA, IgM	0.8	n.a.
	Acute schizophrenia spectrum disorder	[103]	Serum	Fixed CBA (LG11)	144/n.a.	IgG, IgA, IgM	0	n.a.
	Acute bipolar disorder	[103]	Serum	Fixed CBA (LG11)	125/n.a.	IgG, IgA, IgM	0	n.a.
	First episode psychosis	[104]	Serum	Fixed CBA (CASPR2) + IHC + neurons _{**}	61/45	IgG	0	0
				Fixed CBA (LG11) + IHC + neurons _{**}	61/45	IgG	0	0
	Post-partum psychosis	[112]	Serum	Fixed CBA (CASPR2) + IHC + neurons _{*****}	96/64	IgG	0	0
				Fixed CBA (LG11) + IHC + neurons _{****}	96/64	IgG	0	0
NMDAR	First episode psychosis	[94]	Serum	Live CBA (NR1/2b)	228/105	IgG	3	0
	First episode psychosis	[93]	Serum	Live CBA (NR1/2b)	46/n.a.	IgG	6.5	n.a.

Targeted antigen	Disorder	Ref.	Antibody source	Methods	N (patients/controls)	Isotypes studied	IgG prevalence (patients/controls)	Targeted antigen
	Psychotic disorders ₂	[74]	CSF	Fixed CBA (NR1/2b)	125/n.a.	IgG	0.8	n.a.
	Schizophrenia/schizo affective disorder	[95]	Serum	Fixed CBA (NR1) + IHC	1378/1703	IgG, IgA, IgM	0.6	1.2
	Affective disorders	[95]	Serum	Fixed CBA (NR1) + IHC	310/1703	IgG, IgA, IgM	1.9	1.2
	Schizophrenia	[38]	Serum	Fixed CBA (NR1/2b) + IHC	50/n.a.	IgG, IgA, IgM	0	n.a.
	First episode psychosis	[110]	Serum	Flow/live CBA (NR1) + IHC + neurons	43/17	IgG, IgM, IgA	11.6	0
	Schizophrenia spectrum disorder	[107]	Serum/plasma	Fixed CBA (NR1/2b) + IHC	475/n.a.	IgG	0	n.a.
	First episode psychosis	[106]	Serum	Fixed CBA (NR1/2b) + IHC + neurons	80/40	IgG	0	0
	First episode psychosis	[96]	Serum	Fixed CBA (NR1/2b) + IHC	70/34	IgG	0	0
	Schizophrenia spectrum disorder	[109]	CSF	Fixed CBA (NR1/2b)	119/n.a.	IgG	0	n.a.
			Serum	Fixed CBA (NR1/2b)	81/n.a.	IgG	0	n.a.
	First episode psychosis	[105]	Serum	fixed CBA (NR1/2b), Live CBA (NR1/2b)	298/n.a.	IgG	0.7	n.a.
	Schizophrenia	[108]	Plasma	Fixed CBA (NR1) + IHC ₂	298/n.a.	IgG	2.7	n.a.
					312/n.a.	IgG	0	n.a.

Targeted antigen	Disorder	Ref.	Antibody source	Methods	N (patients/controls)	Isotypes studied	IgG prevalence (patients/controls)	Targeted antigen
	Treatment refractory schizophrenia spectrum disorder	[111]	Serum	Live CBA (NR1/2b)	43/n.a.	IgG	7.0	n.a.
	Acute schizophrenia spectrum disorder	[103]	Serum	Fixed CBA (NR1) + IHC	144/n.a.	IgG, IgA, IgM	0	n.a.
	Acute bipolar disorder	[103]	Serum	Fixed CBA (NR1) + IHC _{***}	125/n.a.	IgG, IgA, IgM	1.6	n.a.
	First episode psychosis	[104]	Serum	Fixed CBA (NR1) + IHC + neurons _{**}	61/45	IgG	3.3	0
	Post-partum psychosis	[112]	Serum	Fixed CBA (NR1/2) + IHC + neurons _{*****}	96/64	IgG	2.1	0
AMPA	First episode psychosis	[94]	Serum	Live CBA (GluR1/2)	228/105	IgG	0	0
	Psychotic disorders _±	[74]	CSF	Fixed CBA (GluR1/2)	96/n.a.	IgG	0	n.a.
	Schizophrenia/schizoaffective disorder	[95]	Serum	Fixed CBA (GluR1/2) + IHC	1378/1703	IgG, IgA, IgM	0.1	0.1
	Affective disorders	[95]	Serum	Fixed CBA (GluR1/2) + IHC	310/1703	IgG, IgA, IgM	0	0.1
	First episode psychosis	[96]	Serum	Fixed CBA (AMPA) + IHC	70/34	IgG	0	0
	Schizophrenia spectrum disorder	[109]	CSF	Fixed CBA (GluR1/2)	114/n.a.	IgG	0	n.a.
			Serum	Fixed CBA (GluR1/2)	81/n.a.	IgG	0	n.a.

Targeted antigen	Disorder	Ref.	Antibody source	Methods	N (patients/controls)	Isotypes studied	IgG prevalence (patients/controls)	Targeted antigen
	Schizophrenia	[108]	Plasma	Fixed CBA (GluR1/2)	78/n.a.	IgG	0	n.a.
	Acute schizophrenia spectrum disorder	[103]	Serum	Fixed CBA (AMPA) + IHC***	144/n.a.	IgG, IgA, IgM	0.7	n.a.
	Acute bipolar disorder	[103]	Serum	Fixed CBA (AMPA) + IHC	125/n.a.	IgG, IgA, IgM	0	n.a.
	First episode psychosis	[104]	Serum	Fixed CBA (GluR1/2) + IHC + neurons**	61/45	IgG	0	0
	Post-partum psychosis	[112]	Serum	Fixed CBA (GluR1/2) + IHC + neurons****	96/64	IgG	0	0
GABA _A R	First episode psychosis	[94]	Serum	Live CBA (GABA _A R, α1, γ2)	228/105	IgG	4	1
	First episode psychosis	[96]	Serum	Fixed CBA (GABA _A R) + IHC	70/34	IgG	0	0
	Post-partum psychosis	[112]	Serum	Fixed CBA (GABA _A R) + IHC + neurons****	96/64	IgG	0	0
GABA _B R	Psychotic disorders _±	[74]	CSF	Fixed CBA (GABA _B R)	96/n.a.	IgG	0	n.a.
	Schizophrenia/schizoaffective disorder	[95]	Serum	Fixed CBA (GABA _B R1/2) + IHC	1378/1703	IgG, IgA, IgM	0	0
	Affective disorders	[95]	Serum	Fixed CBA (GABA _B R1/2) + IHC	310/1703	IgG, IgA, IgM	0.3	0
	First episode psychosis	[96]	Serum	Fixed CBA (GABA _B R) + IHC	70/34	IgG	0	0

Targeted antigen	Disorder	Ref.	Antibody source	Methods	N (patients/controls)	Isotypes studied	IgG prevalence (patients/controls)	Targeted antigen
D2DR	Schizophrenia spectrum disorder	[109]	CSF	Fixed CBA (GABA _B R1/2)	112/n.a.	IgG	0	n.a.
				Fixed CBA (GABA _B R1/2)	81/n.a.	IgG	0	n.a.
	Schizophrenia	[108]	Plasma	Fixed CBA (GABA _B R1)	78/n.a.	IgG	0	n.a.
				Fixed CBA (GABA _B R) + IHC	144/n.a.	IgG, IgA, IgM	0	n.a.
	Acute schizophrenia spectrum disorder	[103]	Serum	Fixed CBA (GABA _B R) + IHC	125/n.a.	IgG, IgA, IgM	0	n.a.
				Fixed CBA (GABA _B R) + IHC	61/45	IgG	0	0
	First episode psychosis	[104]	Serum	Fixed CBA (GABA _B R) + IHC + neurons**	96/64	IgG	0	0
				Fixed CBA (GABA _B R) + IHC + neurons****				
	Post-partum psychosis	[112]	Serum	Flow/live CBA (D2DR) + IHC + neurons	43/17	IgG, IgM, IgA	7.0	0
				Fixed CBA (D2DR) + IHC	70/34	IgG, IgM, IgA	0	0
DPPX	First episode psychosis	[96]	Serum	Fixed CBA (D2DR) + IHC	96/64	IgG	0	0
				Fixed CBA (D2DR) + IHC + neurons****				
	Post-partum psychosis	[112]	Serum	Fixed CBA (D2DR) + IHC	1378/1703	IgG, IgA, IgM	0.1	0.1
				Fixed CBA (DPPX) + IHC	310/1703	IgG, IgA, IgM	0	0.1

Targeted antigen	Disorder	Ref.	Antibody source	Methods	N (patients/controls)	Isotypes studied	IgG prevalence (patients/controls)	Targeted antigen
Nicotinic AChR	First episode psychosis	[96]	Serum	Fixed CBA (DPPX) + IHC	70/34	IgG	0	0
	Post-partum psychosis	[112]	Serum	Fixed CBA (DPPX) + IHC + neurons _{8****}	96/64	IgG	0	0
	Schizophrenia	[165]	Serum	ELISA (α7 AChR)	21/17	IgG	23.8	0
	Psychotic disorders	[166]	Serum	RIA (α7 AChR)	508/118	IgG	0.2	0
	Schizophrenia/schizoaffective disorder	[95]	Serum	Fixed CBA (GlyRa1) + IHC	1378/1703	IgG, IgA, IgM	0.1	0.1
	Affective disorders	[95]	Serum	Fixed CBA (GlyRa1) + IHC	310/1703	IgG, IgA, IgM	0	0.1
	First episode psychosis	[96]	Serum	Fixed CBA (GlyR) + IHC	70/34	IgG	0	0
	Post-partum psychosis	[112]	Serum	Fixed CBA (GlyR) + IHC + neurons _{8****}	96/64	IgG	0	0
	Psychotic disorders ₂	[74]	CSF	immunoblot (GAD65)	142/n.a	IgG	0	n.a.
	Schizophrenia/schizoaffective disorder	[95]	Serum	Fixed CBA (GAD65) + IHC	1378/1703	IgG, IgA, IgM	0.6	0.3
GAD	Affective disorders	[95]	Serum	Fixed CBA (GAD67) + IHC	1378/1703	IgG, IgA, IgM	0.1	0.1
				Fixed CBA (GAD65) + IHC	310/1703	IgG, IgA, IgM	0.3	0.3
				Fixed CBA (GAD67) + IHC	1378/1703	IgG, IgA, IgM	0.3	0.1
				Fixed CBA (GAD65) + IHC	70/34	IgG	0	0
	First episode psychosis	[96]	Serum	Fixed CBA (GAD65) + IHC				

Targeted antigen	Disorder	Ref.	Antibody source	Methods	N (patients/controls)	Isotypes studied	IgG prevalence (patients/controls)	Targeted antigen
	Acute schizophrenia spectrum disorder	[103]	Serum	Fixed CBA (GAD65) + IHC _{***}	144/n.a.	IgG, IgA, IgM	0.7	n.a.
	Acute bipolar disorder	[103]	Serum	Fixed CBA (GAD65) + IHC _{***}	125/n.a.	IgG, IgA, IgM	2.4	n.a.
Amphiphysin	Psychotic disorders _{**}	[74]	CSF	Immuno-blot (amphiphysin)	142/n.a.	IgG	0	n.a.
	Schizophrenia/schizoaffective disorder	[95]	Serum	Fixed CBA (amphiphysin)	1378/1649	IgG, IgA, IgM	1.7	1.7
	Affective disorders	[95]	Serum	Fixed CBA (amphiphysin)	310/1649	IgG, IgA, IgM	2.3	1.7
	Schizophrenia spectrum disorder	[96]	CSF	Fixed CBA (amphiphysin)	93/n.a.	IgG	0	n.a.
			Serum	Fixed CBA (amphiphysin)	81/n.a.	IgG	0	n.a.

AChR: acetylcholine receptor; AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; CASPR2: contactin-associated protein 2; CBA: cell based assay; CSF: cerebrospinal fluid; D2DR: dopamine receptor 2; DPPX: dipeptidyl aminopeptidase-like protein 6; GABA_AβR: gamma-aminobutyric acid A/β receptor; GAD: glutamic acid decarboxylase; GlyR: glycine receptor; Ig: immunoglobulin; IHC: immunohistochemistry; LGI1: leucine-rich glioma inactivated protein 1; NMDAR: N-methyl-D-aspartate receptors; RIA: radioimmunoassay; VGKC: voltage gated potassium channel.

*Some patients were selected based on suspicion of organic brain disease.

**Only CBA positive samples were tested by IHC/neurons.

***Positive CBA results were negative when tested by IHC.

****Only IHC positive were tested by neurons/CBA.

An initial study reported serological prevalence of antibodies to VGKCC in first episode psychosis (2.2%) [93], tested by radioimmunoassay (RIA), but lacked a control group. A recent study also using VGKCC RIA reported a similar prevalence of autoantibodies both in first episode psychosis (5%) as well as controls (3%) [94]. Importantly, the same and other studies find much lower prevalences when studying specific IgG autoantibodies targeting LGI1 and CASPR2 by cell based assay (CBA) (0-1%) [95-97]. Antibodies to LGI1 but not to CASPR2 were also detected in 2 out of 144 (1.4%) patients with acute schizophrenia disorders and in 1 out of 125 patients (0.8%) with acute bipolar disorder in serum by CBA. However this study lacked a control group and CBA positive sera gave negative results by immunohistochemistry (IHC) [103]. However, it is important to keep in mind that autoantibodies against VGKCC but not directed against LGI1 or CASPR2, have no clinical relevance in cases of autoimmune encephalitis [98-100].

Variable results have been reported for the prevalence of autoantibodies to NMDAR in patients with psychotic disorders. Several studies have shown high prevalence (~10%) of autoantibodies when including IgG, IgA, and IgM isotypes. However, there were no differences in the distribution of Igs among psychotic patients, other neuropsychiatric disorders and healthy controls; indicating that these isotypes are not disease specific [95,101,102]. When studying only the IgG isotype, these and other studies reported much lower NMDAR prevalences in psychotic patients (0.7-3.3%) [74,94,103-105] or complete absence of these autoantibodies [96,103,106-109]. A recent study also analyzed the three Ig isotypes in serum of different disease cohorts (NMDAR encephalitis, suspected autoimmune encephalitis, schizophrenia, stroke, and dementia), and found that the IgG NMDAR autoantibodies were disease specific for NMDAR encephalitis, with no antibodies present in 50 schizophrenia subjects nor in the other disease groups. In addition, IgG but not IgA or IgM had pathogenic effects *in vitro* [38].

Higher prevalence of NMDAR IgG autoantibodies have been, however, found in special cohorts of psychotic disorders: The first group included children with first episode of acute psychosis. Using a combination of NR1 and D2DR flow cytometry, CBA and primary neuronal cell culture, Pathmanandavel and colleagues found serum NMDAR IgG antibodies in 5 out of 43 of these children (one of the patients had IgG + IgM autoantibodies targeting NR1, one had IgG to NR1 + D2DR IgG autoantibodies, and another one had IgA but not IgG to NR1) [110]. The second group consisted of a cohort of 43 refractory patients with schizophrenia or schizoaffective disorder (with a mean duration of more than 15 years). IgG autoantibodies to NMDAR were found in 7% of the cases by live CBA. None of the patients had MRI abnormalities, nor neurological signs including seizures [111]. However, the lack of a control group, together with the exclusive use of live CBA, known to give higher detection rates [105], make these results difficult to interpret. The third group consisted of patients with post-partum psychosis. Neuronal surface reactivity was detected by brain IHC in serum in 4 out of 96 post-partum patients with psychosis. Two of

these patients specifically presented IgG against the NMDAR by CBA. However, all the patients recovered without any immunotherapy [112]. Effects of the maternal antibodies in the offspring have been so far only described in cases of autism spectrum disorder, mental retardation and/or disorders of psychological development [113], specific language difficulties and dyslexia [69, 114-117], all of them disorders which develop early in life.

The detection of autoantibodies have proven to be more sensitive in CSF compared to serum in cases of NMDAR encephalitis [73,118,119]. However, in psychotic disorder cohorts, there is limited access to CSF samples. NMDAR and VGKCC (LGI1 and CASPR2) autoantibodies were detected in CSF (not tested in serum) in 0.8% for NMDAR antibodies and 2.4% for VGKCC antibodies in psychotic disorder patients [74]. Another study with a similar cohort showed comparable percentages of VGKCC autoantibodies in CSF (2.5% positive for CASPR2) but no antibodies were detected for NMDAR. No neuronal autoantibodies were found in the serum from the same patients [109]. The difficulty of interpreting studies where CSF has been analyzed, is that there are no control cohorts available, and the results have not been cross-validated.

Antibodies against other neuronal antigens have been described for autoimmune encephalitis and are associated with psychotic manifestations. To date autoantibodies to AMPAR, GABA_BR, GABA_AR, D2DR, GlyR, mGluR1/mGluR5, alpha7 AChR, GAD67/GAD67, amphiphysin, neurexin, etc. have been reported as absent or at low prevalence among psychotic disorders (reviewed by [54]).

Interestingly, some studies have reported presence of autoantibodies against unknown neuronal antigens. This is the case of 2 out of 96 post-partum psychosis patients, who showed reactivity by brain immunohistochemistry and immunocytochemistry using live hippocampal neurons [112]. In 17 out of 925 cases, patients admitted to acute psychiatric care, presented immunofluorescence staining on rat and monkey brain sections, without corresponding to a specific antigen [103]. A cohort from the Netherlands Study of Depression and Anxiety (NESDA) revealed that 8 out of 1739 patients but none of the 492 controls, presented with strong hippocampal patterns by rat brain IHC. Five of the positive patients were confirmed by primary hippocampal neurons and all of them shared common clinical manifestations, characterized by anxiety (Zong et al., manuscript in preparation). Similar results have been observed in a cohort of neuropsychiatric disorders, where autoantibodies against neuronal antigens have been detected by brain IHC in 14 out of 621 patients with psychosis, in 1 out of 70 patients with affective disorders and in 5 out of 259 non-disease controls (Hoffmann et al., manuscript in preparation).

4.2. Case reports of patients with strong psychotic manifestations

The increasing awareness of autoimmune encephalitis clinical presentation among clinicians and especially psychiatrists has led to early diagnoses of autoimmune encephalitis with psychotic manifestations. However, there are still a number of case reports with NMDAR autoantibodies predominantly with psychotic symptoms, where a correct diagnosis was complicated. These patients were given provisional diagnoses such as dissociative disorder, or psychotic disorder for several weeks or even years, until the presence of antibodies was finally discovered and treated accordingly with immunosuppression [90,120,121]. A recent case reported a misdiagnosed autoimmune encephalitis with VGKCC antibodies in a 16-year old girl, acutely presenting with isolated psychotic symptoms characterized by mania, psychosis and various behavioral and personality changes with no evidence of any neurological abnormality. Psychotropic medication only aggravated some of the symptoms and resulted in a minimal overall improvement. After 2 weeks, high levels of anti-VGKCC antibodies (but not specifically against LGI1 or CASPR2) were detected in serum (methodology not clearly specified). The patient received several courses of intravenous immunoglobulin (IVIG) and methylprednisolone and gradually recovered to normal status, 6-9 months after the psychotic episode [122,123].

Yet, despite the presence of neurological symptoms, an autoimmune condition can be misdiagnosed as psychotic disorder. Especially, after initiation of antipsychotic medication, typical autoimmune symptoms such as dyskinesias are often assumed to be extrapyramidal side effects [5]. Also, epilepsy patients have shown increased risk of psychosis and psychotic illness [124,125], whilst schizophrenic patients are also more likely to suffer from epilepsy [126]. Therefore, it is not surprising that psychotic manifestations are accompanied in many cases by neurological abnormalities, including seizures and therefore the diagnosis of autoimmune encephalitis can be delayed.

A case of a 17-year old male was reported with acute behavioral and mental disturbances characterized by psychosis, agitation, disorganization in behavior and thought processes, hypermetabolism and catatonia, together with seizures. Upon unresponsiveness to antipsychotic agents, the patient underwent electroconvulsive therapy with a remarkable improvement. After a period of four weeks, CSF analysis revealed the presence of anti-NMDAR antibodies (methodology not specified). IVIG immunosuppressant therapy was started, showing a noteworthy improvement [127]. After a long history of alcohol excess consumption, depression and self-harm, a 58-year old male was admitted to the hospital after presenting with seizures, confusion, slurred speech and ataxia. After psychiatric assessment he was diagnosed with paranoid schizophrenia and given antipsychotic medication. Further tests were performed after a deterioration of his symptoms and epileptic onset. Antibodies to VGKCC were detected in serum but not in CSF (detection assay not specified) and the patient underwent plasmapheresis, in combination with

IVIG and corticosteroids, which caused a substantial improvement of the seizures but with only a minor gain of his cognitive abilities [128].

Post-partum psychosis cases include: i) a 21-year old female presented with psychotic symptoms four weeks after giving birth. After a few weeks, she suffered generalized seizures and had high anti-nuclear antibody levels but no abnormalities in the CSF. A substantial improvement resulted from plasmapheresis and steroids treatment. Later, serum IgGs against VGKCC, specifically targeting LGI1 were identified by RIA and live CBA respectively. The patient recovered completely after immunosuppressive treatment [129]; ii) three months after a normal delivery, a 25 year old female developed insomnia, agitation and thanatophobia (fear of death). Antipsychotic treatment, including electroconvulsive therapy, resulted in generalized seizures. After aggravation of the psychotic symptoms and a suicidal attempt, laboratory tests were performed. No abnormalities were detected in serum but in CSF, where antibodies to NMDAR were found (detection assay not specified). Forthwith she was diagnosed with an ovarian teratoma. Repeated IVIG and intravenous methylprednisolone did not show any relieve of symptoms. However, after tumor resection, the patient had a dramatic and stable improvement with minimal behavioural changes at 6 months after surgery [119]; iii) similarly, three women were hospitalized during pregnancy presenting with headache and malaise; followed by bizarre behavior, anxiety, paranoid delusions, seizures and a decreased level of consciousness. Ovarian teratoma was diagnosed in 2 cases (resulted in pregnancy termination in one of the cases). After the identification of NMDAR antibodies in serum and CSF by CBA, the patients were treated with methylprednisolone, plasmapheresis and/or IVIG, together with ovarian teratoma resection in 2 of the cases. The babies showed no abnormalities after birth [130].

Tumor resection and immunotherapy using first line treatment usually results in partial or complete disappearance of the symptoms. However, in some cases, second line immunosuppressive therapies are required. This is also described in two independent case reports of young women, who developed post-partum psychosis followed by typical signs of NMDAR encephalitis. One was diagnosed with ovarian teratoma and both had NMDAR autoantibodies in the CSF. Tumor removal, intravenous methylprednisolone and plasmapheresis resulted in little improvement, but subsequent rituximab therapy improved the clinical manifestations of both women dramatically [131,132].

All in all, autoimmune encephalitis is estimated to have an annual incidence of around 1.2 per 100,000 cases [133]. In many case reports describing patients with pronounced psychotic manifestations, the methodology used to detect autoantibodies is not carefully detailed, making it very difficult to interpret results. Although we do not expect a higher incidence rate in the psychiatric population, we would still like to highlight the importance of considering the diagnosis of autoimmune encephalitis in patients presenting with psychosis and seizures. This is especially vital in those with no previous history of psychotic

disorders, a rapid progression of the disease, or resistance to antipsychotic medication since the majority of the patients respond well to readily available immunotherapy.

5. Sensitivity and specificity

The interpretation of the results in the literature regarding patients with pronounced psychotic manifestations is, unfortunately, difficult. As described through this review, lack of specified methodological strategies and the choice of the different assays, together with the absence of control groups reduces the impact of the findings.

In a group of patients with psychosis, it is likely that the prevalence of neuronal surface antibodies will be below 1% per specific neuronal antibody, making the reliability of a positive outcome rather small. Thus, the outcome of individual tests would be a poor guidance for counseling immunotherapy and certainly would make simple screening for antibodies in psychosis unfeasible. False results can pose a considerable problem for its interpretation, especially when the probability of specific neuronal antibodies in the test group is low. This is because the reliability of a positive assay outcome is influenced by the prevalence and the number of false positives of the test, as depicted in Fig. 4: the lower the prevalence, the poorer is the reliability with a concomitant increase of the chance of obtaining false positive results.

Fig. 4. Relation between reliability of positive outcomes of antibody tests as a function of the prevalence of the antibody within the targeted group with psychosis. The fractions are expressed as percentages. For specificity three conditions are shown, corresponding to 1%, 3% and 5% false positives assumed for the group of healthy controls. The graph is a hyperbole according to the equation $\text{Reliability} = \text{Pr} / ((\text{Pr} + (1 - \text{Sp})(1 - \text{Pr}))$, where Pr is prevalence of the autoantibody within the psychosis group and Sp the specificity of the antibody assay in the control group. For the equation it is assumed that the likelihood for yielding false positives in the psychosis patients without the antibody is the same as in the healthy control group. The figure shows that the antibody test becomes very unreliable when the prevalence is low in combination with a high percentage of false positives in the human control group. For example, with a prevalence of 1% and a specificity of 95%, only 17% of all positive outcomes are expected positive whereas 83% of them are expected to be false. With 99% specificity, the outcomes are expected to be 50% correct and 50% false positives, respectively.

5.1. Sensitivity and false negatives

The fraction of disease cases that an assay correctly predicts, defines the clinical sensitivity of the assay in question [134,135]. A clinical assay should have around 70% or higher sensitivity to be useful. There are several reasons why an assay could result in negative outcomes for patients genuinely suffering from an autoimmune disease (i.e. with a sensitivity of 70% this would be 30% of all cases);

- 1) Low (undetectable) antibody concentration in blood
- 2) Low affinity of the antibody to the antigen

- 3) High affinity and avidity of the antibody for the antigen *in vivo*, leading to reduced antibody levels in blood (immunoabsorption effect [136])
- 4) Interference between the protein in the sample and the assay (i.e. absence of the antibody in the membrane due to antibody internalization and resulting in unclear or ambiguous results)
- 5) Lack of recognition caused by conformational epitope changes (denaturalization or non-native conformations) or by absence of receptor-associated proteins, required for the original exposure of the antigen (i.e. anchoring the receptor to the membrane and keeping it in its native conformation) [137]
- 6) Hook or prozone effect, caused by the excess of antibodies or the lack of agglutination and formation of immunocomplexes

5.2. Specificity and false positives

The fraction of individuals with absence of disease that an assay correctly predicts defines the clinical specificity of an immunological assay. Clinical specificity should be preferably 95% or higher to be useful. However, there are at least three reasons why a test might not be fully specific (95% specificity implies that there are 5% false positives in the control group). “False positive” outcomes may arise because:

- 1) The test sample does indeed contain an antibody that is directed to the antigen used in the test; but apparently non-pathogenic (e.g. by not reaching the CNS)
- 2) The test sample contains an (non-pathogenic) antibody that is directed to a “related” antigen which cross-reacts with the antigen used in the test (e.g. a similar receptor or ion channel)
- 3) A different protein in the sample interferes with the antigen staining so that an incorrect positive result is read in the laboratory

It has been reported that positive results for NMDAR antibodies obtained by a commercial kit were not confirmed through cross-validation in two other independent laboratories using commercial and in-house CBA as well as IHC [107]. This example highlights the importance of considering the variation across tests (including variability between different batches of the same test), which will result in differences in sensitivity, specificity, or background signal and supports the need for expert and independent approaches evaluating the results in order to reduce variability and inconsistent results [105,107,138].

Changes in sensitivity and, specifically, in specificity between different laboratories (including companies), variations in the protein homology between species used in the assays and conformational modifications by the pre-treatment such as fixation of the tissue are all possible causes for incoherent or

false positives/negatives to occur. Although the homology between human and rodent proteins reaches 95% in most cases, some differences in the extracellular regions can lead to false results, both positive and negative. It has also been observed that embryonic and adult forms of some subunits may differ [139] (i.e. the gamma subunit of the AChR is present in embryonic tissue but is replaced by a homologous epsilon subunit in adult tissue [140]). Different autoantibodies can coexist in the same patient and this phenomenon may occur in up to 10% of the patients with an autoimmune disease. This raises the possibility that part of the false responses are due to the presence of autoantibodies against a variety of antigens, some of which happen to cross-react with the antigen used in the assay.

5.3. Considerations for combining diagnostic assays

Cellular and tissue based techniques are used to diagnose the presence of autoantibodies. However, it is important to mention that when approaching psychotic disorder cohorts where the expected prevalence of autoantibodies is quite low, the methods likely require optimization.

Results from the different assays can be complementary but may not always be comparable. CBA for example is a very specific but not especially sensitive technique [105]. Within this method, variations exist in the experimental conditions, which will highly influence the results. Live CBA is known to better preserve the epitopes which can be damaged when applying fixatives and permeabilizing agents. Live assays, however, notwithstanding the technical disadvantage that they can not be prepared as a kit in advance, have higher rates of positivity [105]. Variable results can also be dependent on the species origin from which the cells in the test are derived and also on the expression of the antigens when composed of different subunits (e.g. the NMDAR CBA can be set up with the NR1 or the NR1+NR2a/b or the NR2 subunit alone). The choice of antigen fragments used can also affect the validity of the CBA results e.g. i) the use of synthetic peptides, ii) parts or the complete antigen and iii) the incorporation of carrier proteins to detect secreted proteins that are naturally anchored to the membrane. This is highly relevant, since most of the antibodies have been shown to target the antigen when expressed in the native conformation *in situ*.

Whether one or another subunit is targeted can matter as this may be translated into different clinical manifestations. For example, the NR1 subunit plays a pathogenic role in NMDAR encephalitis [141] whereas the second subunit of the receptor, NR2a/b, has been related with neuropsychiatric systemic lupus erythematosus [142].

A source of confusion has been the detection of different antibody isotypes and their subtypes. Some studies only report the presence of Igs without any distinction of which type and moreover, the

pathogenic relevance of some of the isotypes is disputed [38,143]. Comparative studies in different disease cohorts showed that IgG was the most prevalent, pathogenic (validated using 3 different methods) and disease specific isotype for NMDAR. Furthermore, the experimental results, due to differences in sample dilution used in the separate assays (i.e. from 1:10 to 1:320 in CBA) are thus of limited comparability across experiments [38].

Some differences could stem from the type of body fluids that are tested, serum, plasma or CSF. Such differences have been reported in the literature, since there is no clear consensus on the most relevant fluid for diagnosis among the different types of encephalitis. Antibodies to GABA_AR were detectable in CSF samples from all patients using live or fixed CBA, while antibodies in serum were mostly visible only with the live version of the CBA [42]. In psychotic disorders where autoimmunity in the CNS could be responsible for the pathology, the analysis of the CSF is a critical point, since a role for Igs in neuropsychiatric disorders is doubtful when autoantibodies cannot be demonstrated in the CSF [144].

A minimum of two different tests in tandem should be used in order to decrease the likelihood of false outcomes. For example, it is recommended to use a combination of CBA's, IHC and/or primary hippocampal cell culture assays or comparing similar tests between different laboratories. At the same time of course, it is also necessary to analyze a group of control samples to confirm that the antibodies are truly disease specific and so the number of "false positives" are reduced to negligible low levels. Screening of autoantibodies in these cases should be done in a panel rather than for individual antigens due to probable coexistence of different target antigens because of the overlap of symptoms in different cohorts [145] and in individual patients [146-148]. Lastly, it is very important to include an exhaustive psychiatric and neurological examination to preselect the patients to be tested, by recognizing particular symptoms that can be associated with specific autoimmune variants of the disease. Discovering such relevant clinical details is therefore the main challenge before we can start treating patients on the basis of individual test assays.

6. Effect of immunosuppression and new therapeutic approaches

When it is not too late, immunosuppressive intervention after immuno-diagnosis might avoid possible brain damage as a result of prolonged exposure of the target to the autoantibodies. Based on the evidence from autoimmune encephalitis, psychotic disorders caused by autoimmunity are expected to be similarly treatable by diminishing the level of the autoantibodies. First line immunotherapy may consist of high intravenous steroid (methylprednisolone), high-dose IVIG and/or plasma exchange. A substantial proportion of autoimmune encephalitis patients improve with these treatments. The non-responders have to follow second line immunotherapies, based on cell therapy, including rituximab and

cyclophosphamide [149]. Innovative therapies such as proteasome inhibitors, targeting plasma cells involved in the production of antibodies with a secretion of more than 10,000 molecules/cell/second [150] have been used to treat autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and myasthenia gravis [151]. Bortezomib, a first generation proteasome inhibitor, has been used as therapy in a small group of patients with treatment-resistant NMDAR encephalitis, showing a potential therapeutic effect of this drug in refractory patients [85]. A period of 4–8 months is required in most cases to recover completely, starting with a normalization of the autonomic dysfunctions, seizures and abnormal movements, followed by the recovery of the memory and attention deficits and of the behavior and executive impairment functions [73]. The effect of prolonged exposure to the autoantibodies synthesized by plasma cells within the CNS and the effect of these antibodies in the synapse can explain why the recovery in patients with encephalitis is slow. Infiltration of plasma cells and autoantibody production in NMDAR encephalitis [52,152] and the observation of the long white matter tracts degeneration, neuronal loss, antibody deposits and decreased expression of the target antigen, support the notion of an effect of plasma cells within the CNS [36,52].

NMDAR encephalitis cases with first episode isolated psychotic symptoms are treatable by immunotherapy and the majority of patients showed a good response [87], with early treatment predicting a better outcome [149]. However, it should be noted that upon recovery the CSF antibody levels decreased but still tested positive in most cases (i.e. 24 out of 28 patients with NMDAR antibodies) [73]. Plasmapheresis, IVIG and corticosteroids have shown good efficacy in autoimmune encephalitis patients with psychotic symptoms, leading to gradual improvement in all cases [90,92,120,121,153,154]. Second line immunosuppressants have been applied in an acute psychotic episode, after NMDAR antibodies were detected. A combination of intravenous steroids and IVIG together with cyclophosphamide and rituximab were administered and showed a slow but almost complete progressive recovery after a period of four months [155]. Immunotherapy in VGKCC antibody-associated encephalopathy, including IVIG or plasma exchange, effectively reduces antibody levels and showed an improvement of cognitive functions, although memory deficits are usually not fully reversible [118,128,129,156-161] which likely implicates some damage to the hippocampus. Complement activation could be responsible for this damage or, alternatively, calcium overload-induced neuronal cell death due to overstimulation, as inhibition of potassium current causes spontaneously high frequency firing. Such firing is observed in neuromyotonia, a peripheral disease with antibodies to VGKCC [162]. Whether or not immunotherapy would be equally beneficial in terms of efficacy and recovery in all autoimmune patients purely with psychotic symptoms is still unclear, since only a few cases (VGKCC and NMDAR positive) have been described up to now (see section on cases report).

7. Conclusions

It is conceivable that an antibody against a neurotransmitter receptor or an ion channel evokes recognizable neurological symptoms in some way. In some cases these symptoms can be subtle and almost insignificant, tending to be overlooked when patients are first seen in psychiatry rather than in neurology departments [5]. It is also possible that a higher level of autoantibodies or a longer exposure time is necessary for neurological symptoms to surface in a patient.

Patients with neuronal autoantibodies diagnosed with a psychotic disorder, although rare, are now recognized primarily due to increasing awareness. However, to date only a few articles have described the presence of these autoantibodies (Table 1). Sample cohorts are usually small and heterogeneous, with an unclear distinction between the different mental disorders and the stage of the disease (especially in psychotic disorders, where the symptoms overlap among the different disorders). It is also clear that in many cases there is a superposition of clinical manifestations between autoimmune encephalitis and psychotic disorders [86]. There is a need to include neurological evaluation and CSF analysis in psychotic patients, since these symptoms can be easily misinterpreted. The use of more sensitive scales for mild symptoms such as the neurological soft signs examination, is intended to detect subtle but observable abnormalities in motor and sensory functions not associated with specific brain regions. Interestingly, several studies have reported lower neurological soft signs scores in healthy controls compared to patients with schizophrenia and bipolar disorder. This makes this scale very promising to identify neurological signs in specific cohorts where these manifestations are not very prominent, such as psychotic disorders [163,164].

The identification of neurological manifestations could serve to better characterize this specific subgroup of autoimmune mediated mental disorder patients where psychotic manifestations are very pronounced, masking the neurological abnormalities. It is also important to mention that several neurological side effects including parkinsonism, akathisia, tardive syndromes, seizures and cerebrovascular events are often observed in patients on antipsychotic drugs. These neurological complaints can overlap with neurological symptoms due to an autoimmune reaction and be mistaken as purely side effects of the drugs.

The methodological differences of the diagnostic assays reviewed are a limiting factor, due to their specificity and sensitivity. There is a need to analyze the prevalence of antibodies targeting novel antigens that are not currently detectable, e.g. those that are not specifically targeting defined antigens, those that are not sufficiently homologous between rodent and human or antigens present on other cell types such as astrocytes and glial cells.

The study of the autoantibodies using *in vivo* and *in vitro* techniques is essential to understand the pathogenic mechanisms underlying these rare autoimmune psychotic cases. This will help to create better diagnostic tools and more specific therapeutic strategies.

In the future, large systematic studies of psychotic patients are needed in order to examine the occurrence of autoimmune-related pathology such as antibody-mediated channelopathies. It is very important to learn how to select clinical phenotypes that help to identify certain patients as a group that runs an increased risk for an autoimmune pathology. Early diagnosis and treatment will reduce the possible sequels generated by the presence of autoantibodies in the target organ and will cut the recovery time of the patients.

Take home message

To date, pathogenic autoantibodies targeting neuronal surface antigens are very rare in psychotic disorders without any form of neurological manifestation.

It is essential that psychiatrists are aware of the existence of symptoms which overlap between autoimmune encephalitis and psychotic disorders in order to prompt early diagnosis and intervention for cases with an atypical disease course.

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Statement of author contributions

All authors participated in the drafting of the manuscript and provided approval of the final submitted version.

Declarations of interest

None.

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03.

Unidentified neuronal surface IgG autoantibodies in a case of Hashimoto's encephalopathy

Marina Mané-Damas¹, Anita Vinke², Carolin Hoffmann¹, Shenghua Zong¹, Mario Losen¹, Peter Molenaar¹, Jan Damoiseaux³, Suzanne Koudijs², Rob P.W. Rouhl^{2,4}, Pilar Martinez Martinez¹

¹*Department of Psychiatry and Neuropsychology, Maastricht University, Maastricht, the Netherlands*

²*Department of Neurology, Maastricht UMC+, Maastricht, the Netherlands*

³*Central Diagnostic Laboratory, Maastricht UMC+, Maastricht, the Netherlands*

⁴*Academic Center for Epileptology Kempenhaeghe/MUMC+, Heeze and Maastricht, the Netherlands*

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Abstract

Hashimoto's encephalopathy is an encephalitis of presumed autoimmune origin characterized by the presence of autoantibodies against thyroid proteins. We present a case of a young patient with pre-existing Hashimoto's thyroiditis and progressive cognitive complaints, absence-like episodes, and sporadic bilateral epileptiform frontal and frontotemporal activity. No abnormalities were observed during the neurological examination and on MRI. Antibodies to thyroid peroxidase (TPO) were elevated and remained positive while the symptoms were present. Levothyroxine and methylprednisolone did not ameliorate the complaints. Subsequent treatment with high-dose intravenous immunoglobulins (IVIG) led to improved cognitive functions and to the disappearance of the absence-like-episodes. Patient's serum, but not CSF, gave a characteristic IgG-specific hippocampal pattern in rat brain immunohistochemistry; this immunoreactivity was maintained after specific and complete depletion of TPO antibodies. Serum IgG bound to primary neurons in cell culture, likely targeting a yet unidentified neuronal surface antigen.

The clinical response to IVIG suggests but does not prove that the circulating novel autoantibodies may induce the encephalopathy. It would be of interest to investigate more patients with Hashimoto's encephalopathy for the presence of neuronal surface autoantibodies, to define their role in the disease and their target antigen(s).

Background

Autoimmune encephalitides are debilitating disorders characterized by a rapid progression of prominent neuropsychiatric manifestations, associated with autoantibodies against neuronal cell-surface proteins, ion channels or neurotransmitter receptors, and a good response to immunotherapy [1].

Hashimoto's encephalopathy, also known as steroid-responsive encephalopathy associated with autoimmune thyroiditis (SREAT), is a rare disorder characterized by a variable presentation of neurological and psychiatric manifestations, the presence of anti-thyroid antibodies and by a clinical response to steroids [1]. However, as thyroid antigens are mainly expressed in the thyroid, this would not explain the presence of cognitive decline and neurological manifestations [2]. Recently, pathogenic autoantibodies to neuronal receptors have been identified, co-occurring in some cases with glutamic acid decarboxylase 65 (GAD65) or thyroid peroxidase (TPO) antibodies. The co-occurrence of both autoantibodies may result in misdiagnosis of the patient.

Here we describe a young patient with suspected autoimmune encephalitis presenting with unidentified neuronal surface autoantibodies and concomitant TPO antibodies, who modestly responded to immunosuppressive treatment.

Case presentation

A 13-year-old boy with a previous history of Hashimoto's thyroiditis presented with muscle pain, dry skin and subtle memory complaints. A scheme of the clinical events of this case report is shown in Figure 1A. Serological analysis at that time, revealed high levels of creatinine kinase (5.105 U/L; normal value <171 U/L), thyroid-stimulating hormone (TSH) (>100 mU/L; normal value 0.50-3.40 mU/L), anti-TPO (426.2 IU/mL; normal value <100 IU/mL), whereas thyroxine levels were low (<5.2 pmol/L; normal value 11.5-17.7 pmol/L). Treatment was started with levothyroxine 100 mcg, once daily, after which his thyroxine levels normalized and muscle pain and skin dryness improved. However, six months after the diagnosis, the cognitive complaints worsened and the patient received a five-day course of methylprednisolone, which did not alter his symptoms. Next, his school performance declined in quality, indicating a further worsening in his cognitive abilities. A neuropsychological test showed no remarkable differences beside a subtle decline in his performance intelligence quotient (Figure 1B). One year after his first memory complaints started, the patient was referred to our academic hospital for further investigation with the suspicion of an autoimmune encephalitis.

Family history was positive for hypothyroidism and high TPO autoantibody levels on the mother's side. Neurological examination showed no focal deficits or other abnormalities, and brain MRI was unremarkable. At time of admission to our pediatric neurology department, the patient was suffering from amnesia and had long lapses of concentration. Generalized absence seizures were suspected. However, repeated EEG tests, including a 24-hour registration, only revealed sporadic bilateral frontal and frontotemporal activity with some epileptiform features, without clinical correlation (Figure 1C). Therefore, the absence-like episodes were not considered to be of epileptic origin. Repeated serological laboratory tests showed normal levels of TSH (0.5 mU/L), presence of TPO antibodies (69 IU/mL) and moderately elevated thyroxine levels (23.4 pmol/L). The lack of response to corticosteroids made a Hashimoto's encephalopathy (SREAT) less likely. Because of the ongoing subjective cognitive decline, an alternative cause of this encephalopathy was considered. Further investigation revealed normal cerebrospinal fluid (CSF) cell count, glucose and protein levels, and autoantibodies known to cause autoimmune encephalitis including N-methyl-D-aspartate receptor (NMDAR), α -amino-3 hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), γ -aminobutyric acid receptor subunits B (GABABR), leucine-rich glioma-inactivated 1 (LGI1) and contactin-associated protein-like 2 (CASPR2), were undetectable in serum and CSF (autoimmune encephalitis Mosaic 1, Euroimmun). Myelin oligodendrocyte glycoprotein (MOG) autoantibodies were negative in serum (Cell Based FACS assay, Sanquin, Amsterdam). The clinical symptoms in combination with these results, led to a working diagnosis of a possible (seronegative) autoimmune encephalopathy. Therefore, the patient was treated with a 5-day course of high-dose intravenous immunoglobulins (IVIG). After the treatment, the cognitive decline stabilized and the absence-like episodes ceased. He finished secondary school (HAVO) and no further deterioration has been observed since the initial manifestations, 5 years ago.

Figure 1. Clinical timeline, IQ and EEG. A) Clinical timeline representing the evolution of serological values of creatinine kinase, thyroid- stimulating hormone (TSH), TPO autoantibodies and thyroxine levels. Treatment intervention is also illustrated as well as qualitative disease progression. B) IQ progression by Wechsler intelligence scale for children. Total IQ (TIQ), verbal IQ (VIQ) and performance IQ (PIQ) at the age of 6, 8 and 13. The average score for the test is 100, and any score between 90 - 109 is considered to be in the average intelligence range. C) EEG showing generalized spike and wave discharge with right frontal dominance during photic stimulation with 50 Hz.

Antibody and antigen characterization

Rat brain sections and primary cell cultures from rat embryo hippocampus were used to detect IgG autoantibodies to neuronal antigens [3]. A strong immunoreactivity of serum antibodies to the middle region of the dentate gyrus and the molecular layer of the cerebellum was identified, with a titer of 1:1,600. Pyramidal neurons and GABA-ergic interneurons in the hippocampus were labelled and the hippocampal neuropil-like pattern was present using sera corresponding to different disease time points (Figure 2, A-D). In contrast, rat brain immunoreactivity of sera from a TPO-epilepsy control serum, and a non-disease control, was low (Figure 2, E-F). To quantify the intensity of the rat brain stainings, we selected different areas of the hippocampus including the stratum oriens, the stratum radiatum, the stratum lacunosum-moleculare, the molecular layer of dentate gyrus and the granule cell layer. The optical density of the patient' s serum immunoreactivity at all investigated time points was consistently high in these hippocampus regions, especially in the molecular layer of the dentate gyrus, the stratum radiatum and the stratum oriens (Figure 2G). Interestingly, this immunoreactivity was highest at the time point corresponding to one year after onset, when the patient was admitted to our neurology department.

Autoantibody reactivity to a surface antigen was confirmed using live rat primary hippocampal neurons (Figure 3). However, live and fixed cell-based assays for NMDA, AMPA, GABA_A, GABA_B receptors as well as LGI1, and CASPR2 membrane proteins and the intracellular proteins GAD65 and GAD67 were negative. The staining using CSF was negative in cultured live rat hippocampal neurons, in the rat immunohistochemistry and in the abovementioned cell-based assays.

Figure 2. Identification and quantification of a novel neuropil-like hippocampal pattern in rat brain sections. A-F) Rat brain immunohistochemistry IgG reactivity of serum. A-D) brain immunoreactivity of sera of the Hashimoto's encephalopathy patient case at different time points: A) At first presentation B) one month after first presentation. C) one year after first presentation and D) after IVIG infusion. E) brain immunoreactivity of serum from a patient with epilepsy and TPO autoantibodies. F) brain immunoreactivity of serum from a non-disease control. The arrows in C and F point at areas shown at higher magnification in C1 and F1, respectively. Note the predominance of a neuropil-like pattern in C1. G) Relative optical density quantification in different areas of the hippocampus, outlined in yellow. SO= stratum oriens, SR= stratum radiatum, SML= stratum lacunosum, ML= molecular layer of dentate gyrus and GL= granule cell layer. Scale bars are 100 μ m and 20 μ m.

Figure 3. Patient' s autoantibodies recognize an unidentified neuronal surface antigen in cultured primary hippocampal neurons. Immunofluorescent photomicrographs of cultured, live primary hippocampal neurons. Human IgGs are stained in green fluorescence, microtubules (MAP2) in red fluorescence, and cell nuclei (Hoechst DNA stain) in blue fluorescence. Serum immunostaining of A) a patient with DPPX antibodies, as a control, B) the Hashimoto' s encephalopathy patient case and C) a non-disease control. A1, B1 and C1 show the merge of nuclear and dendrite staining, A2, B2 and C2 show the merge of nuclear and human IgG staining, A3, B3 and C3 show the merge of all 3 fluorescent stainings. A4 an, B4 and C4 show the immunofluorescence staining of a dendrite at higher magnification. Scale bars are 50 μ m.

The coexistence of additional autoantibodies in the serum besides anti-TPO was confirmed after six consecutive immune-adsorption steps, using TPO-coated microtiter wells from the anti-TPO ELISA (IgG) kit (Euroimmun; EA 1012-0961 G). Anti-TPO IgG levels were reduced from 87.2 IU/mL to 0 IU/mL. However, the TPO antibody depleted serum retained the same brain IgG immunoreactivity pattern (Figure 4).

All procedures were approved by the medical ethical committee of the Maastricht University Medical Center+.

Figure 4. TPO autoantibodies are not responsible for the neuronal reactivity and TPO immuno-adsorption shows co-existence of autoantibodies. Graph on the left panel: TPO antibody levels (IU/mL) at 6 consecutive steps of immune-adsorption using wells coated with recombinant human TPO protein. TPO antibody was depleted from the sera of the following patients: a patient with TPO antibody-positive thyroiditis (blue squares), the Hashimoto's encephalopathy patient (green closed circles), a patient with autoimmune encephalitis and dipeptidyl-peptidase-like protein 6 (DPPX) antibodies (red inverted triangles), and a non-disease human serum control (NHS) (grey triangles). A positive (diamond) and a negative (open circle) calibrator control were included without absorption. **Right panel:** Rat brain immunohistochemistry using non-immuno-adsorbed sera (A1-D1) and immune-adsorbed sera (A2-D2) from A) a patient with DPPX antibodies, B) the Hashimoto's encephalopathy patient, C) a patient with TPO positive antibody thyroiditis and D) a non-disease control. Scale bar is 100 μm.

Discussion

The presence of serum autoantibodies against thyroid proteins, including TPO, is one of the key diagnostic criteria for Hashimoto's encephalopathy [1]. Clinically, patients can present with a broad range of symptoms, including seizures, myoclonus, stroke-like episodes with focal neurological and psychiatric manifestations such as hallucinations, impaired cognition or even dementia. In this particular case, there was a subjective cognitive decline and absence-like episodes, which persisted after adequate supplementation of thyroid hormone and treatment with methylprednisolone.

Hashimoto's encephalopathy is known as steroid-responsive encephalopathy associated with autoimmune thyroiditis (SREAT) due to the high rate of response to steroids [1]. Even though more than 95% of patients show a good response to steroids [4], some patients require additional immunosuppressive therapies including IVIG, cyclosporine, cyclophosphamide and rituximab [5].

TPO is a membrane-bound glycoprotein, mainly expressed in the thyroid. Thyroid autoantibodies are present in more than 10% of the healthy population, as well as in patients with autoimmune encephalopathies, questioning their causative role in the pathology [6]. A study showed that approximately 16% of autoimmune encephalitis patients with GABA_AR antibodies have coexisting TPO autoantibodies [5]. Furthermore, cognitive impairment and other neuropsychiatric manifestations in these patients did not correlate with the presence of TPO antibodies as the fluctuations of the autoantibody levels did not correlate with the clinical manifestations [7].

The presence of TPO autoantibodies in CSF is not included in the diagnostic criteria for Hashimoto's encephalopathy. Hence, CSF of our patient was not initially tested for autoantibodies against thyroid antigens. Only a few studies have analyzed thyroid autoantibodies and circulating immune complexes in the CSF, which were reported in a small cohort of Hashimoto's encephalopathy patients but not in a control group [8]. Furthermore, we did not find nor identify known autoantibodies associated with autoimmune encephalitis in the CSF; moreover, no reactivity of CSF against neuronal antigens was detected by rat brain immunohistochemistry. Undetectable autoantibody levels in the CSF of patients with neuro-psychiatric manifestations could result from an immune adsorption effect within the brain where the autoantigen is expressed [9]. Other factors that could contribute are low autoantibody levels crossing the blood brain barrier, in combination with the use of techniques with high detection limit. Nevertheless, low autoantibody levels in the brain could have an effect when they have high affinity and a wide range of effector functions.

Co-occurrence of TPO autoantibodies with AMPAR, NMDAR, GABA_AR, GABA_BR, LGI1 and MOG autoantibodies has been described in patients with very diverse clinical phenotypes, with a

predominance of epilepsy [5, 10-12]. These findings are supported by this patient's case report, where the TPO autoantibodies were not responsible for the brain-specific IgG (immuno) reactivity, implying the presence of other autoantibodies targeting a neuronal antigen. Lack of reactivity against the currently known neuronal antigens targeted in autoimmune brain disorders and the lack of similarity in the known hippocampal patterns preclude at present the identification of the pathogenic target [1].

Tuzun and colleagues described the presence of autoantibodies against novel targets in two out of eight patients with limbic encephalitis and co-occurring thyroid autoantibodies [10]. Presence of IgG reactivity was also identified in Hashimoto's encephalopathy patient serum using human and mouse brain sections while the results from patients with Hashimoto's thyroiditis were negative [13]. Various studies have described the presence of unidentified autoantibodies against neuronal (surface) antigens with a potential pathogenic role in other pathologies [14] (S. Zong, unpublished results).

The clinical manifestations displayed by the patient described in this case report and the conspicuous hippocampal immunoreactivity pattern of the patient's sera, are novel to the best of our knowledge.

All-in all, TPO autoantibodies in our patient seem to be mere bystanders rather than having a causative, pathogenic role in Hashimoto's encephalopathy. The diagnosis of this encephalopathy remains controversial, since some patients do not meet all diagnostic criteria nor respond to steroids. It would be desirable to study the presence of neuronal surface autoantibodies in both serum and CSF in additional cases of suspected Hashimoto's encephalopathy in order to analyze their putative contribution to the pathology. A better understanding of the pathogenic mechanisms and co-occurrence of autoantibodies will contribute to aiming therapy at the right targets.

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Author Contributions Statement

AV, SK and RPWR provided clinical information. JD provided the samples. MMD, CH, SZ ML and PMM designed the experiments and interpreted the data. MMD performed the research. MMD, AV and PMM drafted the manuscript. All authors revised the manuscript and gave critical feedback.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics statement

The Hashimoto’ s encephalopathy patient’ s samples, data and permit for the publication of this case report were collected with written informed consent by the parents, according to the national and institutional ethical guidelines, in accordance with the Declaration of Helsinki. Additionally, the use of other human material reported within this case report was approved by the medical ethical committee of the Maastricht University Medical Center+ (METC 14-4-157).

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04.

Prevalence of neuronal antibodies in a cohort of early-onset psychotic disorders: the PSYANTIB study

Marina Mané-Damas¹, Anita Vinke², Anna Mané-Santacana³, Nikita van de Burgt¹, Bea Campfort¹, Carolin Hoffmann¹, Shenghua Zeng¹, Peter Molenaar¹, Mario Losen¹, Rob Rouhl², Theresia van Kempsfort^{1,4}, Pilar Martinez-Martinez¹

¹ Department of Psychiatry and Neuropsychology, School of Mental Health and Neuroscience, Maastricht University, Maastricht, the Netherlands

² Department of Neurology, Maastricht University Medical Center, Maastricht, the Netherlands

³ Department of Psychiatry, Hospital del Mar, Barcelona, Spain

⁴ Department of Psychiatry, Maastricht University Medical Center, Maastricht, the Netherlands

05.

Novel treatment strategies for myasthenia gravis with autoantibodies against the AChR

Marina Mané-Damas et al.

*Department of Psychiatry and Neuropsychology, School of Mental Health and
Neuroscience, Maastricht University, Maastricht, the Netherlands*

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06.

Silencing of Dok-7 in adult rat muscle increases susceptibility to passive transfer myasthenia gravis

Alejandro M. Gomez ¹, **Marina Mané-Damas** ¹, Jo Stevens ¹, Peter Molenaar ¹, Hans Duimel ², Fons Verheyen ², Judith Cossins ³, David Beeson ³, Marc H. De Baets ¹, Mario Losen ¹, Pilar Martinez-Martinez ¹

¹ *Neuroimmunology Group, Division of Neuroscience, School for Mental Health and Neuroscience, Maastricht University, the Netherlands*

² *Electron Microscopy Unit, Department of Molecular Cell Biology, Maastricht University, Maastricht, the Netherlands*

³ *Neurosciences Group, Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford, United Kingdom*

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Abstract

Myasthenia gravis (MG) is an autoimmune disease mediated by autoantibodies that target proteins at the neuromuscular junction, primarily the acetylcholine receptor (AChR) and the muscle-specific kinase. Because downstream of kinase 7 (Dok-7) is essential for the full activation of muscle-specific kinase and consequently for dense clustering of AChRs, we hypothesized that reduced levels of Dok-7 increase the susceptibility to passive transfer MG. To test this hypothesis, Dok-7 expression was reduced by transfecting shRNA-coding plasmids into the tibialis anterior muscle of adult rats by *in vivo* electroporation. Subclinical MG was subsequently induced with a low dose of anti-AChR monoclonal antibody 35. Neuromuscular transmission was significantly impaired in Dok-7-siRNA-electroporated legs compared with the contralateral control legs, which correlated with a reduction of AChR protein levels at the neuromuscular junction (approximately 25%) in Dok-7-siRNA-electroporated muscles, compared with contralateral control muscles. These results suggest that a reduced expression of Dok-7 may play a role in the susceptibility to passive transfer MG, by rendering AChR clusters less resistant to the autoantibody attack.

The neuromuscular junction (NMJ) is the synaptic connection between a motor nerve terminal and the skeletal muscle membrane. For efficient neuromuscular transmission to occur, acetylcholine receptors (AChRs) must be densely clustered on the top of postsynaptic membrane folds facing the nerve terminal, in a process primarily mediated by the activation of muscle-specific kinase (MuSK). To be activated, MuSK requires two signals: one from nerve-derived agrin and one from the muscle cytoplasmic protein downstream of kinase 7 (Dok-7) [1,2]. Once released from the nerve terminal, agrin binds to its receptor, the low-density lipoprotein receptor-related protein 4, and this, in association with basal activation by Dok-7, activates MuSK at the muscle's postsynaptic membrane. This ultimately leads to dense AChR clustering by the receptor associated protein of the synapse (rapsyn). In mouse models in which one of the main AChR-clustering proteins (rapsyn, MuSK, Dok-7, or low-density lipoprotein receptor-related protein 4) is knocked down, pups die at birth due to an absence of mature NMJs that results in respiratory failure, [2-5] demonstrating the crucial role of each of these proteins during embryonic synaptogenesis [2,6,7].

During embryonic development, the agrin-MuSK-Dok-7 triad is widely recognized as the main organizer of neuromuscular synapses [2,6,8,9] and, in adulthood, is fundamental for the maintenance and stability of AChR clusters at the NMJ. The latter has been demonstrated by both siRNA down-regulation [10] and conditional inactivation [11] of MuSK expression and, more extensively, by the effects of anti-MuSK antibodies at the NMJ [12-15]. In such studies, the most consistently reported finding was a significant reduction of AChR clustering that follows the decrease in MuSK levels at the endplate, in addition to increased nerve branching, disassembly of endplates, and formation of aberrant NMJs.

Recessive mutations in the DOK7 gene are a frequent cause of congenital myasthenic syndromes [16,17]. Patients with such mutations develop a limb girdle pattern of muscle weakness at an early age. Simplified endplates with reduced postsynaptic folding and impaired neuromuscular transmission are found in muscle biopsies of these patients [18-20]. These observations suggest the possibility that Dok-7 could also have an important role in the pathophysiology (and treatment) of conditions that affect the NMJ in adulthood, such as in the autoimmune disease myasthenia gravis (MG).

In AChR-MG, autoantibodies cause complement-mediated damage at the NMJ, leading to loss of AChR and AChR-associated proteins [21]. Loss of synaptic proteins may impair the clustering of newly synthesized AChR, thus contributing to the severity of symptoms in MG. In this regard, silencing the expression of rapsyn in adult muscles with siRNAs impaired neuromuscular transmission [22], whereas its overexpression protected the NMJ from antibody-mediated damage in the passive transfer MG (PTMG) model induced by injections of anti-AChR monoclonal antibody 35 (mAb35) [23].

To evaluate the role of Dok-7 in adult NMJs, we studied the effects of Dok-7 down-regulation by siRNAs in control and PTMG muscles. Muscles of adult rats were transfected by electroporation with the pSUPER vector, which encodes shRNAs. Our results suggest that reduced Dok-7 expression in the muscle potentiates the susceptibility of the NMJ to the damage caused by PTMG.

Materials and Methods

shRNA Constructs and Evaluation of Silencing Efficiency

Dok-7-siRNA sequence selection was performed using the siRNA design interface from the University of Hong Kong (<http://i.cs.hku.hk/wsirna/software/sirna.php>, last accessed April 30, 2016). A total of five siRNAs targeting rat DOK7 (NM 001130062), plus one scrambled siRNA, were cloned in the pSUPER vector [24] as described [22].

Dok-7 silencing efficiency was measured *in vitro* by cotransfecting the shRNAs or the scrambled constructs with a rat DOK7-green fluorescent protein (GFP) expression vector (rat DOK7, NM 001130062 in a pEGFP-N1 vector, cloned by Geneart AG, Regensburg, Germany) in human embryonic kidney (HEK) 293 cells. HEK cells were transfected with 2.5 µg of each plasmid using the polyethylenimine transfection method [25], in the presence of D-glucose (6.5% w/v). Dok-7 silencing efficiency in transfected cells was estimated by quantification of Dok-7 protein levels in cell extracts by Western blot analysis (see Immunoblot Analysis); each condition was analyzed in triplicate.

In addition, we cotransfected C2C12 myoblasts with Dok-7 shRNAs and DOK7-GFP using a NEON transfection system (Invitrogen, Paisley, UK), and we cultured them in Dulbecco's modified Eagle's medium plus 15% inactivated fetal bovine serum until confluent. Cells were then switched to Dulbecco's modified Eagle's medium plus 5% inactivated fetal bovine serum for differentiation and, once myotubes were formed, AChR clusters were stained with α-bungarotoxin Alexa 594 (B-13423; dilution 1:1000; Invitrogen), as described [17]. A total surface of 4.25 mm² was imaged per condition (in triplicate) with an Olympus IX71 fluorescence microscope (Olympus, Tokyo, Japan), and the average AChR cluster length was estimated with ImageJ version 1.48a (NIH, Bethesda, MD; <https://imagej.nih.gov/ij/>). A minimum length of 3 µm was considered for defining an AChR cluster. From their *in vitro* silencing efficiency, we selected two of the five shRNAs (S-1 and S-5), in addition to the scrambled-shRNA, for muscle electroporation *in vivo*.

S1-forward, 5' -GATCCCCGAGATCAGCTTCCTGTTTTCAAGAGAAAACAGGAAGCTGATCTGCTTTTTGGAAA-3' ; S1-reverse, 5' -AGCTTTTCCAAAAAGCAGATCAGCTTCCTGTTTTCTCTTGAAAAACAGGAAGCTGATCTGCGGG-3' ; S5 forward, 5' -GATCCCCGATCAGCTTCCTGTTTGACTTCAAGAG -AGTCAAACAGGAAGCTGATCTTTTTGGAAA-3' ; S5-reverse, 5' -AGCTTTTCCAAAAAGATCAGCTTCCTGTTTGACTTCTCTTGAAAGTCAAACAGGAAGCTGATCGGG-3' ; scrambled shRNA-forward, 5' -GATCCCCGAGATGGTCCCTGTTTGACAGTCAAGAGACTGTCAAACAGGGA -CCATCTCTTTTAA-3' ; and scrambled shRNA-reverse, 5' -AGCTTAAAAAGAGATGGTCCCTGTTTGACAGTCTCTTGA ACTGTCAAACAGGGACCATCTCGGG-3' .

Immunoblot Analysis

Protein extracts from transfected HEK cells were separated by SDS-PAGE, transferred into nitrocellulose membranes, and incubated with rabbit anti-Dok-7 (H-77; dilution 1:400; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (10R-G109a; dilution 1:3.000.000; Fitzgerald, Acton, MA;) antibodies. Secondary antibodies were donkey anti-mouse IgG IRDye 680 and goat anti-rabbit IgG IRDye 800 (926-32222 and 926-32211, respectively; LI-COR Biosciences, Lincoln, NE), diluted 1:10.000. Stained membranes were imaged with the Odyssey Imaging System (LI-COR Biosciences), and bands were quantified with the ImageJ software version 1.48a (NIH), normalizing for glyceraldehyde-3-phosphate dehydrogenase expression.

Animal and tissue processing

Seven-week-old female Lewis rats were obtained from Charles River Laboratories (Cologne, Germany), with permission from the Committee on Animal Welfare, according to Dutch governmental rules. Statistical power analyses were performed to calculate the minimum number of animals required to achieve statistically significant results, based on statistical parameters obtained from previous animal studies in our laboratory [22,23,26]. For electroporation, induction of PTMG, and electromyography (EMG), animals were anesthetized and later euthanized as described [22]. Frozen tibialis anterior muscles were cut in a cryostat (CM3050 S; Leica Biosystems, Wetzlar, Germany) in 10 µm sections. For electron microscopy (EM) analysis, animals were euthanized as described [23,26], and fixed muscles were sectioned at 500 µm with a VT1200S vibratome (Leica Biosystems).

In Vivo Muscle Electroporation

Dok-7 shRNA plasmids were prepared with the Gigaprep DNA purification kit (catalog no. 12191; Qiagen, Hilden, Germany), according to the manufacturer's manual, and dissolved at a concentration of 2 µg/µL in 0.9% NaCl. For the PTMG experiments, we injected muscles with a combination of 50 µg of each of the two shRNA vectors (S-1 and S-5) and, for the contralateral control leg, 100 µg of the scrambled-shRNA vector, in a final volume of 50 µL, followed by electroporation. Electroporation of the tibialis anterior

muscle was performed as previously described [22,23] using the Electro Square Porator ECM 830 (BTX, San Diego, CA).

Induction of PTMG and Experimental Setup

Tibialis anterior muscles of adult rats were electroporated with shRNA plasmids on days 0, 2, and 4, and PTMG was induced by i.p. injection of mAb35 [27] on day 35, whereas control animals received saline solution. PTMG animals received 5 pmol/100 g body weight of mAb35 (subclinical dose) (Supplemental Figure S1) and were sacrificed 48 hours later (five PTMG and three control rats). Day 35 after electroporation was chosen for induction of PTMG because it has been demonstrated that there is a time-lag between reduced MuSK levels, the main effector protein activated by Dok-7, and a reduction in AChR levels at the NMJ. It has been shown, for instance, that the passive transfer of anti-MuSK antibodies in mice is followed by a reduction of endplate AChR content not earlier than 14 days after the injection of anti-MuSK IgGs [12]. In addition, Cre-Lox conditional inactivation of MuSK in muscle fibers of adult mice requires at least 27 days for inducing a 50% reduction in junctional AChR levels [11].

EMG

Decrement of compound muscle action potentials (CMAPs) was measured simultaneously in both tibialis anterior muscles of five PTMG and three control rats at the day of sacrifice, as described [22,23,28]. Decrement was defined as >10% decrease of the amplitude and area of the CMAPs during 3-Hz stimulation. If no decrement was observed initially, neuromuscular transmission was challenged by a continuous i.p. infusion of a 20 µg/mL solution of curare [(+)-tubocurarine, T2379; Sigma-Aldrich, St. Louis, MO], at a rate of 1 mL/h (0.33 µg curare/min) with a syringe pump. During curare infusion, CMAP measurements were repeated at intervals of 1 minute (measuring one leg at a time, with 30 seconds between a measurement in one leg and a measurement in the other leg), until a reproducible decrement was observed in both muscles, for all animals. The time at which a decrement is observed is directly correlated to the dose of curare injected and to the amount of functional AChR at the NMJ.

Immunofluorescence

Muscle cryosections of 10 µm were stained for b-galactosidase activity as described [22], dehydrated in a series of ethanol dilutions (70% to 100%), immersed in Ultraclear (J.T. Baker, Deventer, the Netherlands), and mounted with Pertex (Histolab Products AB, Gothenburg, Sweden). To ensure evaluation of the whole muscle tissue, we analyzed three different sets of 10-µm sections spaced 500 µm in between, and quantified the number of transfected fibers/total number of fibers per muscle cryosection to define the

percentage of transfection. Images were acquired with an Olympus BX50 microscope and the Stereo Investigator software version 10.40 (MicroBrightField Bioscience, Williston, VT).

To visualize NMJs, 10- μ m frozen muscle sections were fluorescently stained. Cryosections were fixed in cold acetone for 15 minutes at 4 ° C and blocked for 30 minutes with donkey serum [1:500 in 0.05% tris-buffered saline (TBS)-Triton]. Sections were also blocked with an endogenous streptavidin/biotin blocking kit (SP-2002; Vector Laboratories, Burlingame, CA), according to the manufacturer' s instructions. Slides were incubated for 1 hour with the following primary antibodies: rabbit anti-MuSK (dilution 1:1000; kind gift from Steven J. Burden, New York University, New York, NY) and mouse anti-SV2 (against synaptic vesicle protein 2; dilution 1:2000; DS Hybridoma Bank, Iowa City, IA). Alexa 647-conjugated α -bungarotoxin (dilution 1:300; B35450; Invitrogen, Breda, the Netherlands) was used for detection of AChRs. Subsequently, slides were incubated 1 hour with the following secondary antibodies: biotin-conjugated goat anti-rabbit IgG (dilution 1:1000; 111-065-144; Jackson ImmunoResearch, West Grove, PA) and Alexa 594 donkey anti-mouse IgG (dilution 1:300; A21203; Invitrogen), followed by incubation with Alexa 488 streptavidin (dilution 1:2000; S11223; Invitrogen) for another hour at room temperature. Finally, slides were mounted in 80% glycerol-TBS. All antibodies and reagents were diluted in 2% bovine serum albumin in TBS, and all washing steps were performed with TBS-Triton. Because of technical limitations with the currently available antibodies against rat Dok-7, we were unable to stain NMJs of transfected muscles for this protein (data not shown).

Quantitative Immunofluorescence Analysis

The relative concentrations of AChR and MuSK at the NMJ were analyzed as described [22,23] Muscle sections from five PTMG and three control rats (16 muscles in total) were stained for immunofluorescence as described. For each muscle, two sets of sections from two different regions of the tissue were stained, each set containing three muscle sections of 10 μ m. Endplate regions were identified based on their morphologic structure and positive staining for SV2, and they were imaged for the quantification of SV2, AChR, and MuSK intensities with an Olympus Provis AX70 fluorescence microscope. Approximately 50 images per muscle (with 3 to 10 endplates each) were acquired, and the fluorescence intensities were quantified using ImageJ version 1.48a (NIH), in a blinded fashion (A.M.G.). For each endplate, both AChR and MuSK mean intensities were normalized to the SV2 mean intensity. The average of the normalized intensities from all endplates in a set of muscle sections was used for comparison between conditions.

EM

Electron micrographs were obtained from endplate regions of the Dok-7-siRNA-electroporated muscles of five subclinical PTMG rats (48 hours after induction of PTMG) and their respective control animals ($n = 3$). Sections of glutaraldehyde-perfused muscles (500 μm) were processed for EM imaging as described [23,26]. Ultra-thin sections (80 nm) were viewed with a Philips CM 100 electron microscope (Philips, Amsterdam, the Netherlands). At least 15 endplate regions (including one to three endplates each) were imaged from each muscle, and the morphometric analysis of each micrograph was performed using the ImageJ version 1.48a in a blinded fashion (A.M.G.), as described [23,26,29,30].

Statistical Analysis

GraphPad Prism 4 (GraphPad Software, La Jolla, CA) was used to perform statistical analyses. Normally distributed values were compared using either one- or two-way analysis of variance and Bonferroni post hoc tests. $P < 0.05$ was considered significant. Values are expressed as means $\pm$ SEM, unless stated otherwise.

Results

Dok-7 shRNAs Reduce Dok-7 Levels and Induce Smaller and Simplified AChR Clusters in Vitro

To reduce the expression of Dok-7, we designed and cloned shRNAs into the pSUPER expression vector and tested them *in vitro* by cotransfection with DOK7-GFP in HEK 293 cells. After transfection, two pSUPER constructs (S-1 and S-5) efficiently reduced the levels of Dok-7-GFP (between 40% and 90%) compared with DOK7-GFP cotransfected with the scrambled pSUPER vector (negative shRNA control), as quantified by Western blot analysis ($P < 0.001$) (Figure 1, A and B). The combination of these two shRNAs had a minor additive effect (95% reduction in Dok-7-GFP levels compared with the scrambled vector; $P < 0.001$) (Figure 1, A and B).

Figure 1. *In vitro* analysis of shRNA silencing efficiency. **A:** HEK 293 cells were cotransfected with plasmids for the expression of Dok-7-GFP (MW 91 kDa), and Dok-7 shRNAs (S-1, S-5, and S-1 + S-5), or a scrambled-shRNA plasmid (negative shRNA control). Forty-eight hours later, cells were harvested, and protein extracts were separated by SDS-PAGE, followed by immunoblotting for Dok-7 (green band) and GAPDH (red band). **B:** Western blot analysis quantification of Dok-7-siRNA silencing efficiency. Cotransfection of DOK7-GFP with Dok-7 shRNAs (S-1, S-5, and their combination) strongly reduced Dok-7 expression compared with cells transfected with the scrambled-shRNA plasmid. Dok-7 band intensities were measured using ImageJ densitometry software version 1.48a and normalized for GAPDH intensity. **C:** *In vitro* analysis of Dok-7-shRNA silencing efficiency in C2C12 cells. C2C12 myoblasts were cotransfected with a plasmid for the expression of Dok-7-GFP, and either Dok-7 shRNAs (S-1 or S-5) or the scrambled-shRNA expression plasmid. On myotube differentiation, DOK7-GFP-transfected cells have large AChR clusters when no Dok-7 shRNA was cotransfected. AChR clusters in S-5-cotransfected myotubes are small and disperse, and even smaller than in NT cells. Boxed areas in the top row delineate the region enlarged in the bottom row, arrows in the bottom row point to AChR clusters. **D:** The average AChR cluster length increases after cotransfection of DOK7-GFP with the scrambled shRNA or shRNA S-1, but it significantly reduces when DOK7-GFP is cotransfected with shRNA S-5. Cells were stained with Alexa 594-conjugated α -bungarotoxin, and ImageJ densitometry software was used for quantification of cluster length. Data are expressed as the means $\pm$ SEM percentage of Dok-7 intensity compared with the negative control shRNA (scrambled). $n = 3$ independent experiments (B). $^{**}P < 0.01$, $^{***}P < 0.001$, one-way analysis of variance. Scale bars: 100 μ m (C, top row); 30 μ m (C, bottom row). AChR, acetylcholine receptor; Dok-7, downstream of kinase 7; GAPDH, glyceraldehyde-3-

phosphate dehydrogenase; GFP, green fluorescent protein; HEK, human embryonic kidney; Neg, negative; NT, nontransfected.

Because overexpression of Dok-7 leads to increased AChR clustering in muscle cells [2], we tested if Dok-7 shRNAs affected clustering of AChRs in differentiated myotubes of the mouse muscle cell line C2C12 cotransfected with a DOK7-GFP plasmid. Cotransfection of DOK7 with S-5 impaired the Dok-7-induced clustering of AChRs and led to smaller and simplified AChR clusters (45% smaller than in scrambled-shRNA transfected cells; $P < 0.001$) (Figure 1, C and D). In contrast, cotransfection of DOK7 with the scrambled-shRNA or S-1 promoted the formation of larger AChR clusters (32% larger than in nontransfected cells; $P < 0.01$) (Figure 1, C and D). Interestingly, clusters in S-5-transfected C2C12 cells were even smaller than those in cells nontransfected with DOK7 (28%) (Figure 1, C and D), albeit not significantly.

Effects of *in Vivo* Dok-7-siRNA Electroporation in Neuromuscular Transmission

To estimate the *in vivo* transfection efficiency of Dok-7 shRNAs, tibialis anterior muscles of 18 rats were injected with a DNA solution containing 100 μ g of S-1 or S-5, plus 50 μ g of the reporter plasmid pVAX1-LacZ and electroporated. These rats were later sacrificed at 7, 14, or 28 days after electroporation ($n = 3$ per time point). For both shRNAs, β -galactosidase was highly expressed 7 days after electroporation in approximately 60% of the muscle fibers, and it was still clearly detectable at day 28 after electroporation (Figure 2A).

Figure 2. *In vivo* electroporation of shRNA plasmids (S-1 and S-5).

A: The overall transfection efficiency was analyzed using X-gal as a substrate for the reporter gene product β -galactosidase in 10 μ m thick muscle sections. To ensure evaluation of the whole muscle, we analyzed three different sets of sections spaced 500 μ m in between. For both shRNA constructs, β -galactosidase expression was observed in muscle fibers at all analyzed time points (7, 14, and 28 days after electroporation), and in the majority of the tissue (approximately 60% of muscle fibers), indicating stable plasmid expression. Here we show a representative set of sections of an S-5 transfected muscle at days 7, 14, and 28 after electroporation. **B:** Scheme of the experimental set up for investigating the effects of *in vivo* Dok-7-siRNA electroporation in the susceptibility to PTMG. bw, body weight; Dok-7, downstream of kinase 7; PTMG, passive transfer myasthenia gravis.

To evaluate the importance of Dok-7 for the susceptibility to PTMG, we electroporated tibialis anterior muscles with Dok-7 shRNAs, subsequently induced PTMG in some animals by injection of 5 pmol mAb35/100 g body weight (subclinical dose), and sacrificed them and their respective controls 2 days later (Figure 2B). Dok-7-siRNA electroporation in muscles of control animals did not impair neuromuscular transmission, as measured by the resistance to curare with EMG ($n = 3$) (Figure 3, A and B). However, Dok-7-siRNA-electroporated muscles in subclinical PTMG animals were less resistant to curare (26% less) compared with the control contralateral leg ($n = 5$; $P < 0.05$) (Figure 3, A and B). These results imply that Dok-7 levels are important for the resistance of the NMJ to an autoantibody attack.

Figure 3. Dok-7-siRNA electroporation impairs neuromuscular transmission in subclinical PTMG (induced with 5 pmol mAb35/100 g bw), and it does not affect the ultrastructure of the NMJ. A: CMAP was measured bilaterally in transfected muscles by EMG, in the presence of continuous curare i.p. infusion. Dok-7-siRNA electroporation

does not affect the resistance to curare of control animals, but it significantly decreases curare resistance in subclinical PTMG animals. B: Representative decrement measurements in a control (left) and a subclinical PTMG (right) animal. CMAP measurements during repetitive supramaximal nerve stimulation at 3 Hz were performed on control (top) and Dok-7-siRNA-electroporated (bottom) tibialis anterior muscles. Decrement was defined as >10% decrease of both the CMAP amplitude and area and indicated above the measurement when positive (amplitude decrement %/area decrement %). Representative EMG traces of a control and a PTMG rat (for both legs) at a dose of 6.5 μ g of curare are shown. Curare resistance decreases in the Dok-7- siRNA-electroporated leg but not in the control leg of the PTMG rat. C-F: Electron microscopy analysis of endplate morphologic structure. C: Normal endplate region in a control muscle, with an elaborate postsynaptic folding facing the NT. D: Normal endplate region in a Dok-7-siRNA-electroporated control muscle. No particular morphologic alterations were observed in most Dok-7-siRNA-electroporated endplates, in both control and subclinical PTMG animals. E and F: Subclinical PTMG muscles contain both normal endplate regions and endplate regions showing reduction of postsynaptic folding (E, arrow) and widening of the synaptic cleft (F, asterisk). Ultrathin muscle sections from PTMG and control animals were imaged with an electron microscope (at least 15 endplate regions per muscle). n = 3 control animals; n = 5 PTMG animals. *P < 0.05, two-way analysis of variance for comparisons. Scale bars = 1 μ m (C-F). bw, body weight; CMAP, compound muscle action potential; Dok-7, downstream of kinase 7; EMG, electromyography; mAb35, monoclonal antibody 35; NMJ, neuromuscular junction; NT, nerve terminal; PTMG, passive transfer myasthenia gravis.

Effects of Dok-7-siRNA Electroporation in NMJ Ultrastructure

In addition, we examined the ultrastructure of the NMJ by EM in control and PTMG animals 37 days after electroporation with Dok-7 shRNAs. Control animals had morphologically normal NMJs in both Dok-7-siRNA-electroporated and control muscles (Figure 3, C and D). Resembling control animals, most endplates in subclinical PTMG rats also had a normal NMJ morphologic structure in both Dok-7- siRNA-electroporated and control legs. However, some endplates were clearly disrupted at the postsynaptic membrane, with wider junctional folds and enlarged synaptic clefts or simplified postsynaptic folds, which are typical signs of complement-mediated damage (Figure 3, E and F). Such endplates were equally observed in both Dok-7- siRNA-electroporated and control legs. For statistical comparisons between conditions, we performed quantitative morphometric analysis of the folding index (ratio between the postsynaptic and presynaptic membrane length [31]) in control and subclinical PTMG animals (n = 3 and n = 5, respectively; approximately 25 endplates per muscle were analyzed). The folding index was not significantly different either between these two conditions (P = 0.064; averages per group: control = 7; PTMG = 6.1) or between Dok-7- siRNA-electroporated and control legs (P = 0.12; averages per group: PTMG control Z 6.1; PTMG Dok-7- siRNA-electroporated = 5.5). These results suggest that Dok-7-siRNA electroporation does not significantly modify the ultrastructure of the NMJ.

Figure 4. Fluorescence microscopy images of representative endplates and quantitative immunofluorescence analysis. Muscle sections were triple stained for AChR (magenta), MuSK (green), and the presynaptic marker SV2 (red) as a reference. **A:** Representative images of endplates from control and subclinical PTMG muscles. No evident differences in morphologic structure or AChR cluster size are observed between Dok-7-siRNA-electroporated and control legs in control animals. Most endplates are normal in subclinical PTMG muscles, although in Dok-7-siRNA-electroporated legs some endplates are disrupted and have lower AChR intensity (as representatively shown in the panel). **B and C:** Quantification of the relative intensity of AChR (**B**) and MuSK (**C**), normalized for SV2 intensity. The average AChR/SV2 ratio is significantly lower in Dok-7-siRNA-electroporated legs, compared with the contralateral control legs, in Sc PTMG animals but not in control animals. MuSK levels are not significantly affected by Dok-7-siRNA electroporation. n = approximately 300 endplates per muscle; n = 5 PTMG animals; n = 3 control animals. $^{**}P < 0.01$, two-way analysis of variance for comparisons. Scale bar = 10

μm . AChR, acetylcholine receptor; Dok-7, downstream of kinase 7; MuSK, muscle-specific kinase; Sc, subclinical; PTMG, passive transfer myasthenia gravis; SV2, synaptic vesicle protein 2.

Dok-7-siRNA Electroporation Reduces AChR Protein Levels in PTMG Rat Muscle

To further characterize the effects of Dok-7-siRNA electroporation on AChR and AChR-associated proteins, we stained for AChR, MuSK, and SV2 in muscles of subclinical PTMG rats ($n = 5$) and untreated animals (control, $n = 3$). We estimated their relative density at the NMJ by quantitative immunofluorescence 48 hours after induction of PTMG. Most endplates in subclinical PTMG rats had a normal morphologic structure in both Dok-7-siRNA-electroporated and control legs (Figure 4A). The intensities of AChR and MuSK were quantified in approximately 300 endplates per muscle and normalized for their corresponding presynaptic SV2 intensity [22]. In control animals, both AChR/SV2 and MuSK/SV2 ratios were not affected by Dok-7-siRNA electroporation, and they were not significantly different from those in subclinical PTMG ($P = 0.66$ and $P = 0.72$, respectively) (Figure 4, B and C). By contrast, Dok-7-siRNA-electroporated legs in subclinical PTMG animals had a significantly lower AChR/SV2 ratio at their NMJs compared with control legs (25% less; $P < 0.01$) (Figure 4B), whereas MuSK levels were not significantly affected ($P = 0.09$) (Figure 4C). These findings are consistent with the impaired neuromuscular transmission observed by EMG in Dok-7-siRNA-electroporated legs of subclinical PTMG animals but not controls. Altogether, they suggest that functional AChR molecules are more likely to be reduced by the relatively low amount of autoantibodies in subclinical PTMG when there is less Dok-7 available in the muscle.

Discussion

In this study, we have examined the effects of Dok-7 down-regulation to obtain further insight into the function of Dok-7 in the adult NMJ and its possible involvement in the pathophysiology of MG. The main finding is that electroporation with our Dok-7 silencer constructs leads to increased susceptibility to mAb35 in the PTMG model, whereas it does not seem to affect the safety factor for neuromuscular transmission in control animals.

The efficiency of neuromuscular transmission is directly related to the number and degree of clustering of AChRs at the surface of the postsynaptic membrane. The NMJ has a large safety factor because the endplate potential caused by the released ACh is much larger than the threshold potential where the muscle membrane starts firing the action potential [21,32]. Because in rodents the safety factor is about five [33], it is likely that the effects of reduced Dok-7 expression would only become visible when neuromuscular transmission is compromised after challenge by an autoimmune attack or a neuromuscular toxin. Indeed, in our experiment we observed a significant impairment in neuromuscular

transmission due to Dok-7-siRNA electroporation, as judged by the curare test, only after challenge by injection with 5 pmol of mab35 (subclinical PTMG).

Our EMG findings are in good correlation with the quantification of AChRs at the endplate by immunofluorescence. We observed a significant reduction in AChR content in Dok-7-siRNA-electroporated legs of subclinical PTMG animals, further supporting the importance of Dok-7 in the stabilization of AChR clusters at the NMJ. In this regard, previous studies have demonstrated that Dok-7 is particularly relevant for the prepatterning of AChR clusters during development [6,34] and Dok-7 knock-down zebrafish generated fewer and smaller AChR clusters [34]. In addition, our ultrastructural analysis by EM did not reveal morphologic abnormalities at the endplates of Dok-7-siRNA-electroporated muscles. Thus, it seems that Dok-7 does not play a crucial role in maintaining the ultrastructure of the mature NMJ; therefore, morphologic changes are probably not responsible for the increased sensitivity to curare of Dok-7-siRNA-electroporated legs. However, a limitation of this study is that we were unable to properly measure the silencing efficiency of our Dok-7 siRNAs *in vivo*, which was probably less efficient than in cell cultures, due to technical limitations in the quantification of Dok-7 protein levels (data not shown). Taking into account that the overall transfection efficiency in muscles was around 60%, we can speculate that, if we would have either restricted our analysis to the Dok-7-siRNA-transfected fibers, or if we would have achieved a higher transfection efficiency, particularly at later time points, we would have probably observed more pronounced effects on the AChR content and/or NMJ morphologic structure.

A link between Dok-7 and MuSK expression has been demonstrated by several studies. For instance, both proteins share the transcription factor Sp1 [35], and Dok-7 is the limiting factor for the formation of AChR clusters when MuSK is overexpressed in muscle fibers [36]. Furthermore, the two proteins increase their expression on denervation [36,37]. Nevertheless, we did not find significantly altered MuSK levels at the NMJ as a result of Dok-7-siRNA electroporation. Because Dok-7 is required for full activation of MuSK, it seems likely that the additional loss of AChR clusters in Dok-7-siRNA-electroporated muscles of PTMG rats was a result of reduced MuSK activation rather than of reduced MuSK levels.

Conclusions

Our results indicate a relevant role for Dok-7 in the susceptibility to damage at the NMJ and, therefore, implicate it in the maintenance of a functional adult NMJ. Taking into account that several well-characterized mutations of the DOK7 gene in humans have been involved in the development of congenital myasthenic syndromes [17-19], it is tempting to speculate that, in the general population, minor variations in the sequence of DOK7 or its promoter could also mildly impair the levels or activity of Dok-7. Such carriers would then be more susceptible to damage at the NMJ; therefore, Dok-7 could be

a promising marker to predict the severity of conditions that challenge neuromuscular transmission, such as MG.

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Author contribution

J.A.A.S. and M.M.-D. contributed equally to this work.

Declaration of interest

Disclosures: None declared.

Supplemental data

Supplemental figure 1. Assessment of the minimal dose of mAb35 that induces decrement of the CMAP, as measured by EMG, without noticeable clinical effects (subclinical PTMG). Non-electroporated rats were injected with 5, 12.5 or 20 pmol/100 g bw mAb35 (n=3 per dose), and neuromuscular transmission was assessed by EMG 2 days later, in the presence of continuous i.p. injections of curare. Animals that received either 12.5 or 20 pmol/100g bw mAb35 already had a decrement before the injection of curare solution, while those injected with 5 pmol/100 g bw required approximately 3.5 µg curare to show a decremental response. This was significantly less curare than what was needed to induce decrement in control animals (5 µg), indicating that injection of 5 pmol/100 g bw mAb35 significantly impaired neuromuscular transmission, though to a lesser extent than injection of 12.5 or 20 pmol/100 g bw mAb35. Additionally, at 2 days after induction of PTMG, animals that received 12.5 or 20 pmol/100 g bw mAb35 had clinical scores ranging from 0 to 2, while control animals and animals injected with 5 pmol/100 g bw mAb35 had no clinical signs of muscle weakness (score 0, data not shown). Clinical scoring was assessed with the paw-grip test, as described [28]. n= 3 rats per dose of mAb35. *** $P < 0.001$, one-way analysis of variance. bw, body weight; CMAP, compound muscle action potential; EMG, electromyography; mAb35, monoclonal antibody 35; PTMG, passive transfer myasthenia gravis. *** $p < 0.001$; 1-way ANOVA.

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07.

Therapeutic efficacy of MLN9708 (ixazomib) in experimental autoimmune myasthenia gravis and primary thymic cell cultures from myasthenia gravis patients

Marina Mané-Domingas¹, Abhishek Saxena^{1,2}, Gisela Nogales-Gadea^{1,3}, Nick Wilcox⁴, Shannel Vireckar¹, Hans Duimel⁵, Jos G Maessen⁶, Peter C. Molenaar¹, Marc H. De Boets¹, Pilar Martinez-Martinez^{1\$} and Mario Losen^{1\$}

¹ School for Mental Health and Neuroscience, Maastricht University, Maastricht, the Netherlands

current affiliation: Shanghai Institute for Advanced Immunochemical Studies, Pudongxin District, Shanghai, China

current affiliation: Neuromuscular and Neuropediatric Diseases Research Group, Neuroscience department, Institut d' Investigació en Ciències de la Salut Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, Spain

⁴ Department of Clinical Neurology, University of Oxford, UK

⁵ ELMI Unit-CRISP, Department of Molecular Cell Biology, Maastricht University, Maastricht, the Netherlands

⁶ Department of Cardiothoracic Surgery, Maastricht UMC+, Maastricht, the Netherlands

\$ Equal contribution

08.

General discussion, limitations and conclusions

Antibody-mediated autoimmune diseases are, in many cases, chronic debilitating illnesses that affect more than 2.5% of the general population, contributing substantially to morbidity, mortality, and annual health care cost [1-3]. Over the past decades, this percentage has increased worldwide.

The disruption of balance within the immune system results in the production of antibodies that recognize and attack self-antigens. Autoimmune diseases present a wide variability of clinical manifestations, targets and pathogenic processes [4, 5]. Symptoms are usually associated and directly correlated with the presence of autoantibodies. However, the damage generated by these autoantibodies and the concomitant inflammatory response can have long term sequelae. As a consequence, rapid diagnosis and early treatment are important [5, 6]. Traditional therapies aim to control the overreaction of the immune system and to reduce the inflammation. While they are highly efficient, around 10% of the patients do not achieve complete remission or have a poor response to these therapies. The benefit of many of immunosuppressive drugs is counterbalanced by their toxicity and serious side effects following long term/chronic administration and also because of the lack of specificity.

This thesis addresses the study of well-known as well as novel, antigens that drive human autoimmune diseases. Furthermore, it aims to identify the inherent limitations to current therapies and to elucidate the disease mechanisms in order to develop more specific targeted strategies with special focus on enhancement of efficacy and tolerability.

After a brief introduction, in **CHAPTER 2** we address the matter of autoimmunity in psychotic disorders. Besides the strong economic burden, mental disorders have a devastating societal impact as a result of the high dependency of the sick and the low rate of re-integration of the patients in society. Therapeutic options aim to ameliorate the symptoms, but they have often a poor response, or no response for some of the symptoms such as cognitive decline or serious side effects. Unfortunately, despite the great efforts of the scientific community, little is known about the aetiology of these disorders. There is evidence which shows the involvement of the immune system in mental disorders. A decade ago, a novel disease named NMDAR encephalitis was discovered to be caused by autoantibodies against neuronal antigens having neurological as well as psychotic manifestations. Neurological abnormalities can be subtle, however, or absent in some cases, leading to underdiagnosis. Awareness of psychiatrists of the existence of autoimmune encephalitis and the overlapping symptoms is essential to guarantee a prompt diagnosis and adequate therapeutic intervention. In our review, we summarize the results of several studies addressing the question whether in some patients, psychosis could have an autoimmune origin. At the starting point of our research, variations of specificity and sensitivity in the screening methods had led to controversial results in the field. We recommend the use of combined standardized assays to validate the results so as to reduce the number of false positive or negative outcomes. Further, it should be realised

that cerebrospinal fluid (CSF) analysis is essential for a definitive diagnosis of psychotic disorders of autoimmune origin. Cross-validated results confirmed that pathogenic autoantibodies targeting neuronal surface antigens are very rare in psychotic disorders without any form of neurological manifestation. The number of cases with isolated psychosis of autoimmune origin is apparently very limited. To have a clear vision of the potential role of autoimmunity in psychotic disorders, larger, homogeneous and systematically described cohorts need to be studied, with paired analysis of both plasma and CSF.

Subsequently, we continued contributing to the aforementioned research question and investigated the presence of neuronal autoantibodies in patients with neuropsychiatric manifestations. In **CHAPTER 3** we describe the case of a young patient with Hashimoto's thyroiditis with thyroid peroxidase (TPO) autoantibodies, progressive cognitive complaints and absence-like episodes. The persistence of the symptoms after levothyroxine and methylprednisolone raised the suspicion of an autoimmune encephalopathy. We discovered a novel IgG-specific hippocampal pattern by rat brain immunohistochemistry. This conspicuous pattern was maintained after the depletion of TPO autoantibodies. Binding to primary neurons in cell culture, indicated that the coexisting autoantibodies target a yet unidentified neuronal surface antigen. Finally, the clinical response to IVIG suggested that the circulating novel autoantibodies may be responsible for the encephalopathy. With this case report, we underline the importance of investigating additional patients with Hashimoto's encephalopathy for the presence of neuronal surface autoantibodies. Moreover, it is important to characterize the autoantibody pathophysiology and their target antigen(s).

CHAPTER 4 describes the preliminary results on the presence of hippocampal autoantibodies in patients with psychotic disorders. While this is not the first study to address this research question, some aspects have been paid little or no attention previously such as the characteristics of the recruited patients, the detailed neuropsychiatric characterization, the neurological examination and the CSF analysis. Rat brain immunohistochemistry revealed immunoreactivity, IgG specific, in the serum of 7% of the patients. Novel hippocampal patterns have been found in 7 of the 8 positive samples. An anti-Hu-like pattern, with strong immunoreactivity in the stratum oriens connecting to the stratum lacidum and in the granule cell was observed in the last sample. All the positive samples belonged to patients with severe psychotic manifestations, strong degree of functioning impairment and shorter duration of the disease. In a sub analysis, including only patients with first episode psychosis and strong disease manifestations (cohort 2), the percentage of brain immunoreactive patients is significantly higher: 15.4%. The number of positive samples becomes then relatively higher than previously reported studies and control/non-disease individuals. However, most of these studies included patients with a longer duration of the disease and mild psychotic manifestations, without neurological examination and no CSF analysis. In the few studies

that included patients with a severe phenotype, the reduced cohort size, the lack of assays cross-validation and the lack of reproducibility makes it difficult to establish a conclusion. With our preliminary results, we would like to make a case for continuing investigations of the presence of (novel) neuronal autoantibodies in patients with severe psychotic manifestations, soon after their first episode, who may experience a poor response to antipsychotics. We thus encourage the use of standardized validated screening assays, including the analysis of CSF and recommend the confirmation of the results with other methods or other laboratories. Patients with psychotic disorders with an autoimmune origin could possibly directly benefit from an early diagnosis and treatment.

Limitations of the previously mentioned studies include the relatively small cohort size. In the case report, the identification of a single patient with a novel hippocampal pattern gives an opportunity for other researchers and physicians to find similarities with their patients thereby encouraging a collaborative effort to unveil potential disease mechanisms. For the diagnosis of autoimmune encephalitis it is recommended that abnormalities in the CSF are identified [6]. Moreover, autoimmune encephalitis is unlikely in view of the lack of antibody activity in the CSF of the patient and the potential pathogenicity of autoantibodies found in his serum is questionable. However, pleocytosis or protein/IgG levels were not measured in the single sample of CSF. In this case, it is possible that the level of autoantibodies in the CSF at that time point was low or that autoantibodies were immunoadsorbed by an antigen present in the brain [7]. Unfortunately, the lack of identification of the antigen precludes any speculation regarding the pathogenicity of these autoantibodies. In the PSYANTIB study, the broad inclusion criteria might be “diluting” the prevalence of autoantibodies in psychotic patients. As matter of fact, preliminary results indicate that patients with characteristics of strong psychotic manifestations, high functioning impairment and a short duration of the disease have more autoantibodies detected by rat brain immunohistochemistry. Narrowing the inclusion criteria would probably help to identify these patients. In addition, neuronal surface autoantibodies in chronically ill patients have been found to be very rare. Besides that, not all patients had donated CSF, since it was optional in our protocol due to the ethical restrictions. Differences in the neurological and psychiatric examination between both centers could also be a source of variability of interpretation. Imaging techniques like MRI or EEG that could help to identify other abnormalities for follow up and treatment response would significantly promote the relevance of the study.

In **conclusion**, we think it important to stress the value of screening in mental disorder patients for the presence of autoantibodies against known but also unknown antigens. Acquiring more knowledge on the pathophysiology of psychotic disorders with an autoimmune origin will help to understand the disease mechanisms, guarantee an accurate diagnosis and eventually to design and develop specific treatment strategies. This concept links to the following chapters of this thesis, which focuses on one of

the best-characterized antibody-mediated autoimmune diseases, namely myasthenia gravis (MG). In MG, the study of the disease mechanisms has been shown most helpful to design and to develop novel treatment strategies and to repurpose the use of drugs with similar disease mechanisms.

CHAPTER 5 describes novel treatment strategies for MG with autoantibodies against the AChR. After a brief overview on the established treatments, we reveal the need of innovative-targeted immunotherapies and symptomatic therapies for antibody-mediated disorders. Symptomatic relief therapies, antibody competition, complement modulation, FcRN antagonisms and cell therapies are approached in detail. We highlight the relevance of understanding the different disease mechanisms, which might translate in the efficacy of certain therapeutic strategies but lack of response in others. A clear example of it are plasma cell targeting therapies, specifically the use of proteasome inhibitors, shown to have beneficial effects in a subgroup of patients with AChR-MG but not MuSK-MG, where B cell targeting therapies seems to be more suitable. Additionally, after the extensive description of the mechanisms of action and the rationale behind these novel therapies we speculate on the changes of autoreactive antibody levels following different therapeutic strategies. Even though the review focuses on AChR-MG, the rationale behind it applies also to many other antibody-mediated autoimmune disorders.

In **CHAPTER 6**, we describe the consequences of down-regulating Dok-7, a protein required for the correct activation of muscle-specific kinase (MuSK) and consequently the dense clustering of AChRs, essential for successful neuromuscular transmission. Using a passive transfer animal model for MG, the silencing of Dok-7 significantly reduced the number of AChRs available at the neuromuscular junction. This reduction made the neuromuscular transmission weaker and more susceptible to the attack of the pathogenic autoantibodies. Mutations in the Dok-7 sequence of promoters, altering the expression of the protein could increase the susceptibility to damage at the neuromuscular junction. All together, these results could serve as a proof of principle for the development of novel therapeutic strategies aiming to upregulate the expression of Dok-7 at the neuromuscular junction. Agrin is a protein that contributes to the assembling and aggregation of AChRs by binding to LRP4 and activating MuSK/Dok-7. NT-1654, a new agrin agonist, improved in an animal model of MG neuromuscular transmission, promoted the clustering of AChRs and reduced the MuSK loss at the neuromuscular junction, which all resulted in improved muscle force and diminished fatigue in the animals [8].

A serious *limitation* of the Dok-7 study was that we could not properly measure the silencing efficiency of our Dok-7 siRNAs *in vivo*, which was likely to be less efficient than *in vitro*. Taking into account that the overall transfection efficiency was around 60%, we can speculate that, if we could have either restricted our analysis to the Dok-7 siRNA-transfected fibers or if we could have achieved a higher transfection

efficiency, particularly at later time-points, we would have probably obtained more pronounced effects on the morphology and the AChR content of the neuromuscular junction. It would be interesting as a next step also to study the effect of silencing Dok-7 in an animal model for MuSK-MG.

Understanding the role of plasma cells in antibody-mediated autoimmune disorders and the capacity of proteasome inhibitors to deplete not only malignant but also normal plasma cells, resulted in the following research. In **CHAPTER 7**, we describe the efficacy of the proteasome inhibitor MLN9708, also known as ixazomib, for treatment in AChR MG. Ixazomib has been demonstrated to have less side effects in multiple myeloma patients compared to first generation proteasome inhibitors [9, 10]. In primary thymic cell cultures from MG patients, ixazomib led to an almost complete elimination of plasma cells and halted IgG and AChR antibody production. In experimental autoimmune myasthenia gravis (EAMG), the administration of ixazomib resulted in a reduction of 75% total plasma IgG levels and in a 94% reduction of AChR antibody levels. No differences were found between preventive and therapeutic treatment strategies and no signs of neurotoxicity were observed. Treatment with ixazomib resulted in a protective effect against weight loss, which is as a result of muscle weakness and fatigue in EAMG. These results have also been observed in another antibody-mediated animal model for systemic lupus erythematosus [11]. This study provides the basis for a possible short-term clinical trial in AChR-MG patients. In addition to MGFA scores, such a study should include the quantitative analyses of autoantibody levels and single fiber electromyography of patients.

The lack of electrophysiological studies as well as curare resistance, *limits* the comparability with previously reported drug efficacy studies for MG [12]. Definitive studies on the effect of the complete Freund' s adjuvant (CFA) in animal models will help to subtract the effect of such a reagent from the potential neurotoxic effect of the studied drugs.

In *summary*, elucidating the pathogenic mechanisms underlying autoimmune neuromuscular diseases is clearly of great importance. Our knowledge, together with the inherent limitations of the current therapies provide the basis for novel or repurposed therapies, with increased specificity, circumventing the effects of systemic immune suppression and increasing tolerability. In addition, combination therapies should be explored to identify synergistic responses that will exploit the therapeutic effects of the administered drugs, limiting their side effects. Tailor-made therapeutic approaches will most likely have a therapeutic benefit in different disease settings. We are on the cusp of translating this new knowledge to treat antibody-mediated human diseases.

Lastly, the *perspectives and future directions* of the studies conducted during this thesis are recapitulated as follows:

- More studies including severely ill psychotic patients, using a robust screening strategy encompassing different detection methods, CFA analysis, and neurological examination will help to describe the presence or absence of neuronal surface autoantibodies in these disorders.
- It is important to study known but also novel antigenic targets, which may play a role in neuropsychiatric disorders. Currently, commercially available tests include such a possibility. However, in practice, antigen-specific tests are more commonly used in the clinic for diagnostic purposes. For this reason, the use of different techniques including antigen unspecific assays will help to avoid underdiagnosis of patients with novel autoantibodies.
- After the identification of novel as well as known autoantibodies with distinctive clinical presentation in patients with neuropsychiatric disorders, it is essential to confirm the pathogenicity and study their mechanisms of action.
- Using classical animal models for autoimmune disorders such as myasthenia gravis is very valuable for the study of the mechanisms of action of the pathogenic autoantibodies.
- Gaining perspective into the pathophysiology of autoimmune disorders will help to understand the disease mechanisms and to identify potential novel therapeutic targets.
- *In vitro* and *in vivo* studies on the safety and tolerability and the therapeutic efficacy of novel and repurposed drugs provide the basis for future “proof of principle” human clinical trials.
- Lastly, in-depth characterization of different drugs and its therapeutic outcome in patients will allow us to understand whether combination therapies could have a synergic effect with reduced side effects. These findings could then be further optimized towards a tailor-made treatment.

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The background of the slide is a light gray with a complex, abstract pattern. It features a mix of geometric shapes, including triangles, circles, and spirals, overlaid with thin, dark gray line art. Some of the line art depicts human figures in various poses, while others are more organic, resembling leaves or flowing lines. The overall effect is a textured, layered design.

09.

Valorization

Chronic disorders are defined as persistent lifelong health conditions that usually require ongoing clinical support. They significantly affect the activity and daily life of the patients and their families. Examples of the most common chronic diseases in the world include Alzheimer's disease, cancer, coronary heart disease, autoimmune diseases such as diabetes, rheumatoid arthritis, multiple sclerosis, and mental disorders. These diseases usually affect different parts of the body and develop in waves, with periods of remission and relapse that can vary in intensity. Enormous progress has been made in the diagnostics, monitorization and treatment of these disorders. While symptoms can partially be suppressed and sometimes even eliminated, no cure exists to date. Besides the consequences of the disability experienced by the patients and the societal burden, chronic disorders represent a stunning economic affliction on society and health care resources.

Importantly, the immune system plays a crucial role in a significant number of chronic disorders, while in many others its contribution is still under investigation. The studies described in this dissertation have mainly focused on the potential role of the immune system in psychotic disorders, the pathogenic mechanisms and the efficacy of novel therapeutic strategies in myasthenia gravis.

It is estimated that 1 in 5 adults is suffering from a mental illness, and a total cumulative of 43% of the population experience a form of mental illness at least once in their lifetime [1,2]. The spectrum of mental disorders is extremely diverse and the underlying pathogenic mechanisms remain a mystery, making the diagnosis and treatment extremely difficult. Antipsychotics and mood stabilizers focus on the suppression of the symptoms but are not efficient at reducing the cognitive decline experienced by many patients or at stagnating disease progression. Besides long-term periods where medication is required, sometimes for life, the treatment-associated side effects and the lack of treatment efficacy which in some cases leads to institutionalization is very common in the active phases of the disease. The public stigmatization and the negative connotation associated to these disorders challenges the integration of these individuals into society, meaning that they can experience difficulties finding and maintaining a job and also struggle to live an independent life.

It has been demonstrated that there is a clear link between the immune system and mental disorders [3]. Its exact role, however, is not yet well-defined. In autoimmune encephalitis antibodies against neuronal surface channels and receptors trigger neuropsychiatric manifestations which could, in some cases, be incorrectly diagnosed as schizophrenia or bipolar disorder.

Lately, the prevalence of antibody-mediated autoimmune disorders, those caused by antibodies that mistakenly recognize and attack organs, tissues and cells of the body, is increasing significantly [4, 5]. Nowadays, 2.5% of the global population suffer from an antibody-mediated autoimmune disease [6].

Some of the most common are multiple sclerosis, diabetes, systemic lupus erythematosus, celiac disease, psoriasis and myasthenia gravis. Myasthenia gravis is a neuromuscular disorder characterized by fluctuating muscle weakness and fatigue. Antibodies against different proteins of the neuromuscular junction are the direct cause of the disease, and the underlying pathogenic mechanisms have been intensively studied.

In this dissertation, I have reviewed the literature on psychotic disorders (including schizophrenia and bipolar disorder) and the potential role of the immune system in the development of these disorders. Interestingly, the methodological differences among the different screening assays raised a controversial variability in the prevalence of these autoantibodies in psychotic disorders. Thanks to the dissemination of scientific studies, patients with a subtle and uncommon disease course, treatment resistance or relapses and/or subtle neurological abnormalities, where the probability of autoimmunity is enhanced, are nowadays diagnosed in most of the cases. The use of standardized detection assays, and the analysis of cerebrospinal fluid, along with an extensive neurological examination indicate that the percentage of patients with pure psychosis and an autoimmune origin is very low. Early diagnosis and treatment are essential for a complete and fast recovery of the patients. However, it is important to be critical when it comes to the analysis of the results, since treatment administration could also have severe consequences for the patient when the disease is not correctly diagnosed.

Including different detection assays, I was able to identify novel autoantibodies in a young adult with Hashimoto's thyroiditis, progressive cognitive complaints and subtle neurological abnormalities. After testing negative for neuronal surface autoantibodies by the commercial kit, I revealed the presence of coexistent autoantibodies against a protein expressed in the brain, specifically, in the membrane of pyramidal hippocampal neurons. Although I have not been able to identify the target yet, the clinical response to immunosuppressive IVIG treatment suggests but does not prove that these novel autoantibodies may induce the encephalopathy. Even though this is a single case report and the expected prevalence of autoimmunity in these patients is very low, reporting our findings could help other researchers to identify similar cases. In an extended cohort of newly recruited patients with a recent onset of psychosis, 7% of the patients presented neuronal autoantibodies. Thanks to the extensive neuropsychiatric description, I was able to identify the phenotype of these patients, characterized by severe manifestations and impairment of cognitive functioning and, interestingly, a shorter time between the last psychotic episode and the moment of the visit. This will help to narrow the characteristics of these patients with higher probabilities to have an underlying autoimmune cause and facilitate their diagnosis and further treatment.

Insight into the pathogenic mechanisms of psychotic disorders will help to understand the role of the different components involved in the disease. Autoantibodies could then be used as biomarkers that could serve not only for diagnosis but also to monitor the disease progression and might be instrumental in developing specific therapies that will not only mask the symptoms but treat the disease at the source. If successful, this will cause a true landslide in psychiatry with genuine healing effects on patients. The impact on patients, their families, society and economy would be substantial.

Even though antibody-mediated autoimmune disorders, especially myasthenia gravis, are better characterized and we know more about the disease mechanisms, important gaps remain in our understanding of the disease.

Using an animal model for myasthenia gravis, I showed that changes in the expression of Dok7, involved in the clustering of the acetylcholine receptors (AChRs), essential for the correct muscle contraction, directly affects the disease susceptibility. Elucidating the role of the different proteins involved in the disease not only answers a basic research question about the pathogenic mechanisms, but also would be most helpful for designing new therapies. In the case of agrin, a protein involved in the upstream activation of MuSK via its interaction with LRP4, the administration of an agrin fragment has shown binding to LRP4 and stimulation of the neuromuscular junction. Together with Dok7 and rapsyn, they assure the correct anchoring and dense clustering of the AChRs at the membrane, essential for a correct neuromuscular transmission. Manipulating the signalling path of these proteins by enhancing MuSK activation, one could improve the motor function and correct neuromuscular defects not only in myasthenia gravis but also in many other disorders of the neuromuscular junction such as amyotrophic lateral sclerosis, spinal muscular atrophy, nerve injury and sarcopenia. In combination with other disease-modifying therapies, they can help to reduce the damage at the neuromuscular junction and accelerate its recovery.

Antibody-mediated autoimmune diseases are in most cases successfully treated with broad-spectrum immunosuppressive drugs, known as first line therapeutic strategies. However, the lack of complete remission, the presence or development of treatment-resistance and the high incidence of unwanted side effects reinforce the unmet medical need to find and design novel therapeutic strategies. Using myasthenia gravis as a template, I described the novelties in the field of pharmacological intervention and highlight the relevance of treatment combination. Interlacing different drugs can enhance the effect or reduce the side effects. An additional value comes from the bivalency aspect of the newly developed therapies, which could also apply to other disorders where the immune system is involved such as cancer. Treatment strategies are moving towards personalized treatment, i.e. an exhaustive study of the disease mechanisms developed in each patient and the subsequent selection of the most

efficient therapies that alone or in combination, following a specific timeline, will help patients to reach disease remission that will be maintained over their lifetime.

Besides the design of novel drugs, this knowledge gives new opportunities to use drugs already available on the market, whose effects can be beneficial for other pathologies. Proteasome inhibitors, initially developed to treat multiple myeloma, a plasma cell malignancy, not only eliminate malignant plasma cells but all plasma cells. Plasma cells produce high amounts of antibodies and are significantly involved in antibody-mediated autoimmune disorders. However, they are rather insensitive to regular immunosuppressant therapies. I proved the therapeutic effect of ixazomib, a second generation proteasome inhibitor, in various models of myasthenia gravis. Additionally, no signs of neurotoxicity were observed, which was one of the bottlenecks of previous proteasome inhibitors. Animal research is essential for the application of novel or repurposed human therapies as they provide mechanistic information and give evidence of the safety and effectiveness in different diseases. These results are the proof of principle used to develop a clinical trial with ixazomib in patients with myasthenia gravis. Eliminating the plasma cells is a step further towards the cure of not only myasthenia gravis, but many other antibody-mediated autoimmune diseases since these cells are responsible for the antibody production and in consequence are responsible for the development of the disease.

All in all, the studies described in this dissertation are relevant for a substantial proportion of society considering the patients who currently have or will develop mental illnesses or autoimmune disorders which also have impact on members of their family. The results presented will influence the diagnostics and treatment of these patients with a direct impact in health care costs and new opportunities for pharmaceutical industries.

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The background of the page is a light gray abstract pattern. It features a grid of squares, some of which are filled with a darker gray. Overlaid on this grid are various thin, black line-art elements, including circles, spirals, and organic, flowing shapes that resemble leaves or branches.

10.

Curriculum vitae

Marina Mané Damas was born on March the 15th in 1990 in Tarragona, Spain. She enrolled in the Bachelor degree programme in Biotechnology at the Universitat Rovira i Virgili (2008) in Tarragona, Spain. During her Bachelor internship, she joined the team of Prof. Dr Isabel Illa, Sendra at the Experimental Neurology laboratory at the Research Institute at the Hospital de la Santa Creu i Sant Pau. Under the supervision of Dr Gisela Nogales-Gadea she studied the presence of autoantibodies against neuronal surface receptors in rare neuromuscular disorders. During this period she got fascinated by the nuts and bolts of the laboratory assays, as well as the fruitful discussions with other team members, which were wrapped up and presented for her internship thesis, which was scored with the highest grade in the class. After she graduated (2012), she enrolled in the Neuroscience Master-programme at the University of Barcelona. For her master internship, she continued in the research team of Prof. Dr Isabel Illa and followed up on the identification of novel antigenic molecules enrolled in the pathogenesis of CIDP and developed novel assays to identify the presence of autoantibodies in the serum of patients, which resulted in several publications. Before successfully completing her master studies (2013), she was appointed as research assistant at the Inflammatory Arthritis Group lead by Dr Juan Cañete in a joined project with the group of Innate Immunity at the under the supervision of Prof. Dr Francisco Lozano at IDIBAPS research institute at the Clinic Hospital in Barcelona. She was involved in several projects investigating the potential role of molecules of the innate immune system in different immune mediated disorders. At the end of 2014, she moved to Maastricht and started her PhD project at the department of Psychiatry and Neuropsychology at Maastricht University, under the supervision of Dr Mario Losen and Prof. Dr Pilar Martinez. Throughout her PhD, she worked at two main projects, the first project was aimed at the investigation and the elucidation of the role of autoantibodies in psychotic disorders. Intrigued by the controversial results in the field, she established several *in vitro* assays and started a human study to define the prevalence of neuronal autoantibodies in patients with psychosis. She joined the lab of Prof. Dr Dalmau, who is a key figure in the field of autoimmune encephalitis. In the second project, she contributed to the understanding of the role of different proteins in the pathogenic mechanisms of a well-known antibody-mediated autoimmune disease: myasthenia gravis. Following previous studies, she proved the therapeutic effect of ixazomib, a second generation proteasome inhibitor. A clinical trial with this drug in myasthenia gravis patients will soon start to which the outcome of her study has strongly contributed. These results have been presented in several national and international conferences. Over the past years she has supervised numerous bachelor and master students and has participated in the coordination of different courses. Before finishing her PhD, Marina obtained a Kootstra postdoctoral fellowship to continue her research. Currently, she is interested in the immune subsets involved in autoimmune disorders, unveil the *in vitro* and *in vivo* effects of human derived autoantibodies from patients with antibody-mediated autoimmune disorders to gain knowledge and clear the path towards tailor made therapeutic strategies.

11.

List of publications

Publications

- Mané-Damas M, Vinke A, Hoffmann C, Zong S, Losen M, Molenaar P, Damoiseaux J, Koudijs S, Rouhl R, Martinez Martinez P. **Unidentified neuronal surface IgG autoantibodies in a case of Hashimoto encephalopathy** (*accepted, Frontiers in Immunology*)
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- Mané-Damas M, Tan A, Molenaar P, Hoffmann C, Zong S, Losen M, Martinez-Martinez P. **Autoimmunity in psychosis.** Psychotic Disorders: Comprehensive Conceptualization and Treatments. Clinical Medicine Books, Oxford University Press USA (*in press*)
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- Correia Hoffmann C, Zong S, Mané-Damas M, Stevens J, Malyavantham K, İsmail Küçükali C, Tüzün E, De Hert M, van Beveren N.J.M, González-Vioque E, Arango C, Damoiseaux J.G.M.C, Molenaar P, Losen M, Martinez-Martinez P. **The search for an autoimmune origin of psychotic disorders: Prevalence of autoantibodies against hippocampus, glutamic acid decarboxylase and antinuclear antigens.** (*submitted to Schizophrenia research*)
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experimental autoimmune myasthenia gravis and primary thymic cell cultures from myasthenia gravis patients. *(in process, Journal of Autoimmunity)*

- Mané-Damas M, et al. **Novel treatment strategies for myasthenia gravis with autoantibodies against the AChR** *(in process, Autoimmunity reviews)*
- Mané-Damas M, et al. **Hinge-deleted IgG4 blocker therapy for acetylcholine receptor myasthenia gravis - *in vitro* data of competition efficacy** *(in preparation)*

12.

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