# Autoantibodies in disorders of the brain

# Autoantibodies in disorders of the brain: expanding the spectrum

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# Autoantibodies in disorders of the brain: expanding the spectrum

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# Chapter 1

## General introduction

Brain, as part of the central nervous system (CNS), is once thought to be an immune-privileged organ, which is now known to be incorrect. Actually, current evidence indicates a tight relation between the immune system and the brain [1]. This is not only supported by the fact that immune cells of the CNS have been found contributing to the maintenance of the normal neurogenesis but also the immune system can attack the brain and cause so-called autoimmune brain diseases, including autoimmune encephalitis, autoimmune-related epilepsy, CNS vasculitis, and neuromyelitis optica, etc [2, 3]. The spectrum of autoimmune brain diseases is still growing nowadays. Although not all the exact pathogenic mechanisms in these diseases are clearly understood, autoantibodies to neuronal cell surface antigens are believed to be pathogenic in many cases.

**The spectrum of neuronal autoantibodies**

Autoantibodies to neuronal cells in the central nervous system (CNS) can be divided into two groups according to the location of their targeting antigens: 1) autoantibodies to intracellular neuronal proteins, such as Hu, Yo, Ri and 2) autoantibodies to neuronal surface proteins (NSAbs) [4]. The first group of autoantibodies are also known as classic paraneoplastic antibodies which are not pathogenic themselves but associated with a variety of neurological manifestations, occurring as a result of an underlying tumor, usually breast or lung cancer. While in the second group, autoantibodies are believed to play a pathogenic role in the associated neurological disorders with or without tumor. This research is rapidly expanding with new autoantibodies identified annually [4, 5]. These NSAbs target neurotransmitter receptors, ion channels or associated proteins on the membrane of neuronal cells and most of their pathogenic effects have been demonstrated (the first reported time is indicated), including metabotropic glutamate receptor 1 (mGluR1) (2000) [6], N-Methyl-D-aspartate receptor (NMDAR) (2007) [5], α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) (2010) [7, 8], leucine-rich, glioma inactivated 1 (LGI1) and contactin-associated protein-like 2 (Caspr2) (2010) [9], GABAB receptor (GABABR) (2010) [10], metabotropic glutamate receptor 5 (mGluR5) (2011) [11], dipeptidyl aminopeptidase-like protein 6 (DPPX) (2013) [12-14], GABAA receptor (GABAAR) (2014) [15-17]. Besides, certain autoantibodies targeting intracellular antigens such as autoantibodies to glutamic acid decarboxylase (GAD-Abs) are usually not associated with tumors and their pathogenicity is questioned [18]. Nevertheless, these autoantibodies are associated with various neurological disorders including limbic encephalitis, neuromyotonia, Morvan's syndrome, epilepsy as well as a subgroup of first-episode psychosis [19-22]. Different autoantibodies may relate to distinct clinical syndromes and confer a broad clinical spectrum. Currently, our knowledge in this is still growing and it coincides with an increased interest in screening for NSAbs in psychiatric disorders.

**Neuronal autoantibodies and psychiatric disorders**

The etiology of psychiatric disorders is heterogeneous and still poorly understood. A set of biological changes and risk factors have been identified for the different diagnoses and immune dysregulation is one of them. The basic evidence is based on that psychiatric disorders occur more often amongst people suffering from autoimmune diseases than in healthy individuals [23, 24]. It is not clear whether patients with autoimmune diseases have more mental complaints due to somatic discomforts or the dysregulated immune system directly targets the brain and causes mental disturbances. To study the latter situation, many researchers have

focused on inflammation and cytokines. A few studies also show that certain autoantibodies targeting intracellular antigens, such as anti-nuclear antibodies, anti-ribosomal P proteins, and anti-thyroid peroxidase, are associated with psychiatric symptoms in systemic autoimmune disorders [25-27]. However, those autoantibodies to intracellular antigens are normally considered as an indication of immune dysregulation and have not been proved to play a causative role in the disease.

Another biological change shared among different neuropsychiatric disorders is the alteration of synaptic transmission or dysfunction of ion-channels, including hypofunction of NMDAR, dopamine receptor or voltage-gated potassium channel, are. In the past decades, studies have shown that those proteins are actually the target of the immune system in neurological disorders with psychiatric symptoms. Here anti-NMDAR encephalitis is used as a paradigm to show this connection between NSAbs and psychiatric disorders. In 2007, anti-NMDAR autoantibodies were initially detected in a group of patients with an ovarium teratoma with psychiatric symptoms followed by neurological manifestations including seizures, movement disorder, and dysfunction of the autonomous nervous system [5]. Thereafter, it has become clinical practice to investigate if patients might have NMDAR encephalitis in a subgroup of psychotic patients [28]. The concept that NSAbs can cause psychotic symptoms is based on the fact that two-thirds of anti-NMDAR encephalitis patients present initially with psychiatric manifestations before presenting with neurological complains and in 5% of these cases, isolated psychotic episodes occur without simultaneous neurologic involvement [29, 30]. This is not surprising since the NMDAR is crucial for glutamate signaling, and the hypo-function of the system has been previously linked to schizophrenia [31]. To date, most NSAbs target receptors that play important roles in neural signal transduction. All in all, this leads to the hypothesis that a subgroup of patients with neuronal autoantibodies can present with only isolated psychiatric symptoms and thus mimic schizophrenia or psychosis (Figure 1) [32, 33]. Furthermore, the same hypothesis could also be applied to other psychiatric disorders, including mood disorders (bipolar, depression and anxiety), autism spectrum disorders, obsessive-compulsive disorders, and attention-deficit/hyperactivity disorders, which have not been well studied yet [34].

**Pitfalls of neuronal autoantibody detection**

Several studies have focused on the prevalence of neuronal autoantibodies in schizophrenia or first-episode psychosis with controversial results [35-39]. The main finding is that anti-NMDAR autoantibodies are more common only in patients with first-episode psychosis, yet the exact prevalence is not consistent [37, 40]. Three main points might have contributed to the different results in those studies: 1) differences in the biological fluid analyzed, serum or cerebrospinal fluid (CSF); 2) differences in the screening methods used: cell-based assay (CBA), rat brain tissue-based immunohistochemistry (IHC) and staining on live neurons. 3) The dilution of the tested sample ranged from 1 in 20 to 1 in 320.

Immunohistochemistry (IHC) was used in early studies to identify autoantibodies that gave neuropil staining on rat brain tissue [41, 42]. Then staining on live neurons was performed at the same time to confirm that those autoantibodies targeted membrane proteins. Later when the exact antigens were identified, CBA using fixed and permeabilized human embryonic kidney cells with transfected antigens were developed, which has become the standard detection method in the clinic. However, CBA only detects autoantibodies to known antigens

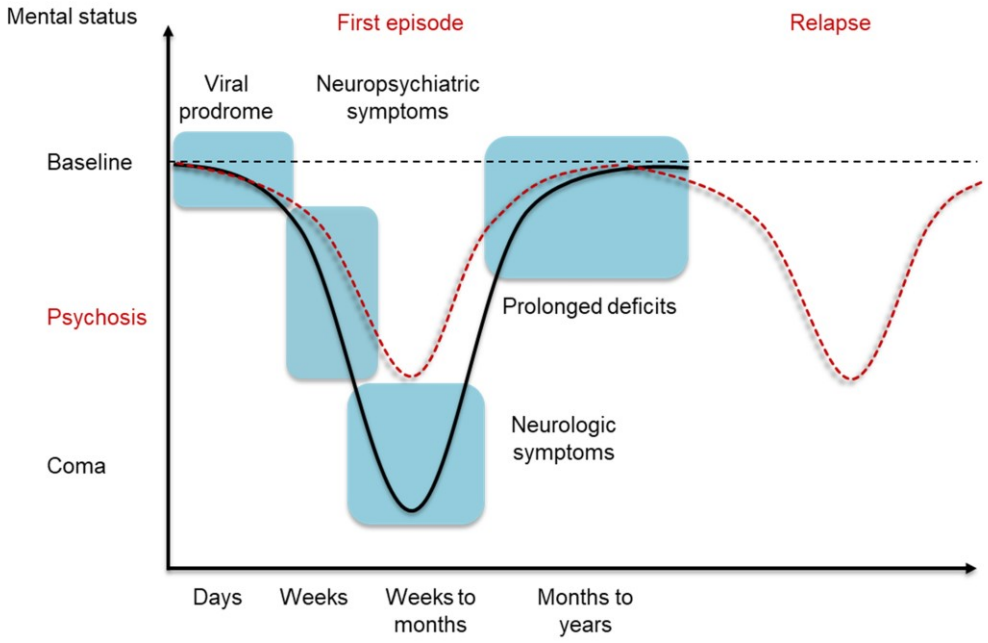


**Figure 1. The typical clinical course of illness in NMDAR encephalitis (black trend line) and a hypothesis of autoimmune psychosis (red trend line).** The clinical course of NMDAR encephalitis often starts with viral-like symptoms including lethargy, headache, upper respiratory symptoms, nausea, diarrhea, muscle pain, and fever. This stage can last several days. Then more severe psychotic symptoms will develop such as anxiety, paranoia (similar to first-episode psychosis), and short-term memory loss. Later, severe physical and behavioral changes take place including seizures, movement abnormalities, and hyperventilation. It takes time to return to their baseline function, with cognitive deficits and behavioral changes lasting from months to years. In a few cases, isolated psychotic symptoms without movement abnormalities have been reported. Even if in some cases the symptoms relapse, they might be misdiagnosed as psychotic disorders such as schizophrenia.

that are included in the assay. The fixation and permeabilization steps may also lead to false-negative or false-positive results, as conformational surface antigens might be destroyed or modified, and some intracellular antigens would be exposed. In some laboratories, live CBA is used where the patient sample is incubated on transfected HEK cells before fixation to avoid conformational changes and to improve sensitivity. Due to the lack of a systematic comparison between IHC, live and fixed CBA in practice, it is not easy to interpret results from different laboratories when different methods were used. Our previous study showed that the exclusive use of a single method may yield clinically irrelevant, false-positive results, especially in high-throughput screening with low prior probability [43], which is the case in patients with psychiatric disorders.

Thus, in the investigation I conducted in neuropsychiatric disorders, I made use of multi-methods including IHC, live and fixed CBA and staining on live neurons to better cover a broader range of known autoantibodies as well as lead the discovery of novel neuronal autoantibodies.

**Aims and outline of this thesis:**

In this thesis, the aim is to summarize the current knowledge of autoantibodies in neuropsychiatric disorders, to search for known and novel pathogenic neuronal autoantibodies in a broader range of neuropsychiatric disorders, and to compare different neuronal autoantibodies detection methods.

**Chapter 2** reviews evidence in the recent literature for the role of NSAbs as well as related systemic autoantibodies in five neuropsychiatric disorders. It evaluates the techniques used, discusses how results can be interpreted and identifies the research gaps.

**Chapter3** reviews the recent evidence for the occurrence of NSAbs in mood disorders with a special focus on depression. It discusses how those NSAbs could potentially be related to neuropsychiatric disorders with a special focus on their putative pathogenic role in depression.

**Chapter 4** assesses the prevalence of NSAbs in the plasma of patients with depression or anxiety by using IHC, CBA and staining on live neurons to analyze if they are more common in patients compared to controls. This is a large cohort-control study including 1739 depression or anxiety patients and 492 non-mental disorder controls.

**Chapter 5** investigates the prevalence of neuronal and bystander autoantibodies in the sera of psychotic disorders. It is a large case-control study including 621 patients with psychotic disorders, 70 individuals with affective disorders, 41 with other mental disorders and 257 controls.

**Chapter 6** compares different methods (ELISA, CBA, and IHC) for the detection of GAD-Abs. It is also investigated if other NSAbs rather than GAD-Abs are present in patients with suspected autoimmune brain diseases.

**Chapter 7** summarizes the key findings of this thesis, discusses the limitations and outlines further research directions.

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# Chapter 2

## Autoantibodies in neuropsychiatric disorders

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**Abstract:**

Little is known about the etiology of neuropsychiatric disorders. The identification of autoantibodies targeting the *N*-methyl-D-aspartate receptor (NMDA-R), which causes neurological and psychiatric symptoms, has reinvigorated the hypothesis that other patient subgroups may also suffer from an underlying autoimmune condition. In recent years, a wide range of neuropsychiatric diseases and autoantibodies targeting ion-channels or neuronal receptors including NMDA-R, voltage-gated potassium channel complex (VGKC complex), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA-R), γ-aminobutyric acid receptor (GABA-R) and dopamine receptor (DR) were studied and conflicting reports have been published regarding the seroprevalence of these autoantibodies. A clear causative role of autoantibodies on psychiatric symptoms has as yet only been shown for the NMDA-R. Several other autoantibodies have been related to the presence of certain symptoms and antibody effector mechanisms have been proposed. However, extensive clinical studies with large multicenter efforts to standardize diagnostic procedures for autoimmune etiology and animal studies are needed to confirm the pathogenicity of these autoantibodies. In this review, we discuss the current knowledge of neuronal autoantibodies in the major neuropsychiatric disorders: psychotic, major depression, autism spectrum, obsessive-compulsive and attention-deficit/hyperactivity disorders.

## 1. Introduction

Schizophrenia, major depressive disorder (MDD), and bipolar disorder (BD) were classically seen as psychiatric disorders or mental illness, which classifies a disturbance of the "mind". This classification developed within the paradigm of dualism in which mind and body are separated [1]. As such, psychiatric disorders were distinguished from the neurological diseases which have a demonstrable pathology. With today's understanding of both fields, the distinction is only based on symptomatology because both classifications have detectable biological causes. Due to these developments in psychiatry, the subspecialty of neuropsychiatry is growing. Accordingly, in neuropsychiatric disorders, both psychiatric symptoms (affecting emotions, thoughts, and behaviors) and neurological symptoms (movement disorders, epileptic seizures, and cognitive impairment) can be identified. The occurrence of one of these symptoms does not necessarily yet leads to the diagnosis of a certain psychiatric or neurological disease; an isolated epileptic attack is not epilepsy and an isolated psychotic episode is not schizophrenia. Only when symptoms are persisting over a certain time, this diagnosis will be made. Notwithstanding that the knowledge of biological psychiatry is advancing, the diagnosis of these syndromes is still based on behavioral phenotypes following the classification from the Diagnostic and Statistical Manual of Mental Disorders (DSM, currently version 5) and the International Statistical Classification of Diseases and Related Health Problems (ICD, currently version 10). These diagnoses are still not trivial because the rating of psychiatric symptoms is challenging (although important efforts have been made to objectivize the diagnosis [2]) and many neuropsychiatric disorders have overlapping symptoms. Consequently, classification guidelines keep changing during the years.

The etiology of neuropsychiatric disorders is very diverse and still poorly understood but a set of biological changes and risk factors have been identified for the different diagnoses. Some of these disease mechanisms are overlapping between different neuropsychiatric disorders, with the major biological changes being the alteration of synaptic transmission, including hypofunction of dopamine receptor (DR) and *N*-methyl-D-aspartate receptor (NMDA-R) and, also, dysfunction of the voltage-gated potassium channel (VGKC) [3–5]. Inflammation is associated with neuropsychiatric etiology, probably caused by infections or autoimmune diseases. In recent years, the discovery of certain autoantibodies targeting the central nervous system (CNS) such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA-R), γ-aminobutyric acid (GABA-R), and the metabotropic glutamate receptors (mGluR) could be an important breakthrough in neuropsychiatry. Autoantibodies targeting mainly neuronal membrane proteins have now been revealed to potentially alter memory, behavior, and cognition or cause psychosis, seizures, and abnormal movements [6,7]. These autoimmune encephalopathies also have an implication in psychiatry as some of these autoantibodies (such as anti-NMDA-R) are seen in patients with psychotic symptoms that have not previously been considered to have an autoimmune origin [8]. Thus, psychotic disorders and autoimmune encephalitis have overlapping symptoms. During the last years, effort has been made to better understand the autoimmune mechanisms that can induce neuropsychiatric disorders. Here we review antibody-mediated autoimmunity against neuronal (membrane) proteins in five major neuropsychiatric disorders: psychotic, major depressive (MDD), autism spectrum (ASD), obsessive-compulsive (OCD) and attention-deficit/hyperactivity (ADHD) disorder. Table 1 summarizes the characteristics, prevalence, and etiology of these disorders.

## 2. Indications for Autoimmune Mechanisms in Neuropsychiatric Disorders

**Table 1.** Description of the characteristics, prevalence, and etiology of mental disorders.

| Disorder | Characteristics | Prevalence | Etiology |
|---|---|---|---|
| Psychotic disorders | Delusions, hallucinations, disorganized speech and behavior, and other symptoms. Social or occupational dysfunction. | Estimates of the prevalence vary greatly. The median European prevalence is ~5.3%, with an interquartile range of 1.9%–14.4% [9]. | Environmental and genetic factors; about 80% of heritability [10–13]. |
| Major depressive disorder (MDD) | Feelings of persistent sadness and anhedonia that affect thoughts and behavior. Leading to physical problems. A major cause of morbidity worldwide [14]. | Prevalence is up to 15% of the population. | Environmental and genetic factors; possibly autoantibody involvement [15–19]. |
| Autism spectrum disorder (ASD) | Social communication deficit, restricted interest, repetitive behaviors with high sensitivity to changes in the environment. Difficulty to establish human affective and interpersonal relationships [20]. | Prevalence of 1.47% in 2010 [21], increased over time, males being 5 times more affected than females [22]. | Environmental and genetic factors; ~90% heritability [23]. |
| Attention-deficit/hyperactivity disorder (ADHD) | Inattention, hyperactivity, and impulsivity like excessive talking, fidgeting, or an inability to remain seated in appropriate situations. Incapability to focus and organize tasks and activities. | Most prevalent chronic neurodevelopmental disorder in school-age children, affecting 2-18% [24,25] and being more frequent in males than in females. | Strong genetic link as well as environmental factors [25]; heritability ~76% [26]; post-infectious autoimmunity [27]. |
| Obsessive-Compulsive disorder (OCD) | Anxiety, recurrent unwanted thoughts (obsessions) and repetitive behaviors (compulsions). | Affects 1%–3% of the worldwide population [28–30]. | Genetic and environmental factors [31]; heritability of ~50% in children [32] post-infectious autoimmunity [33,34]. |

Genetic studies of large sample sizes have revealed several gene variants that increase the risk of neuropsychiatric disorders, including genes encoding for neurotransmitter receptors and ion channels. In the Psychiatric Genomics Consortium, single nucleotide polymorphisms (SNPs) in two L-type voltage-gated calcium channel subunits, voltage-gated calcium channel subunit alpha1 C (CACNA1C) and calcium voltage-gated channel auxiliary subunit beta2 (CACNB2),

were identified as common risk factor among all studied diagnosis including ASD, ADHD, BD, MDD and schizophrenia [35].

In addition, variants from the human leukocyte antigen (HLA) region (major histocompatibility complex; MHC molecules) which are involved in antigen presentation have not only been associated with the risk of developing autoimmune diseases, but also with the risk of developing several neuropsychiatric disorders. A C4B null allele, a deficient form of the HLA C4B gene (no C4B protein produced), was reported to be more frequent in ASD, ADHD, and dyslexia [12]. Another locus called the HLA DRB1 was implicated in schizophrenia and ASD as well as autoimmunity [36,37]. Taken together these findings suggest that neuroinflammation and autoimmunity may play a role in neuropsychiatric disorders [13].

Autoantibodies in neuropsychiatric disorders cause mainly a loss rather than a changed pattern of channel activity, possibly associated with neuroinflammation and neurodegeneration [38–40]. To demonstrate autoimmune pathogenicity according to Witebsky's postulates, four conditions have to be met: (1) the autoantibody must be present with the clinical manifestation and detectable in the blood and/or affected tissue; (2) autoantibodies should target a receptor, ion channel, or other protein expressed on the membrane surface; (3) antibody transfer can replicate the disease in an animal experimental model or in humans (maternal transfer); and (4) elimination or suppression of the autoimmune response by therapy can prevent disease progression or improves the clinical manifestations.

## 3. Ion Channels and Receptor Functions

Autoantibodies in neuropsychiatric disorders commonly target neuronal ion channels or associated proteins. For an in-depth understanding of how autoantibodies against these molecules cause disease, it is necessary to comprehend the functions of neuronal ion channels and receptors, which largely determine the inter-neuronal communication and properties of neurons. These channels are facilitating the depolarization, hyperpolarization and also repolarization of neurons and thus are essential to the signal transmission and functioning of the brain [41]. The basis of the transmission of electric currents is the membrane potential, which is the difference in electrical charge between the inside and outside of neurons. This difference is produced by ion pumps that create high extracellular $Na^+$, $Cl^-$ and $Ca^{2+}$ concentrations and high $K^+$ inside of the neuron. During an action potential, depolarization induces a rapid influx of $Na^+$ followed by a slightly slower opening of $K^+$ channels that induce repolarization and thereby enable a relatively fast subsequent activation of the neuron. Within the nervous system, different neurons possess a unique mixture of a wide variety of ion channels, which characterize their electrophysiological properties. The different types of synapses are largely defined by the neurotransmitter that is used for signal transduction, which are acetylcholine (ACh), noradrenaline (NA), dopamine, glutamate (Glu), serotonin (5HT), γ-aminobutyric acid (GABA, inhibitory), glycine (Gly, inhibitory), nitric oxide and a series of peptide neurotransmitters including endorphin.

Neurotransmitters can activate these receptors by either inducing a direct opening of an ion channel (ionotropic receptor) or altering the concentration of intracellular metabolites via GTP binding proteins (metabotropic receptors). Due to their mode of action, ionotropic receptors, such as the NMDA-R, promote rapid signal transduction and are responsible for the majority of neuronal communication in the CNS and peripheral nervous system (PNS). On the other hand, metabotropic receptors, including the metabotropic Glu receptors (mGluRs) and the D2

dopamine receptor (D2DR), act via second messengers and therefore have a slower effect but also a longer duration of action and can lead to long-term changes such as synaptogenesis.

Conceptually, it makes sense that binding of autoantibodies to these receptors which have the potency to interfere with the action of these fundamental signaling processes can induce severe neurological and psychiatric symptoms. This is further supported by the fact that substances with an inhibitory effect on neurotransmitter receptors, such as ketamine, acting on NMDA-R or lysergic acid diethylamide on 5HT receptors (5HT-R) are potent hallucinogens.

## 4. The Role of Blood-Brain Barrier Integrity on Autoantibody Effects

Despite tight immune surveillance of the CNS, antibodies cross in low numbers through the blood-brain barrier (BBB). Once they reach the cerebrospinal fluid (CSF), the turn-over is about four times per day. This dynamic equilibrium results in immunoglobulin G (IgG) levels in the brain that amount to about 1% of the plasma levels, and in about 10% of the total protein in the CSF [38,42,43]. In autoimmune encephalitis, it is known that several autoantibodies cross the BBB and can be detected in the CSF. However, the mechanism of how antibodies cross to the CSF is not very well understood. The permeability of the BBB is altered upon damage and inflammation of the brain. Additionally, an impaired function of apolipoprotein E (ApoE) has been shown to reduce the barrier function of tight junctions [44]. This knowledge was used to study whether an impaired BBB would change the effect of peripherally administered human NMDA-R antibodies in a mouse model. Hammer *et al.* claim that only in ApoE knock out mice but not in wild type mice, human NMDA-R antibodies cause psychosis-related behavioral perturbation [45]. The same study also relates the effects of the autoantibodies to the patients' history of birth complications or neurotrauma indicating possible BBB insufficiency. This hypothesis is further supported by the findings that an increased prevalence of psychiatric comorbidity in diseases is associated with BBB dysfunction, including systemic lupus erythematosus (SLE) [46–48], stroke [49–52], epilepsy [53,54] and autoimmune encephalitis [45,55]. An increased albumin ratio in CSF to serum in patients with MDD and schizophrenia further suggests increased BBB permeability [56]. On the other hand, circulating B cells cross the BBB during normal immune surveillance [57] which might include antibody-producing cells. CD138+ plasma cells were found in post-mortem and biopsy tissue of NMDA-R encephalitis patients [58]. Intrathecal antibody production was also described in Sydenham chorea (SC) patients with anti-lysoganglioside GM1-specific IgG [56] and in a case with autoantibodies against the GluN1 subunit (also known as NR1) of the NMDA-R where the patient did not respond to plasmapheresis treatment, while plasma antibody levels dropped but CSF levels remained high [59]. Some groups report that in patients with encephalitis autoantibodies against NMDA-R, AMPA-R, metabotropic or B class of the GABA-R (GABAB-R), dipeptidyl-peptidase-like protein-6 (DPPX), mGluR1 or mGluR5 can always be found in the CSF whereas other autoantibodies, such as autoantibodies to leucine-rich glioma inactivated-1 (LGI1), to contactin associated protein-2 (CASPR2), to glycine receptor (GlyR) and to the ionotropic or A class of the GABA-R (GABAA-R) may, in rare instances, be identified only in serum [7]. If no autoantibodies can be detected in the CSF, it is unclear how they can have central effects and thus if they are pathogenic. However, if the autoantibodies are present but immuno-absorbed by the antigen in the brain, they might not be detectable in the CSF [60].

In addition, T cells might have a role in BBB integrity and thus antibody penetration. Recently, Dileepan and colleagues described that T-helper 17 cells activation caused by group A *Streptococcus* infection disrupt the integrity of the BBB, and facilitate circulating autoantibodies to enter the brain [61].

## 5. Transfer of Autoantibodies via the Placenta

Transfer of maternal IgG antibodies to the fetus is a protective mechanism during the period in which the infant has an undeveloped humoral immune response [62]. IgG antibodies are the only Ig isotype that crosses the placenta and they do so via neonatal Fc receptors (FcRn) on syncytiotrophoblast cells. The amount of IgGs passing to the fetus is altered dependent on e.g. maternal levels of specific antibodies, the period of gestation, placental integrity, and type of antigen. If the mother has IgG autoantibodies in the blood, these will also be transferred to the neonate where they can induce pathogenic effects. Additionally, it has been seen in a rat model that in the fetus, the IgG penetration to the brain is higher than in the adult [63], indicating that these autoantibodies might reach and bind neuronal receptors in the fetus. Such an example is autoantibodies targeting the acetylcholine receptor (AChR) located at the neuromuscular junction (NMJ) which is composed of five subunits. Receptors are either of the embryonic form, composed of α1, β1, γ and δ subunits, or of the adult form composed of α1, β1, δ and ε subunits. Mothers carrying autoantibodies specifically against the gamma subunit (AChRγ) are frequently asymptomatic [64,65]. Maternal antibodies of this sort can impair skeletal muscle development and cause fixed joint contractures and other deformities called arthrogryposis multiplex congenita. In other neurodevelopmental disorders such as autism [66–68] and dyslexia [69], a role of maternal autoantibodies has been suggested (see the section on ASD later). In SLE, a pathogenic transfer of maternal antibodies has been described [70] and maternal antibodies have been hypothesized to cause long-term cognitive changes since children born to mothers with SLE display a high incidence of learning disorders [71–73]. In a mouse model with high maternal autoantibody levels targeting double-stranded DNA (dsDNA) and cross-reacting with GluN2a/2b subunits of NMDA-R, cognitive impairments in adult offspring have been detected due to histological abnormalities in the fetal brain [74]. Taken together, these studies suggest that *in utero* exposure to neurotoxic/inflammatory autoantibodies generates developmental abnormalities with long-term consequences. In some cases, the effects of neonatal autoantibody exposure might only present later in life and potentially only with certain environmental exposures which make it very difficult to study these disease mechanisms. In case that the presence of maternal autoantibodies can be detected, these complications are treatable during pregnancy with intravenous IgG (IVIg) that competes with the endogenous autoantibodies, saturate FcRn and increase IgG turnover [75].

## 6. Autoantibody Effector Mechanisms

Autoimmune diseases are induced by complex immune dysfunctions of T-cells, B-cells, and other immune cells, but can be simply classified as T-cell or antibody-mediated. It is still largely unknown which mechanisms are involved in autoimmune neuropsychiatric disorders; however, most studies point towards an antibody-mediated pathology. We will, therefore, focus here on the IgG antibody-mediated disease mechanisms, which can be summarized as follows:

(a) Complement deposition and inflammation is a common mechanism in autoimmune diseases. The complement system is part of the innate immune system and can be activated by

antibody-antigen complexes, which leads to the activation of complement proteins amplifying its effector mechanisms [39,76]. Effects of complement are (i) opsonization and engulfment by phagocytes with receptors for complement; (ii) chemo-attraction and activation of phagocytes and (iii) formation of the so-called membrane attack complex in cell membranes leading to lysis, extensive tissue damage and loss of tissue architecture, including receptors and ion channels.

For example, complement activation is important in Rasmussen's encephalitis, where autoantibodies anti-GluR3 subunit of the AMPA-R have been detected [77]. Peripherally, anti-AChR autoantibodies (of IgG1 and IgG3 isotype) from myasthenia gravis (MG) patients [78,79] activate the classical complement pathway. This causes complement deposition in the NMJ, where the antigen is located, resulting in morphological damage and loss of the AChR in the postsynaptic membrane [39].

(b) Stimulation or inhibition of receptor function can be induced upon binding of the autoantibody without further activation of the immune system. Examples for this mechanism are autoantibodies targeting the folate receptor (FR) which have a very high binding affinity and thereby block binding and uptake of folic acid [80]. This inhibits the transport of folic acid into the CSF and causes cerebral folate deficiency leading to infantile-onset neuropsychiatric symptoms including psychomotor retardation, cerebellar ataxia, dyskinesias and in some cases, seizures. The autoantibodies found in SC patients alter the D2DR function by reducing adenylate cyclase levels at a comparable level to the inhibitory effect by dopamine [81]. By targeting the receptor the autoantibodies can also interfere with the intracellular signaling pathways activated by the calcium/calmodulin-dependent (CaM) kinase II, an enzyme involved in cognition and neurotransmitter synthesis and release [82–84]. The activation of this enzyme has been correlated with an increase of dopamine release in the brain [85].

(c) Antigen internalization (or antigenic modulation) is a mechanism in which binding of autoantibodies induces internalization and commonly degradation of the antigen. The two arms of the antibody can bind each separately to an antigen leading to clustering or cross-linking of the antigens in the membrane. Antibodies against the NMDA-R are thought to cause cross-linking and selective internalization of receptors as shown in cultured neurons [86,87]. Reduction in DR levels has also been observed in the presence of SC patient autoantibodies [81]. In the PNS, specifically in MG, anti-AChR autoantibodies accelerate the internalization of the receptor [88,89], a mechanism that can be blocked by overexpression of the AChR anchoring protein, rapsyn, in an experimental passive transfer MG model, showing the important role of anchoring proteins in the resistance to the autoantibody attack [90].

(d) Loss or block of receptor associated proteins can also significantly alter the function of ion channels. One of the known antigens associated with the VGKC is LGI1 [91,92]. LGI1 autoantibodies can cause a disruption of the ligand-receptor interaction of LGI1 with scaffolding proteins ADAM22 or ADAM23, which is interfering with the trans-synaptic complex that includes presynaptic Kv1.1 potassium channels and post-synaptic AMPA-R [93,94]. It has also been observed in the PNS that the AChR internalization and complement damage produces a loss of scaffolding proteins associated with the receptor like muscle-specific kinase (MuSK), rapsyn, docking protein 7 (Dok-7), LDL Receptor Related Protein 4 (Lrp4) or agrin, altering the endplate organization and, in some cases, aggravating the symptoms and delaying the repairing mechanisms [39].

## 7. The relevance of Intracellular Antigens as Target of Autoimmunity

Considering the pathologic mechanisms described above, autoantibodies are unlikely to be pathogenic if they target intracellular antigens such as Hu, Yo or Ri, as commonly seen in paraneoplastic syndromes [95]. Instead, diseases with intracellular antigens are thought to be T-cell mediated [96] and are not within the scope of this review. The pathogenicity of a few autoantibodies targeting intracellular antigens e.g. amphiphysin, glutamic acid decarboxylase (GAD), ribosome P proteins (Rib-P) and anti-dsDNA, is still controversial. Amphiphysin is a synaptic vesicle protein which might be exposed to autoantibodies in the membrane during synaptic vesicle uptake [7] and has been described as an antigen affected by autoantibodies in Stiff Person syndrome. Amphiphysin autoantibodies induced structural disorganization in GABAergic synapses and changed presynaptic vesicle pools [97]. In addition, autoantibodies to GAD, the enzyme synthesizing the inhibitory neurotransmitter GABA, are related to many neurological disorders e.g. Stiff Person Syndrome, cerebellar ataxia, limbic encephalitis (LE), epilepsy and oculomotor dysfunction [98]. Gresa-Arribas and collaborators observed that anti-GAD autoantibodies are not internalized by neuronal cell cultures, indicating that the antibodies are unlikely to interact with GAD on live neurons [99]. Epitope specificity overlaps between different syndromes with GAD autoantibodies and thus cannot explain the differences in symptoms [98,99].

If the anti-GAD autoantibody is the causative factor, other antibody properties or interacting factors such as environmental factors must also play a role. The study of these autoantibodies is of clinical relevance as a potential diagnostic marker, because GAD autoantibodies in classical paraneoplastic syndrome are indicative for identification of tumors and often coincide with other autoantibodies that target neuronal antigens such as GABA-R [100,101] and GlyR [102,103]. Studies in rats showed that injection of IgG from patients with GAD autoantibodies and neurological symptoms lead to motor dysfunction and impaired NMDA-R signaling but not when injecting IgG from GAD positive diabetes patients without neurological presentation [104], which was interpreted as the pathogenic role of autoantibodies related to neurological symptoms. Other authors claim that these changes were evoked by accompanying other autoantibodies [101]. Interestingly, the repertoire of antibodies to different immunodominant regions in the GAD antigen is wider in the CNS than systemically [99].

Autoantibodies against Rib-P have been proposed to be involved in the neuropathogenic in psychiatric SLE, e.g., anti-Rib-P autoantibody titers were correlated to depression in the onset of SLE [105]. A murine model illustrates the ability of anti-Rib-P autoantibodies to induce depressive-like symptoms [106,107]. Moreover, Matus *et al.* showed that these antibodies could cross-react with a novel neuronal surface protein causing $Ca^{2+}$ influx and apoptosis. However, there exists in the literature some controversy in the association of anti-Rib-P with CNS involvement and neuropsychiatric manifestations in SLE [108,109], which may be due to the great variation in detection assays concerning the purity of the anti-Rib-P autoantibodies, the use of synthetic peptides, or parts/complete antigen as well as the carrier proteins used. Anti-dsDNA autoantibodies have been proposed to cross-react with the GluN2 subunit of the NMDA-R and are responsible for excitatory, non-inflammatory cell death and altered neuronal function [110].

In Table 2 the presence of autoantibodies in neuropsychiatric diseases targeting membrane and intracellular proteins is summarized; the latter will be discussed in the following section.

**Table 2.** Autoantibodies in neurologic diseases with psychiatric symptoms.

| Antigen Target | Subunit/ Associated Protein | Related Disease | n+/n Patient | n+/n Control | Age Range * | Ig Type | Ref. |
|---|---|---|---|---|---|---|---|
| **Autoantibodies to neuronal surface antigens **** | | | | | | | |
| VGKC complex | n.s. *** | Limbic encephalitis | 4/15 | n.t.*** | 47–69 | IgG | [113] |
| NMDA-R | GluN1 | NMDA-R encephalitis with psychiatric symptoms | 50/485 | n.t | 17–44 | IgG | [114] |
| | | | 100/100 | n.t | 5–76 | IgG | [115] |
| | | | 250/250 | 0/100 | n/a | IgG | [116] |
| | | | 6/505 | n.t | 18–35 | IgG | [117] |
| | | NMDA-R encephalitis (isolated psychiatric episodes) | 571/571 | n.t | 12–62 | IgG | [8] |
| | | NMDA-R encephalitis (schizophrenia, and autism) | 1/1 | n.t | 9 | IgG | [118] |
| | | Autoimmune encephalitis in postpartum psychosis | 2/96 | 0/64 | 25, 31 | IgG | [119] |
| | GluN2a/2b | Progressive cognitive dysfunction of unclear etiology | 7/24 | n.t | 49–81 | IgA | [120] |
| | | Herpes simplex encephalitis | 5/44 9/44 9/44 | n.t | 24–79 | IgG IgM IgA | [121] |
| | | Limbic encephalitis, narcolepsy | 3/5, 3/5 | n.t | 18–59, 24–61 | IgG | [122] |
| | GluN1/ GluN2a/2b | NMDA-R encephalitis associate with ovarian teratoma | 12/12 | 0/200 | 14–44 | IgG | [123] |
| AMPA-R | GluA1, GluA2 | Limbic encephalitis | 22/62 | n.t | 23–81 | n/a | [124] |
| GABA-R | Type B | Encephalitis with opsoclonus, Ataxia, Chorea and Seizures | 1/1 | n.t | 3 | IgG | [125] |
| | $\alpha$1/$\beta$3 subunits | Encephalitis with refractory seizures, status epilepticus, | 6/140 | 0/75 | n/a | IgG | [125] |
| | $\alpha$1/$\beta$3 subunits | Encephalitis with thymoma | 1/1 | n.t | 45 | IgG | [126] |
| GlyR | $\alpha$1 | Progressive encephalomyelitis with rigidity and myoclonus (PERM) | 52/779 | n.t | 1–75 | IgG | [102] |
| mGluR | mGluR5 | Encephalitis (Hodgkin lymphoma, Ophelia syndrome) | 2/2 | n.t | 15, 46 | IgG | [127] |
| Kv4.2 | DPPX | Encephalitis (subacute onset of neuropsychiatric symptoms) | 4/4 | 0/210 | 45–76 | IgG | [128] |
| D2DR | D2 | Basal ganglia encephalitis **, Sydenham's chorea **, Tourette's syndrome ** | 12/17, 10/30, 4/44 | 0/67 | 1–15, 2–17, 2–13 | IgG | [129] |
| Folate receptor | - | Cerebral folate deficiency syndrome | 25/28 | 0/28 | 2.5–19.3 | n/a | [130] |
| **Autoantibodies to (neuronal) intracellular antigens **** | | | | | | | |
| Rib-P | P1, P2, P3 | SLE with Depression | 22/100 | n.t | 23–36 | IgG | [105] |
| GAD | n.s. | Non-paraneoplastic limbic encephalitis | 2/2 | n.t | 20,47 | IgG | [131] |

* Age range of the positive subjects; ** Anti-basal ganglia antibodies (ABGA) can bind to either neuronal surface or intracellular antigens and are related to basal ganglia encephalitis, Sydenham's chorea, Tourette's syndrome, OCD and ADHD. For details see OCD and ADHD sections; *** n.s. = not specified; .n.t. = not tested; VGKC complex = voltage gated potassium channel complex; NMDA-R: *N*-Methyl-D-Asparte receptor; AMPA-R = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; GABA-R = γ-aminobutyric acid; GlyR = glycine receptor; mGluR= metabotropic glutamate receptor; Kv4.2 = Potassium channel, voltage dependent, Kv4.2; DPPX = Dipeptidyl-Peptidase-Like Protein-6; DRD2 = dopamine-2 receptor; Rib-P = ribosome P protein; SLE = Systemic lupus erythematosus; GAD = glutamic acid decarboxylase.

Table 3 gives an overview of autoantibodies targeting membrane proteins and intracellular antigens only in psychiatric disorders.

## 8. Autoimmune Encephalitis

Encephalitis is an inflammation of the brain characterized by memory alterations, behavioral and cognitive changes, and seizures, where immune-mediated mechanisms have been related [111]. Paraneoplastic events are very common in encephalitis patients and antibodies to intracellular onconeuronal antigens and cytotoxicity mechanisms have been described [112]. In Table 2, the autoantibodies involved in neurological diseases with psychiatric symptoms are summarized.

A few years ago, NMDA-R autoantibodies were described for the first time in a group of encephalitis patients with ovarian teratoma with the peculiar fact that they suffered psychotic symptoms. Autoantibodies were identified in serum and CSF using a cell-based assay (CBA) (HEK293 cells expressing single subunits or dimers of the GluN1 and GluN2a/2b) and rat brain immunohistochemistry (IHC) [123]. Importantly, about 80% of these patients had full or substantial recovery after treatment with immunotherapy and removal of the tumor if present, which indicates that the antibody is an important, if not, the only cause of symptoms. This further implies that subgroups of patients with neuropsychiatric disorders are treatable with immunotherapy. These findings were confirmed in other encephalitis cohorts [122,132] and in individual patients [133] with a case example of a young lethargic encephalitis patient, who had high NMDA-R autoantibodies (higher in CSF than in serum) and responded well to immunosuppressive therapy [134]. For NMDA-R autoantibodies, higher sensitivity and specificity was found in studies using CSF [116,118,134]. In contrast, using a fluorescent immunoprecipitation assay anti-GluN1, IgG autoantibodies were found in higher levels in serum than in CSF in 10% of cases [114]. The GluN1 subunit of the receptor was identified as the main antigenic epitope in the classic full spectrum NMDA-R encephalitis cases [117,135], specifically a small region in the amino-terminal domain [136]. Upon binding, the autoantibodies are thought to cause cross-linking and selective internalization of NMDA-R as shown in cultured neurons [86]. It has also been proposed that in the extrasynaptic compartment, autoantibodies significantly reduce the surface diffusion of NMDA-R, likely facilitating their internalization and degradation [87]. An important contribution to verify the effect of these autoantibodies was recently given by Planagumà and colleagues who showed that passive transfer of NMDA-R autoantibodies by continuous intraventricular infusion of CSF from patients with NMDA-R encephalitis causes memory and behavioral deficits in mice. The passive transfer model was also able to reproduce the downregulation of total and synaptic NMDA-R density observed in the disease in humans. After discontinuing the patient CSF infusion, the NMDA-R clusters in the hippocampus, and the total NMDA-R protein amount was recovered gradually, supporting the reversible effect of these autoantibodies [137,138].
Patients with autoantibodies against the VGKC complex have been reported in many neurological disorders (including LE, epilepsy, neuromyotonia) as immunotherapy-responsive [139]. Radioimmunoassay (RIA) and IHC techniques were used to test VGKC reactivity in serum and CSF. Higher percentages (17%–26%) of autoantibodies that were thought to be anti-VGKC were found in LE patients [113,140,141]. The autoantibodies were actually targeting proteins that were in complex with the ion channel. In the following study, LGI1 was identified as the real antigen in VGKC positive patients by CBA. CASPR2 which forms part of the scaffold required to anchor the VGKC [142] was also described as an antigen [93,143].

Other neuronal surface antigens have been identified in LE patients such as autoantibodies to AMPA-R with concomitant psychotic symptoms and good response to immunotherapy [124]. The main epitopes are in the AMPA-R subunits GluR2 (6/12) followed by the GluR1 (3/10) and a GluR1/GluR2 conformational epitope (1/10) [124]. No autoantibodies against the GluR3 subunit were found. The autoantibodies bound in 91% of the cases to GluR2 in cluster with GluR3, produced a reduction in the number of GluR2 subunit in the AMPA-R clusters at the synapsis. GABAB-R autoantibodies have been recently related to an aggressive course of autoimmune encephalitis in a young patient [125]. In contrast, GABAA-R autoantibodies have been found in severe forms of encephalitis [125] e.g., in combination with anti-LGI1 in a patient presenting a subacute onset of memory loss, confabulation, and behavioral changes [126]. The D2DR has been first identified in 12 out of 17 basal ganglia encephalitis patients, an autoimmune disorder characterized by movement and psychiatric symptoms. In this study, 3 out of 12 IgG positive patients presented paranoia, psychosis, and hallucinations. These autoantibodies have also been described in other neuropsychiatric disorders [129].

**Table 3.** Autoantibodies related to psychiatric disorders.

| Antigen Target | Subunit/ Associated Protein | Related Disorders (D) | n+/n Patient | n+/n Control | Ig Type | Age Range * | Ref. |
|---|---|---|---|---|---|---|---|
| **Autoantibodies to neuronal surface antigens**** | | | | | | | |
| VGKC complex | LGI1, CASPR2 | Psychotic D | 3/125 | n/t*** | IgG | n/a*** | [144] |
| | n.s.*** | Psychotic D (schizophrenia) | 1/46 | n/t | IgG | 22 (pp) | [145] |
| NMDA-R | GluN1 | Psychotic D and major depressive D. (n.s.) | 81/1688 | 74/1703 | IgM | 26–56 (p) | [146] |
| | | | 92/1688 | 76/1703 | IgA | | |
| | | | 14/1688 | 20/1703 | IgG | | |
| | GluN2a/2b | Psychotic D (schizophrenia) | 4/51 | n/t | IgG | 26–53 (pp) | [122] |
| | | Psychotic D (schizophrenia) | 3/46 | n/t | IgG | 19–28 (pp) | [145] |
| Muscarinic AChR | M1,M2 | Schizophrenia | (n/a)/21 | (n/a)/25 | IgG | 25–56 (p) | [147] |
| | | Psychotic D, bipolar and depressive D | 42/122 | 0/52 | n/a | 24–63 (p) | [148] |
| Nicotinic AChR | $\alpha$7 | Schizophrenia | 5/21 | 0/17 | IgG | 46–61 (pp) | [149] |
| D2DR | D2 | Bipolar and major depressive D | 6/122 | 0/52 | n/a | 30–63 (p) | [148] |
| Opioid receptor | OPRM1 | Psychotic D, bipolar and major depressive D | 16/122 | 0/52 | n/a | 30–63 (p) | [148] |
| | | Major depressive D | 2/27 | n/a | IgG | n/a | [150] |
| 5HT receptor | HTR1A | Psychotic D, Major depressive D | 9/63 | 0/52 | n/a | 30–63 (p) | [148] |
| | | Autism spectrum D (Autism) | n/a | n/a | n/a | <10 (pp) | [151] |
| FR | - | Autism spectrum D (Autism) | 70/93 | n/t | n/a | 3–18 (t) | [152] |
| DAT | - | Attention-deficit/hyperactivity | n/a/46 | n/a/15 | IgG | 4–16 (t) | [153] |
| **Autoantibodies to (neuronal) intracellular antigens**** | | | | | | | |
| GAD | GAD 65 | Psychotic D (Schizophrenia) | 1/1 | n/t | n/a | 19 (pp) | [154] |
| | | Autism spectrum D (Autism), Attention-deficit/hyperactivity D | 3/20, 4/15 | 0/14 | IgG | 8–11 (pp) | [155] |

* Age range: (pp) the age range belongs to the patients tested positive in the assay, (p) the age range belongs to the total patients cohort tested in the assay, (t) the age range belongs to all the subjects (including controls and patients) tested in the assay; ** Anti-basal ganglia antibodies (ABGA) can bind to either neuronal surface or intracellular antigens and are related to OCD, ADHD. For details see paragraph on OCD and ADHD; *** n.s. = not specified; n/a = not available. n/t = not tested; VGKC complex = voltage-gated potassium channel complex; Ig = immunoglobulin G; NMDA-R: *N*-Methyl-D-Asparte receptor; AChR = acetylcholine receptor; DRD2 = dopamine-2 receptor; OPRM1 = opioid receptor, mu 1; 5H = serotonin; HTR1A = 5-Hydroxytryptamine (Serotonin) Receptor 1A; FR = Folate receptor; DAT = Dopamine transporter; GAD= glutamic acid decarboxylase.

## 9. Psychotic Disorders

Psychotic disorders are difficult to conceptualize and thus many misconceptions still exist on what psychosis and schizophrenia are. These disorders share common symptoms that can be divided into five main categories: (i) psychosis (encompassing delusions and hallucinations—also called the positive-symptom dimension); (ii) alterations in drive and volition (the negative-symptom dimension); (iii) alterations in neuro-cognition (cognitive-symptom dimension); and (iv and v) affective dysregulation (giving rise to depressive and manic (bipolar) symptoms) [11]. The different diagnoses will be dependent on the duration and intensity of these different symptoms. Schizophrenia is the most common diagnosis within the psychotic disorders and applies to a syndrome characterized by long duration, bizarre delusions, negative symptoms, and few affective symptoms (non-affective psychosis). Other diagnoses of psychotic depression or bipolar disorder (affective psychosis) represent patients who present with a psychotic disorder with fewer negative symptoms, but with higher levels of affective (depression and mania) symptoms previous to psychosis.

The immunological involvement in psychotic disorders was already hypothesized in 1930 when some immunological signs were detected in patients with schizophrenia [156]. In Table 3, the studies involving autoantibodies in psychiatric disorders are described in detail, including an overview of the specific role of the neuronal antigens in these pathologies.

As it has been already described, NMDA-R encephalitis presents usually with an early psychotic phase and subsequent seizures, movement disorders and autonomous dysfunction, but about 4% of patients develop only isolated psychotic episodes [8,157]. NMDA-R autoantibodies have been detected in schizophrenia [122] and in some case reports, such as a patient with the pure typical psychotic syndrome who recovers after immunotherapy [145] and patients with a first psychotic episode post-partum [119]. Some children also show isolated psychiatric symptoms, such as a case of a 9-year-old individual diagnosed with early schizophrenia, who presented high autoantibody titers in CSF compared with serum and responded well to immunosuppressive therapy [118]. These cases are not typical because, other than in adult cases of NMDA-R encephalitis, symptoms in early life are mainly neurologic rather than psychiatric [158].

After the antigenic epitope was defined, the immunoglobulin isotype frequency was studied in a schizophrenia cohort [159]. IgGs against the GluN1a subunit were described in two first episode catatonic schizophrenic patients (probably misdiagnosed NMDA-R encephalitis), while two other paranoid schizophrenia patients presented IgGs against GluN1a/2b, in lower titers, which declined during remission also shown in other studies [115,141,145]. Curiously, only the two patients with anti-GluN1 IgG autoantibodies presented IgG positive titers in CSF, a controversial result based on the 3.2% reactivity to neuronal surface antigens in CSF found in a psychotic disorder cohort (0.8% to NMDA-R and 2.4% to VGKC complex) [144]. Another study showed GluN1 IgG autoantibodies in 5 out of 43 children with the first episode of acute psychosis, screened by a more objective variety of the CBA using flow cytometry [160]. This subunit was also recognized by IgA or IgM [160] but not specifically related to schizophrenia since they were also present in other pathologies and in control individuals [121,159]. IgA autoantibodies to NMDA-R (but not IgG) were described in a cognitive dysfunction cohort where they are thought to induce decreased NMDA-R expression and NMDA-R mediated currents in neuronal cell cultures [120].

The frequency of autoantibodies to NMDA-R and VGKC complex in different studies ranged from 0% to 10% in cohorts of first-episode psychosis or schizophrenia

[38,122,144,159,161,162]. Nevertheless, the results are controversial, since a number of other studies did not find autoantibodies in neuropsychiatric cohorts [146,159,162,163] or not specific to the disease [45,122]. New and more objective techniques, like flow cytometry CBA, are being introduced to the research routine [129,160], but a standardized procedure to screen patients for neuronal surface antigens needs to be defined to reduce results variability.

No serum autoantibodies against GluR1/GluR2 subunits from the AMPA-R were detected in a schizophrenia cohort (also not in other neuropsychiatric disorders and controls) [159]. On the other hand, the presence of autoantibodies to any class of the GABA-R has not been studied in psychotic disorders to our knowledge. The D2DR autoantibodies have been described in a cohort of first-episode acute psychosis children (3 out of 43 IgG and 1 out of 43 IgM subtypes) [160].

Autoantibodies against muscarinic AChR (mAChR), specifically against the mAChR in the cerebral cortex, have been identified in a small percentage of schizophrenia patients [147,164]. Similarly, autoantibodies against the α7 adult subunit of the nicotinic AChR (nAChR) were found in five out of 21 (23%) schizophrenia patients [149].

Autoantibodies to GAD have been studied in a schizophrenia cohort where no autoantibodies were found in any of the 180 CSF samples [144]. Only a single case has been reported, where a schizophrenic patient presented elevated serum titers against GAD [154].

Psychiatric manifestations consist of a broad spectrum of symptoms that can occur during the course of different disorders that do not only include classical psychiatric disorders. We expect that some patients diagnosed with psychotic disorders like schizophrenia will in the future fall under the diagnosis of autoimmune encephalitis, yet in most cases, the causative role of these autoantibodies remains to be proven. Due to the heterogeneous phenotype of mental disorders, it is important to maintain diagnostic guidelines as homogeneous, standardized and as international as possible.

## 10. Major Depressive Disorders (MDD)

Depression is a major cause of morbidity worldwide [14]. The prevalence is up to 15% of the population in industrialized nations and by 2030 it is projected by the World Health Organization to be the leading cause of disease burden globally [165]. MDD is characterized by a state of low mood and anhedonia, affecting the person's thoughts, behavior, feelings, and sense of well-being [166,167]. Patients may also present with diverse symptoms including lethargy, insomnia, social withdrawal and sexual dysfunction among a range of others.

In MDD variations in ion channel and neurotransmitter receptor function are associated with the risk to develop the disorder. Serotonergic neurotransmission plays an important role in the etiology of depression [168]. The serotonin transporter (SERT) is the only known high-affinity transporter that primary regulates 5HT levels in the brain and is considered a key target for widely used antidepressant drugs [17]. So alterations in SERT levels have been implicated in behavioral and neuropsychiatric disorders including MDD [17,18,169]. Genetic studies also support that polymorphisms within genes that encode for receptors or proteins involved in the serotonergic and dopaminergic systems including SERT, 1A serotonin receptor 5HT-1A, dopamine transporter (DAT) and D4 DR are associated to the risk of MDD [19].

Autoantibodies targeting neurotransmitter receptors or other neuronal antigens have been reported in association with MDD [15,16]. Roy *et al.* were the first to report high reactivity of anti-opioid receptor (OPRM1) IgG autoantibodies in 3 out of 27 patients with MDD [170]. In a later study by the same group, patient serum IgGs were isolated by affinity chromatography

and analyzed for reactivity on rat brain tissue [150]. The results suggested that autoantibodies to neuronal receptors might contribute to psychiatric impairment. Susumu *et al.* replicated this study examining the presence of autoantibodies against not only OPRM1 but also 5HT-1A or 5-hydroxytryptamine receptor 1A (HTR-1A), DRD2 as well as muscarinic cholinergic receptor 1 (CHRM1) [148]. Serum IgG from patients suffering from a range of neuropsychiatric disorders, including mood disorders, was analyzed by RIA. Autoantibodies against CHRM1, in particular, were significantly higher in neuropsychiatric patients than in healthy controls. However, there was no significant difference between different neuropsychiatric disorders nor any obvious correlation was found between the titer of the antibody and psychiatric symptoms [148]. The data on autoantibodies in MDD may suggest autoimmune abnormalities within the brain, however, it is still unclear if they play a pathological role or they are merely bystanders. Anti-NMDA-R antibodies have also been found in patients with MDD [110,171]. In contrast to anti-GluN1 autoantibodies present in autoimmune encephalitis, Larissa *et al.* proposed that anti-GluN2a antibodies are associated with the depressive mood in SLE patients [171]. As described before, anti-GluN2 autoantibodies are thought to be a subset of anti-dsDNA antibodies, which could cause apoptosis of neurons *in vivo* and *in vitro* [110,172].

## 11. Autism Spectrum Disorder (ASD)

At the beginning of the 20th century, Dr. Asperger described the term “autistic”, referring to patients who from the beginning of their lives have difficulties to establish human affective and interpersonal relationships [20]. Nowadays, the last edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) includes four separate disorders under the term Autism Spectrum disorder (ASD): autistic disorder, Asperger’s disorder, childhood disintegrative disorder and the catch-all diagnosis of pervasive developmental disorder not otherwise specified. The symptomatology described in these disorders is characterized by social communication deficit and restricted interest/repetitive behaviors with high sensitivity to changes in their environment, which can be developed in different degrees of severity.

Autoimmune diseases such as rheumatoid arthritis, SLE and type 1 diabetes are strongly associated with ASD family members and ASD patients [173,174]. In a subgroup of ASD patients’ maternal autoantibody transfer may play a role in the disease. The autoantibodies are targeting the GluN2 subunits, which are enriched in female fetuses and make them more vulnerable and severely affected in the fetal state than their male siblings [175]. The infusion of serum or IgGs from mothers with ASD children in pregnant mice [176–178] or non-human primates [68,179] reproduced the ASD-like pathology. Subsequently, neuron-reactive maternal antibodies were studied [180–183] and a pair of 37/73 and 39/73 kDa of fetal brain proteins have been specifically described as maternal antibody targets in mothers with ASD children [180,181]. In contrast, autoantibodies targeting these antigens were not detected in ASD patients [184]. Recently, seven proteins expressed in the developing brain, which are not neuronal receptors but are intracellular, extracellular and/or secreted, have been identified as fetal brain targets and they are currently used as biomarkers to predict ASD risk [185].

As discussed above, most ASD related autoantibodies that are known are transferred from the mother, yet some studies have also identified autoantibodies produced by ASD patients. These are anti-5HT receptor IgG autoantibodies [151,186], and also autoantibodies targeting non-identified antigens in the basal ganglia, prefrontal and temporal cortex, cingulate gyrus, and cerebellum [187,188] specifically against the Purkinje cells [155,189]. IgGs and IgMs against brain endothelial cells were increased in ASD patients measured by (enzyme-linked

immunosorbent assay) ELISA [190]. These findings were confirmed in another study by reactivity on brain endothelial cells where IgG autoantibodies were increased in ASD patients (50%) whereas none of the healthy children showed positive autoantibody staining and children without neurological illnesses to a lower degree (2 out of 21) [191]. A 45/62 kDa cerebellar protein was identified as a possible autoantigen by Western blot using rhesus macaque cerebellum homogenate. The presence of the autoantibodies correlated with a lower adaptive and cognitive function and aberrant behavior in children [192]. A 52 kDa protein located in cerebellar Golgi cells was identified in 21% of the ASD patients analyzed [193]. Later on, the same sera cohort was studied by IHC using the rostrocaudal extent of the macaque brain. A specific subgroup of GABAergic interneurons located in the V1 layer was identified as the specific target [194]. Controversially, another study suggested that this staining might be unspecific because immunoreactivity was detected using the serum of both healthy controls and ASD patients with the intriguing fact that the IgG seropositive ASD patients presented more severe behavior and emotional problems compared to the IgG seronegative ones [195].
Folate receptor autoantibodies have been detected by RIA in 75.3% of ASD patients [152]. Treatment with folic acid (leucovorin) has been shown to significantly improve the ASD symptoms in at least 1 out of 3 of the individuals with ASD. The role of parental autoantibodies remains unclear since the presence of FR autoantibodies in ASD children is not always related to the presence of the autoantibodies in the parents [196].

## 12. Obsessive-Compulsive Disorder (OCD) and Attention-Deficit/Hyperactivity Disorder (ADHD)

The mental disorders explained below are two of the most common neuropsychiatric diseases in early life patients. OCD patients develop pathological hoarding behaviors characterized by obsessions (recurrent intrusive thought) and/or compulsions or tics (repetitive or serotyped behaviors) like recurrent skin-piking resulting in skin lesions. ADHD is the most prevalent chronic neurodevelopmental disorder in school-age children, affecting 5%–8% [197,198] and being more frequent in boys than in girls. In two-thirds of these cases, the disease coexists with other conditions like tics or Tourette syndrome. It is characterized by hyperactivity, impulsiveness and long-lasting inattention.
The relationship between post-streptococcal infections immunity and OCD and ADHD has been widely studied. Obsessions and compulsions were observed in post-streptococcal infection and pediatric autoimmune neuropsychiatric disorders, then commonly referred to as pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) [199–201]. An animal model was established to study if these antibodies cause neuropsychiatric symptoms [202]. Plasma exchange affects the disease and removal of the autoantibodies could cause an improvement of symptoms in OCD and tic disorders in childhood [203]. In recent years, more studies revealed that anti-basal ganglia antibodies (ABGA) actually could target neuronal surface antigens. Kirvan *et al.* found that autoantibodies in PANDAS could bind to neuronal surface and caudate-putamen, which could activate CaM kinase II and cause behavioral disorders [83,204]. Brimberg *et al* first reported that autoantibodies could bind to DRD1 and DRD2 after the immunization with a streptococcal antigen which leads to neuropsychiatric symptoms in the rat animal model [205]. Lately, Lotan and colleagues reported that rats exposed to group A streptococcal antigens developed compulsive-like behavior. Serum IgG from group A streptococcal-exposed rats reacted with DRD1 and DRD2 and 5HT-2A and 5HT-2C serotonin receptors *in vitro* (determined by ELISA

and Western blot). *In vivo*, IgG deposits in the striatum of infused rats colocalized with specific brain proteins such as DR and SERT (by IHC), suggesting that the autoantibodies are the cause of the compulsive-like and motor dysfunction behavior observed in the animals [206]. Autoimmunity is, arising from an abnormal immune response, probably due to the high mimicry found between pathogens and neuronal surface epitopes involved in the dopaminergic and the serotonergic system. These findings link post-infectious autoimmunity to the onset of both OCD and ADHD. Giana *et al.* found elevated IgG autoantibody titers against DAT in the serum of ADHD children by ELISA ($n = 61$) [153], which again suggests that the dysregulation in the levels of dopamine neurotransmitter may be caused by autoantibodies in these disorders.

## 13. Conclusions/ Future Directions

NMDA-R autoantibodies can cause neuropsychiatric symptoms which can range from purely psychiatric to encephalitis with neurological symptoms. Due to the overlap in symptoms with psychotic disorders, some patients might be misdiagnosed as first-episode psychosis or even schizophrenia. We see the need for systematic screening of neuronal autoantibodies in psychotic patients, especially in the early phase of the disease to improve the diagnosis of autoimmune psychotic disorders. Preferably, hereby not only serum but also CSF would be screened because antibody titers might be low in the blood and only detectable in CSF (e.g. due to intrathecal antibody synthesis). The diagnosis of autoimmune neuropsychiatric diseases could be challenging for many clinicians and the acceptance and implementation of these diagnostic procedures differ widely between countries. The communication between the disciplines of neurology and psychiatry is of high importance in these cases because not only antibody testing but also neurological testing might improve the diagnosis of these patients. Mild neurological symptoms are frequent in patients with psychotic symptoms [207] but could be missed because they are (a) difficult to examine in the presence of acute psychiatric symptoms; (b) less attention is paid on these examinations and (c) neurological symptoms are attributed to the side effect of psychopharmacological therapy. Psychiatrists thus need to be aware of this new diagnosis in a subset of psychotic patients and education has to be provided to also perform neurological examinations or form a multidisciplinary team with neurologists (also for CSF sampling).

Still, the prevalence of these autoantibodies in cohorts of schizophrenia, bipolar disorder or other mental illnesses is not determined due to high variation in the current research results. The variable results in the field might be caused by small sample sizes, the heterogeneity in patient cohorts, the unclear distinction between the different mental disorders (especially in psychotic disorders), stage of the disease and also by methodological differences. The most commonly used diagnostic methods are the CBA and IHC on rat brain. Nevertheless, these methods are still relatively new with room for improvement. Adequate training is necessary to interpret the results and to avoid false conclusions as they have been reported before [163,208]. The IHC is limited by high background with serum stainings. The other neuronal autoantibodies described here are still lacking the final proof to confirm that they are causing a neuropsychiatric autoimmune disease. Indications for autoimmunity are that several of these autoantibodies targeting VGKC complex, mAChR and DRD2 receptor are present with the clinical manifestation of psychotic disorders including MDD, that they are detectable in the blood and CSF and also that they target a receptor, ion channel, or other protein expressed on the cell surface which is related to symptoms. To confirm the pathogenic role of isolated autoantibodies according to Witebsky's postulates, (monoclonal) antibody transfer should be

shown to replicate the disease in an animal experimental model or in humans (maternal transfer) and that elimination or suppression of the autoimmune response by therapy can prevent disease progression or reduces the clinical manifestations. To this end, larger systematic, multicenter clinical studies are necessary to reveal the prevalence of different neuronal autoantibodies in neuropsychiatric disorders and whether these patients react to immunotherapy. Additionally, it is especially important to not only analyze sera but also CSF (see above) [116]. Table 4 highlights the relevance of the discussed autoantibodies in neuropsychiatric diseases and summarizes what remains unknown. Presumably, a number of antigens involved in neuropsychiatric disorders are still not well understood demanding further work to identify novel autoantibody targets and help to better diagnose autoimmune patients' subgroups and understand disease mechanisms.

**Table 4.** Evidence of autoantibody-mediated mechanisms in neuropsychiatric disorders.

| Disorders | Targets of the Autoantibodies | Prevalence * | | *in Vitro* * | | *in Vivo* * | | Immunotherapy * | |
|---|---|---|---|---|---|---|---|---|---|
| **Psychotic** | NMDA | +/− | [122,145,146] | + | [86,114,120] | + | [137] | + | [145] |
| | VGKC complex | +/− | [144,145] | + | [93] | n/a ** | | + | [145] |
| | AMPA-R | − | [144,159] | + | [209,210] | + | [211] | n/a | |
| | D2DR | + | [148,160] | + | [129] | n/a | | n/a | |
| | HTR-1A | + | [148] | n/a | | n/a | | n/a | |
| | mAChR | + | [147,148] | + | [147,164] | n/a | | n/a | |
| | nAChR | + | [149] | n/a | | n/a | | n/a | |
| | GAD | +/− | [144,154] | − | [99] | n/a | | + | [154] |
| | FR | + | [202,212] | n/a | | n/a | | n/a | |
| **Major depressive** | OPRM1 | + | [148,150] | n/a | | n/a | | n/a | |
| | D2DR | + | [148] | n/a | | n/a | | n/a | |
| | HTR-1A | + | [148] | n/a | | n/a | | n/a | |
| | mAChR | + | [148] | n/a | | n/a | | n/a | |
| | NMDA-R | + | [146] | + | [86] | + | [137] | n/a | |
| | Rib-p | n/a | | + | [213] | + | [106] | n/a | |
| **Autism** | HTR-1A | + | [151] | n/a | | n/a | | n/a | |
| | FR | + | [152] | n/a | | n/a | | n/a | |
| | GAD | + | [155] | n/a | | n/a | | n/a | |
| **Obsessive-compulsive** | Basal ganglia | +/− | [34,214,215] | + | [204] | + | [202,206] | + | [203] |
| | D2DR | n/a | | + | [216] | + | [205] | n/a | |
| **Attention deficit hyperactivity** | Basal ganglia | +/− | [217,218] | + | [204] | n/a | | n/a | |
| | GAD | +/− | [155,219] | n/a | | n/a | | n/a | |

* All autoantibodies mentioned above have been reported in biological fluids in human subjects with neuropsychiatric disorders. Data shown here does not include autoimmune encephalitis. The pathological role of the autoantibodies has not been demonstrated for all cases; Coding is done as follows: Column for prevalence (Pre): Antibody is more frequent in the specific patients' cohorts than in healthy individuals (+), not (−), or not available (n/a); *in vitro*: Autoantibody shows toxicity to cells *in vitro* or could change the antigen function (+), not (−), or not available (n/a); *in vivo*: Animal studies show the autoantibody could cause neuropsychiatric behavior (+), not (−), or not available (n/a); Immunotherapy: Patients would benefit from immunotherapy (+); if immunotherapy was not beneficial (−), if data not available (n/a); ** n/a = not available; NMDA-R = *N*-Methyl-D-Asparte receptor; VGKC complex = voltage gated potassium channel complex; AMPA-R = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; DRD2 = dopamine-2 receptor; HTR1A = 5-Hydroxytryptamine (Serotonin) Receptor 1A; AChR = acetylcholine receptor; GAD = glutamic acid decarboxylase; FR = Folate receptor; OPRM1= opioid receptor, mu 1.

*To take home…*

- Neuronal surface autoantibodies cause neuropsychiatric symptoms in a subgroup of the patients.

- Antibody screening and neurological examinations should be implemented to improve the diagnosis of autoimmune psychotic disorders.
- Limited sample sizes, differences in patient cohorts and stage of the disease but also methodological differences generate high variation in current results.
- Common techniques, more sensitive and reproducible, are required to standardize the diagnostic tools for the different neuronal antigens across-laboratories.
- It is important to implement CSF analysis in neuropsychiatric disorder diagnosis routine since some autoantibodies are only detectable in CSF.
- More animal studies are needed to unravel the pathogenic effect of the autoantibodies in the CNS.

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**Abbreviations**

The following abbreviations are used in this manuscript:

| | |
|---|---|
| 5HT | serotonin |
| 5HT-R | serotonin receptor |
| ABGA | Anti-basal ganglia antibodies |
| ACh | acetylcholine |
| AChR | acetylcholine receptor |
| AChRγ | gamma subunit of the peripheral neuronal ACh receptor |
| ADHD | attention-deficit/hyperactivity disorder |
| AMPA-R | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor |
| ApoE | apolipoprotein E |
| ASD | Autism Spectrum disorder |
| BBB | blood-brain barrier |
| BD | bipolar disorder |
| CASPR2 | contactin associated protein-2 |
| CBA | cell based assay |
| CHRM1 | muscarinic cholinergic receptor 1 |
| CNS | central nervous system |
| CSF | cerebrospinal fluid |
| D | Disorder |

| | |
|---|---|
| DAT | dopamine transporter |
| DPPX | Dipeptidyl-Peptidase-Like Protein-6 |
| DRD2 | dopamine-2 receptor |
| dsDNA | double stranded DNA |
| FcRn | neonatal Fc receptors |
| FR | folate receptor |
| GABA | γ-aminobutyric acid |
| GABAA-R | A class of the GABA-R |
| GABAB-R | B class of the GABA-R |
| GABA-R | γ-aminobutyric acid receptor |
| GAD | glutamic acid decarboxylase |
| Glu | glutamate |
| Gly | glycine |
| GlyR | glycine receptor |
| HLA | human leukocyte antigen |
| HTR-1A | 5-hydroxytryptamine receptor 1A |
| IgG | immunoglobulin G |
| IHC | immunohistochemistry |
| LE | limbic encephalitis |
| LGI1 | leucine-rich glioma inactivated-1 |
| mAChR | muscarinic AChR |
| MDD | major depressive disorder |
| MG | myasthenia gravis |
| mGluR | metabotropic glutamate receptor |
| MHC | major histocompatibility complex |
| nAChR | nicotinic AChR |
| NMDA-R | *N*-Methyl-D-Asparte receptor |
| NMJ | neuromuscular junction |
| OCD | Obsessive-Compulsive disorder |
| PNS | peripheral nervous system |
| RIA | radioimmunoassay |
| Rib-P | ribosome P protein |
| SERT | serotonin transporter |
| SC | Sydenham chorea |
| SLE | systemic lupus erythematous |
| VGKC complex | voltage gated potassium channel complex |

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# Chapter 3

## Neuronal surface autoantibodies in neuropsychiatric disorders: are there implications for depression?

**Corresponding publication:**

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**Abstract:**

Autoimmune diseases are affecting around 7.6-9.4% of the general population. A number of central nervous system disorders, including encephalitis and severe psychiatric disorders, have been demonstrated to associate with specific neuronal surface autoantibodies. It has become clear that specific autoantibodies targeting neuronal surface antigens and ion channels could cause severe mental disturbances. A number of studies have focused or are currently investigating the presence of autoantibodies in specific mental conditions such as schizophrenia and bipolar disorders. However, less is known about other conditions such as depression. Depression is a psychiatric disorder with complex etiology and pathogenesis. The diagnosis criteria of depression are largely based on symptoms but not on the origin of the disease. The question which arises is whether, in a subgroup of patients with depression, the symptoms might be caused by autoantibodies targeting membrane-associated antigens. Here, we describe how autoantibodies targeting membrane proteins and ion channels cause pathological effects. We discuss the physiology of these antigens and their pathogenicity in relation to depression. Finally, we summarize a number of studies detecting neuronal surface autoantibodies with a special focus on those that the cohorts include depression diagnosis and/ or show depressive symptoms.

## Introduction:

Neuronal surface autoantibodies (NSAbs) have been described mainly in autoimmune encephalitis, a group of newly defined neuroimmunological disorders [1]. Those autoantibodies target essential neurotransmitter receptors, ion channels or associated proteins on the membrane of neuronal cells, such as N-Methyl-D-aspartate receptor (NMDAR) [2], α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) [3, 4], metabotropic glutamate receptor 1 (mGluR1) [5], metabotropic glutamate receptor 5 (mGluR5) [6], GABAB receptor (GABABR) [7], GABAA receptor (GABAAR) [8-10], leucine-rich, glioma inactivated 1 (LGI1) and contactin-associated protein-like 2 (Caspr2) [11], dipeptidyl aminopeptidase-like protein 6 (DPPX) [12-14], and dopamine receptor D2 (D2R) [15]. Antibody positive cases are associated with a spectrum of neurological disorders including limbic encephalitis, neuromyotonia, Morvan's syndrome, epilepsy and psychiatric disorders [16-19].

Depression is a psychiatric disorder with complex etiology and pathogenesis. The International Classification of Diseases (ICD) and The Diagnostic and Statistical Manual of Mental Disorders (DSM) are widely used for the diagnoses of this disorder, based on symptoms but not on the cause of the disease. There are several theories about the causes of depression and immune dysregulation is one of them. The relationship between the immune system and depression has been widely discussed. To date, most research has focused on pro-inflammatory cytokines and a few reviews also propose a direct link of autoantibodies and depression [20, 21]. Studies investigating the presence of autoantibodies in depression have focused on those targeting peripheral organs like the thyroid and intracellular antigens such as anti-nuclear antibodies and ribosomal-P antibodies [21-25]. During the past decade, it has become clear that NSAbs could cause severe neuropsychiatric disorders. Since some of the NSAbs interfere with neurotransmission pathways related to depression [26-28], a subtype of depression may be caused by antibody-mediated autoimmunity and therefore might potentially respond to immunotherapy. In the current review, we summarize the literature about NSAbs in autoimmune encephalitis and psychiatric disorders, with special focus on what is known regarding NSAbs in depression, evaluate the techniques used and how results can be interpreted and identify research gaps. Together, we aim to provide insight into the potential role of NSAbs in depression based on the function of relevant neurotransmitter receptors and ion channels as well as autoantibody effector mechanisms.

## How NSAbs reach the central nerves system

Because neuronal surface proteins are the target of the autoantibodies discussed in this review, it is important to first understand how those autoantibodies get access to the central nerves system (CNS). Now it is widely accepted that the CNS is targeted by the immune system, yet the mechanism of how autoantibodies go through the blood-brain barrier (BBB) is still unclear. Under normal conditions, immunoglobulins go through the BBB at a very low rate; a good example is immunoglobulin G (IgG). IgG concentration in the cerebrospinal fluid (CSF) is approximately 1% of the levels in peripheral circulation [29-31]. This indicates that once the autoantibodies reach the CNS they can cause disease as it has been observed in autoimmune encephalitis. In certain situations, like inflammation, for example, during the group A Streptococcus infection, specific Th17 cells could migrate into the brain through the cribriform plate along olfactory sensory axons. The Th17 cells expressed IL-17A which induced

endothelial tight junction breakdown, increasing BBB permeability and facilitating the penetration of IgG in the brain [32]. Additionally, the BBB may become leaky because of stroke, brain trauma, hemorrhages, microangiopathy or brain tumors, an antibody penetration rate might increase. In this regard, a study has reported that autoantibodies to NMDAR (anti-NMDAR) seropositive schizophrenia patients with a history of neuro-trauma or birth complications had more severe neurological symptoms than seronegative patients. And intravenous injections of extracted Ig fractions (IgG, IgA, or IgM) from anti-NMDAR seropositive patients to BBB leaky (ApoE−/−) mice could induce psychosis-related response [33]. A further study confirmed that APOE4 carrier status and anti-NMDAR seropositivity together was significantly associated with schizoaffective disorder [34]. Those results indicate the importance of the BBB for anti-NMDAR mediated pathology.

Besides, intrathecal synthesis is another possible source for autoantibodies in the CNS. B-cells can migrate to the brain and produce autoantibodies locally [35-37]. This is also important to keep in mind when thinking about therapy because any potential drug against B cells has to pass the BBB to be effective. The evidence is mainly from studies analyzing autoantibodies in serum and CSF from encephalitis patients. It has been reported that in some encephalitis patients, autoantibodies targeting the NMDAR, AMPAR, GABABR, DPPX, mGluR1 or mGluR5 were found only in the CSF [38]. A post-mortem study showed the presence of CD138+ plasma cells in the brain of NMDAR encephalitis patients, which support the intrathecal synthesis of antibodies [36]. Intrathecal antibody synthesis was also described in a case with autoantibodies against the mGluR1 where the patient did not respond to immunotherapy, while serum antibody levels dropped but CSF levels were still high [39]. Other NSAbs, such as autoantibodies to LGI1, Caspr2, glycine receptor (GlyR) and GABAAR may, in rare instances, be identified only in serum but be absent in CSF [38]. However, if the autoantibodies are immuno-absorbed by the antigen in the brain, they might still have effects and play a pathogenic role even they are not detectable in the CSF [40].

## IgG effector functions

Antibodies are Igs produced by B cells of the adaptive immune system. They are defined as IgM, IgG, IgA, IgD and IgE isotypes according to heavy chain C domains. Though different types of NSAbs (IgM, IgA, IgG) have been found so far, IgG type is considered the most pathogenic related [1, 10, 33]. IgG, composed of two paired heavy chain and light chain, is the major antibody in body fluid and a crucial player in the humoral immune response. In humans, four different IgG isotypes (IgG1-4) containing similar amino acid sequences exist, which have different abilities to activate the complement system. IgG1-3 mediate pro-inflammatory activities, while IgG4 has anti-inflammatory activities [41]. IgG effector functions in myasthenia gravis (MG) and other well studied autoimmune disorders are explained as a paradigm (Figure 1).

### 1. Antigenic modulation

Antibodies of the IgG1-3 subtypes are able to crosslink the antigens because of their bivalent nature whereas the IgG4 subtype loses this ability after the fab-arm exchange with other unrelated IgG4 molecules [42]. Cross-linking autoantibodies are believed to bring the antigens to close together on the cell membrane and promote the degradation of the ligand-receptor complex [43]. In the case of MG, anti-acetylcholine receptor autoantibodies (anti-AChR), mainly IgG1 and IgG3, are able to cross-link adjacent AChR molecules and lead to rapidly

internalization by endocytosis and then are degraded [44, 45]. Previous studies indicated that anti-NMDAR, IgG1-3, led to a reduction in the synaptic and extra-synaptic receptors and further decreased the synaptic plasticity and transmission [46-49]. Anti-GABAAR, IgG1, and IgG3, had a similar effect with a reduction of GABAAR clusters in both synaptic and extra-synaptic areas [8-10]. Application of anti-AMPAR (GluR1/2) to neuronal cultures significantly decreased the number of AMPAR clusters also at synaptic and extra-synaptic areas by increasing the internalization of AMPAR clusters, which could be reversed after autoantibody removal, yet the IgG subclasses were not analyzed in these studies [4, 50].

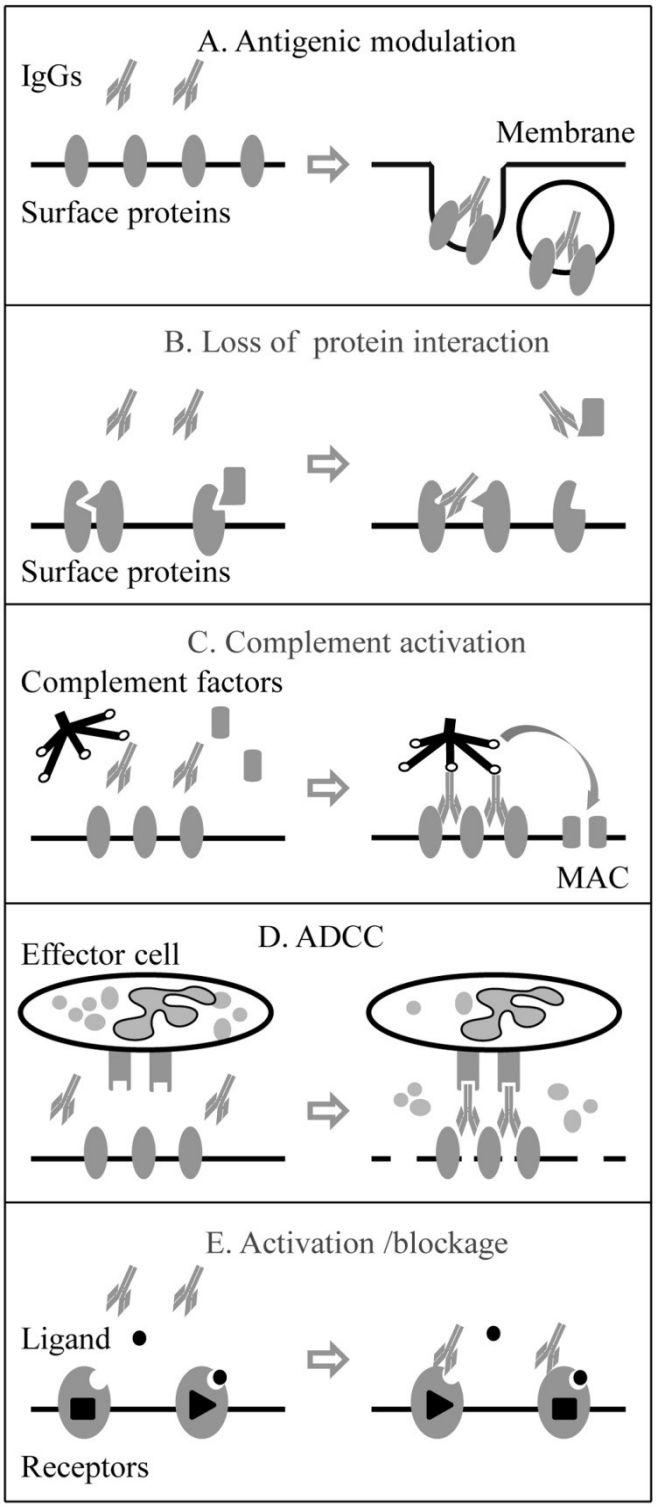


**Figure 1.** IgG autoantibody effector mechanisms. Neuronal surface proteins like G-protein coupled receptors, ion-channels and associated proteins can be the target of autoantibodies. (A) Autoantibodies can directly target surface proteins and induce their internalization by cross-linking of the antigens. (B) Autoantibodies can also target associate proteins and block protein-protein interaction. (C) Autoantibodies (IgG3>IgG1>IgG2) can activate the complement system and form the membrane attack complex (MCA) leading to damage of the membrane. (D) Autoantibodies bind to effector cell with certain Fc receptors (FcRs) can trigger antibody-dependent cell-mediated cytotoxicity (ADCC). (E) In addition, autoantibodies can be agonists or antagonists and activate or block the function of membrane receptors.

## 2. Complement activation

IgG1-3 can activate the complement system by forming the membrane attack complex (MAC) and leading to membrane damage of targeted cells. Still, in MG, anti-AChR binding to AChRs, which are densely packed in the folds of the postsynaptic membrane of the neuromuscular junction, results in a very high density of AChR bound autoantibodies and hence, a very tightly packed Fc region. The complement system is activated with high efficiency and as a result, MAC is formed in the postsynaptic membrane. Together with antigenic modulation, complement activation causes severe endplate membrane damage [44, 51]. Brain biopsy findings support that complement activation and MAC deposition happens associated with acute neuronal cell death in anti-VGKC complex encephalitis and Rasmussen's encephalitis [52, 53].

## 3. Antibody-dependent cell-mediated cytotoxicity

Antibody-dependent cell-mediated cytotoxicity (ADCC) is the process when cytotoxic effector cells (immune cells) kill the antibody-targeted cell by releasing cytotoxic granules or cell death-inducing molecules. The process is activated when the Fc receptors (FcRs) on the effector cell surface bind to the Fc region of target-bound antibodies (IgG, IgA or IgE subtypes). Those effector cells include natural killer (NK) cells, monocytes, macrophages, neutrophils, eosinophils, and dendritic cells. In humans, the IgG1 subtype has the ability to trigger ADCC and is used widely in therapy for certain types of cancer [54, 55]. Neuromyelitis Optica (NMO) is a severe inflammatory demyelinating disease in CNS and autoantibodies against aquaporin-4 (anti-AQP4), a water channel on astrocyte play a role in the pathology of NMO by triggering complement activation and ADCC [56]. In vitro, NMO patient serum and CSF IgG induced ADCC of glial cells transfected with AQP4 [57]. In vivo, anti-AQP4 produced large NMO lesions in mice, with loss of AQP4 and GFAP immunoreactivity, inflammation and demyelination. Those pathologies were largely reduced when injection of AQP4-IgG and complement to mice lacking the Fc receptor, and to normal mice injected with Fc receptor blocking antibody [58].

## 4. Loss of receptor or ion-channel associated proteins

Autoantibodies can target receptor or ion channel associated proteins. As a result, protein-protein interaction between the receptor and the associated protein is interrupted with the consequence that those receptors or ion channels become dysfunctional. Autoantibodies to muscle-specific kinase (anti-MuSK) are another type of autoantibodies involved in the pathogenicity of MG. Anti-MuSK (predominant IgG4) binds to an extracellular epitope on MuSK at the neuromuscular junction, inhibits the pathway involved in the clustering of the AChRs in the membrane and leads to failure of neuromuscular transmission [59]. Autoantibodies to LGI1, a VGKC complex associated protein, play a similar role, resulting in reduced VGKC function at CNS synapses and increased cell excitability [60]. Besides, anti-LGI1 also interferes with other surface receptors. LGI1 interacts with the ADAM22/23, epilepsy-related transmembrane proteins, and regulates AMPAR mediated synaptic transmission in the hippocampus [61, 62]. Additionally, an in vitro study shows that anti-LG11 from encephalitis patients block the binding of LGI1 to ADAM22 by neutralizing the ADAM22-binding domain of LGI1. The loss of LGI1-ADAM22 interaction could further reduce synaptic AMPAR, which indirectly associates with ADAM22 [63]. Importantly, this indicates that besides their direct effect in ion channel-receptors, autoantibodies may interfere

with protein-protein interaction and have consequences in synapse formation, function, and maintenance.

## 5. Activation, inactivation and functional receptor blockage of the receptors

Autoantibodies may activate, inactivate or block ion-channels and neurotransmitter G-protein coupled receptors [64]. Serum IgG from MG patients has been shown to block the ACh-binding sites in cultured mammalian muscle cells [65] and cased acute and severe muscle weakness in rodents inflammation or necrosis [66]. Autoantibodies against the γ subunit of the AChR which only present in embryonic forms of the receptor have been reported in some cases to block the AChR function and cause arthrogryposis congenital [67]. Anti-AMPAR (GluR3B subunit) autoantibodies (anti-AMPA-GluR3B) can activate AMPAR that contains the GluR3B subunit, leading to the spontaneous occurrence of ion currents [68, 69]. In an animal study, anti-AMPA-GluR3B produced following immunization with the GluR3B peptide, bonded cultured neurons, evoked GluR ion channel activity and killed neurons by 'excitotoxicity'[70]. When autoantibodies target G-protein coupled receptors, they can interfere with signaling pathways, which might lead to slow effector responses. An example is Graves' disease, where autoantibodies against the thyroid-stimulating hormone (TSH) receptor stimulate the synthesis of thyroid hormone which is produced in excess and results in hyperthyroidism. Additionally, there are anti-TSH receptor antibodies that block the signal transduction and consequently reduce thyroid hormone production by targeting different epitopes of the receptor [71].

## The targets of NSAbs are relevant in the pathology of depression

Monoamine imbalance is the main biochemical postulate of depression. Both serotonergic neurotransmission and dopaminergic neurotransmission play important roles in causing depressive symptoms [72]. Genetic studies suggest that certain polymorphisms within genes that encode for receptors or proteins, 1A serotonin receptor (5HT-1A) and D4 dopamine receptor, increase the risk of major depressive disorder (MDD) [73]. 5-HT 1A [74, 75] and D2DR [76, 77] levels are decreased in this disorder and both are the targets of several antidepressants [78].

Increasing evidence supports that glutamatergic and GABAergic systems are also involved in depression [27, 28]. Glutamate is the predominant excitatory neurotransmitters in the CNS [79, 80]. Blockade of glutamate uptake from the synapse has been reported to reduce sensitivity to reward, a symptom of depression [81]. Ketamine and other NMDAR antagonists have antidepressants effects [82]. Antidepressants such as imipramine can enhance the synaptic expression of GluR1, a subunit of AMPAR [83].

Interestingly, GABA concentration is reduced in cortical brain and CSF in MDD and this deficit could be reversed by chronic treatment with selective serotonin reuptake inhibitors and electro-convulsive therapy [84-86]. Studies reported that cortical $GABA_AR$ affinity and/or number were reduced in MDD. Additionally, in mouse models heterozygous for the γ2 subunit of $GABA_AR$ ($\gamma2^{+/-}$), which showed unaltered $GABA_AR$ numbers but loss of $GABA_AR$ benzodiazepine binding sites, animals exhibited a modest functional deficit in $GABA_ARs$ and anxious-depressive behavior [87, 88].

Thus, if the above-mentioned neurotransmitter receptors or relevant proteins are targeted by autoantibodies, including ion channels and associated proteins, they could potentially cause depression-like symptoms. Below, we summarize NSAbs that target antigens that are relevant in the pathology of depression (For an illustration see Figure 2).

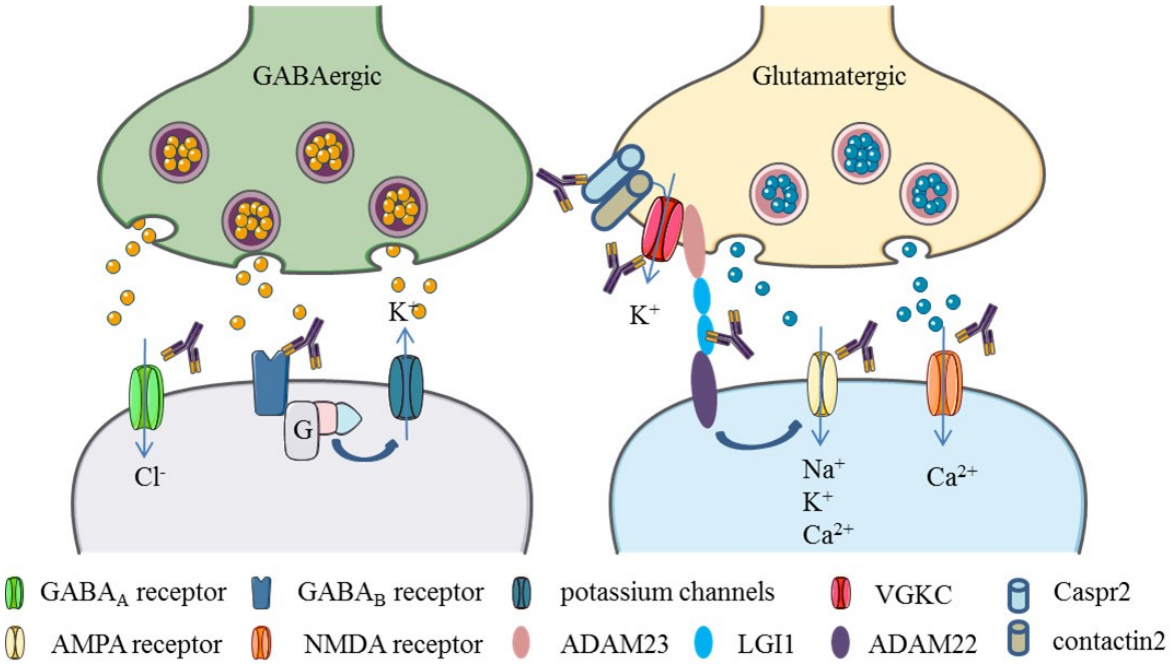


**Figure 2**. Neuronal surface autoantibodies target neuronal receptors, ion channels and/or associated proteins that commonly affect GABA and Glutamate transmission in the brain.
1. GABA receptor activation causes chloride anions influx and potassium flow-out, resulting in the hyperpolarization of the post-synaptic neurons. Autoantibodies to GABAA or GABAB receptors cause internalization of those membrane proteins and block the GABA transmission, leading to excitation of the post-synaptic neurons.
2. Glutamate receptors activation causes polarization of the post-synaptic neurons by positive ions (Ca2+, Na+, K+) influx. Autoantibodies to NMDA and AMPA receptors drive internalization of those receptors and block the glutamate transmission.
3. Potassium channels can be activated by GABA B receptors through G proteins. Some proteins like LG11 and caspr2, contactin2, ADAM22, and ADAM23 are associated with voltage-gated potassium channels. LGI1 can enhance AMPA receptor-mediated synaptic transmission by bind to ADAM22. Autoantibodies target those associated proteins would cause voltage-gated potassium channels or AMPA receptor dysfunction.

## Evidence of NSAbs in depression

### Anti-glutamate receptor autoantibodies

### Anti-NMDAR

The NMDAR, as an ionotropic glutamate receptor, contains two GluN1 and two GluN2 (A-D) subunits (alternatively called NR1 and NR2) forming heterotetramers. The subunit GluN2 can be replaced by the GluN3 (A/B) subunit, which has an inhibitory effect on receptor activity [89, 90]. NMDAR has a variety of physiological roles and any dysfunctions, either enhanced or decreased activity, may result in neuropsychiatric disorders such as schizophrenia, bipolar disorder, major depressive disorder, substance-induced psychosis, Huntington's disease, Alzheimer's disease, and neuropsychiatric systemic lupus erythematosus (NPSLE) [91]. In addition, higher gene expression levels of NR1 and NR2 (A-D) are detected in female patients with MDD [92]. Prolonged inhibition of the NMDAR by phencyclidine leads to memory loss, thought disorder, depression, and personality changes [93]. Antagonists of the NMDAR like

ketamine also have rapid antidepressant effects [94, 95]. All in all, these studies suggest that NMDAR plays a critical role in psychiatric disorders including depression.

Anti-NMDAR in autoimmune encephalitis was first described in 3 patients with ovarian teratoma and commonly presenting with psychiatric symptoms followed by neurological manifestations including seizures, movement disorder and dysfunction of the autonomous nervous system [2]. The methods used for detection were immunohistochemistry (IHC) on rat brain tissues, immunocytochemistry on live hippocampal neurons and fixed cell-based assay (CBA). The autoantibodies identified were present both in CSF and serum. Later studies revealed that the extracellular N-terminal domain of the NR1 subunit is the main epitope of those autoantibodies [96]. A case series showed that in more than two-thirds of cases with NMDAR encephalitis patients were initially seen by psychiatrists or admitted to psychiatric centers because they showed prominent psychiatric symptoms including anxiety, agitation, bizarre behavior, delusional or paranoid thoughts, and visual or auditory hallucinations [97]. Consequently, researchers broaden the search for anti-NMDAR to psychiatric disorders, mainly first-episode psychosis. Bipolar and major depressive disorders were usually included as psychiatric disorder controls. One meta-analysis indicated higher odds of anti-NMDAR in psychotic and affective disorders[98]. An affective disorder cohort consisted of 148 patients was screened for anti-NMDAR, in which 24 (16.2%) were seropositive (5 were IgG, 15 IgA, and 7 IgM). The prevalence in this cohort was higher than in healthy controls (10.8%) [34]. In this study, the method used was fixed CBA and the dilution of serum used was from 1 in 10 and titers for positive cases were double-determined in two laboratories. The results have been argued because of the much higher prevalence of anti-NMDAR in healthy control than in other groups' study results [34, 99, 100]. Further complementary investigations, using a dilution of 1:320, identified a lower percentage of positive individuals in a cohort of depression patients. Anti-NMDAR (IgG, IgA, and IgM) were found 4.1% in depression, still higher than healthy control (1.7%) at a significant level [33, 98]. The author explained the increased number of seropositive anti-NMDAR cases in affective disorder cohort by the fact that the mean age of the affective disorder group was higher than in the control group (autoantibody prevalence is generally increasing with age) [33]. Another study using the same methods found 10.6% (1.9% IgG) positive for anti-NMDAR affective disorder cohort (n=310) but no significant difference to healthy control [101]. Additionally, another study analyzed a depression cohort (n=70) and found 2 (2.9%) seropositive patients for NMDAR (both IgA) and 1 seropositive (0.4%) (IgM) result in healthy control (n=230) but none of them were IgG [100]. Repeat experiment was performed and higher seropositive cases were found both in health and disease group[102]. Early studies by Dickerson et.al [103] (ELISA, Using a peptide of NR2, n=28) and Zandi et.al [104] using variations of the methodology (live CBA) did not report any positive results in a depression cohort. Passive transfer of anti-NMDAR (NR1) to mice could cause depressive-like symptoms [105]. However, the correlation of symptoms in animal models with those observed in humans needs to be further demonstrated [106].

In contrast to anti-NMDAR in autoimmune encephalitis which mainly targets the NR1 subunit, Lapteva and colleagues found that autoantibodies targeting the NR2 subunit of NMDAR were associated to depression in systemic lupus erythematosus (SLE) patients [107]. In fact, anti-NR2A/B autoantibodies were thought to be a subset of the anti-double-stranded DNA (dsDNA) antibodies [108]. The epitope identified to be targeted by the antibodies in this study was a pentapeptide Asp/Glu-Trp-Asp/Glu-Tyr-Ser/Gly. This sequence present on the NR2A/B subunit is a mimotope of anti-dsDNA. This was confirmed by showing that affinity-purified

antibodies from SLE patients targeting this peptide also bind to dsDNA [108, 109]. Moreover, those autoantibodies mediated apoptotic death of neurons in vivo and in vitro [108]. Several studies have investigated the role of anti-NR2 in NPSLE and found that the antibody may lead to dysfunction of NMDAR *in vitro*, and that passive transfer of anti-NR2 in animals induced neuronal apoptosis and affects animal memory and cognitive ability [110, 111].
Anti-NMDAR autoantibodies in depression are still questionable since most of these studies considered the depression cohorts as a control group and numbers were relatively small. Variations in the methodology make it difficult to compare results from different groups, which is a common fact that should be kept in mind through this review. In particular, the methodology varies among studies (CBA or ELISA), or the same methodology is used following different experimental conditions (fixed or live CBA) by different groups, different subunits of the antigens are employed (NR1, NR1, and NR2a/b together in CBA, NR2 peptide in ELISA), different body fluids (serum, plasma or CSF), different immunoglobulins detected (IgG, IgA, and/or IgM) and different dilutions of the sample used (from 1:10 to 1:320) [17].

**Anti-AMPAR**

AMPAR is another ionotropic glutamate receptor which mediates the fast excitatory neurotransmission in the CNS [112]. The majority of AMPAR are tetramers composed of two GluR2 and either two GluR1, 3 or 4 subunits that combine in a brain region-dependent manner [113, 114]. GluR1/2 and GluR2/3 receptors are highly expressed in the synaptic CA3-CA1 areas of the hippocampus. Besides, they are also expressed in the cerebellum and caudate-putamen [115].
Lai and colleagues first reported autoantibodies to AMPAR (GluR1 and GluR2 subunits) in limbic encephalitis [4]. The clinical feature of this type of autoimmune encephalitis is short-term memory deficits, emotional/behavioral changes, and seizures, often paraneoplastic, treatment-responsive, and has a tendency to relapse[4]. GluR3 has been identified as an autoantigen in Rasmussen's encephalitis which the clinical characteristics of these patients were mainly epilepsy and language problems [116, 117]. An anti-AMPAR (GluR1) positive case was reported with breast ductal infiltrating adenocarcinoma that showed behavioral changes, depressed mood, and memory loss during the process of the disease without seizures [3]. In contrast, screening for anti-AMPAR (GluR1 and GluR2) in a depression cohort (n<380) by fixed CBA using 1:10 diluted serum did not report any positive cases [100, 101].

**Anti-GABA receptors autoantibodies**

**Anti-$GABA_AR$**

$GABA_AR$ is an ionotropic receptor and GABA is the ligand. There are several subunit isoforms (α, β, γ) for the $GABA_AR$, which determine the receptor's agonist affinity, the chance of opening, conductance, and other properties. Subunits of $GABA_AR$ have a different distribution in the brain and may respond with different sensitivity to GABA, leading to a different function. A decline in $GABA_AR$ signaling triggers hyperactivity in neurological disorders such as insomnia, anxiety, and epilepsy.
Autoantibodies to $GABA_AR$ were recently identified in autoimmune encephalitis. The clinical feature varies in different studies. Petit-Pedrol et al. reported a series of 18 patients with anti-$GABA_AR$, of whom 6 had high titer antibodies detected both in blood and CSF and showed

severe encephalitis and refractory seizures [8]. The other patients with lower titers in serum had different diagnoses. Six showed encephalitis with seizures, four had the stiff-person syndrome, and two had opsoclonus-myoclonus. Anti- $GABA_AR$ in lower titers was also found in 5 of these 12. The autoantibodies targeted α1 and β3 subunits and caused selective reduction of the synaptic $GABA_AR$ [8]. 2 anti-$GABA_AR$ encephalitis patients were reported and their autoantibodies targeted the β3 subunits [9]. Later, a case study identified the main antigens as α1/γ2 in a group of patients with seizures and cognitive or neuropsychiatric problems. Some of these patients had mood changes (2 in 11 showed depression symptoms and the autoantibodies targeted to α1 or undefined; 3 showed anxiety and the autoantibodies targeted to α1, γ2 or undefined subunits) [10]. A cohort of purely depression disorders has not been tested so far.

**Anti-$GABA_BR$**

$GABA_B$ receptors are metabotropic transmembrane receptors that are linked to G-proteins gated potassium channels [118]. There are two $GABA_B$-receptor subtypes, $GABA_{B1}R$, and $GABA_{B2}R$, assembling into functional heterogenic complexes [119, 120]. $GABA_{B1}R(-/-)$ mice, which lack functional GABA(B) receptors, showed more anxiety and decreased immobility (antidepressant-like behavior) and $GABA_BR$ selective antagonist CGP56433A showed anti-depressant effects as well [121].
Autoantibodies to the $GABA_BR$ (anti- $GABA_BR$) were reported in limbic encephalitis (15 in 410 cases) [7]. In all patients, autoantibodies to $GABA_BR$ targeted the $GABA_{B1}R$ and only one targeted $GABA_{B2}R$ [122, 123]. If anti-$GABA_BR$ inactivates synaptic and extra-synaptic $GABA_BR$, it could potentially cause anxiety but not depression. Additionally, one anti-$GABA_BR$ (B1/B2) positive was found in a depression cohort ($n<310$) by fixed CBA using 1:10 diluted serum with all the controls being seronegative ($n>1693$) [101]. To date, there are only limited studies that focus on this antigen and further investigations should be performed to extend the knowledge about autoantibody effector mechanisms.

**Anti-Monoamine receptors autoantibodies**

**Anti-5HT 1A receptor and Anti-D2 antibodies**

5-HT1A receptor is a subtype of serotonin receptor expressed widely in the limbic system and has implications in the control of mood, cognition, and memory [124]. D2R is a dopamine receptor and has long isoforms (located mainly on the post-synaptic membrane) and short isoforms (mainly on the pre-synaptic membrane), coded by alternative splicing of the same DRD2 gene [125]. It's highly expressed in basal ganglia and also cortex, hippocampus and in the area of the substantia nigra and is involved in synaptic plasticity and memory formation [126]. Both receptors are coupled with G-proteins that inhibit adenylyl cyclase, as well as other second messenger cascades [124, 127].

The presence in serum of IgG autoantibodies against 5HT-1A (anti-5HT1A) and dopamine receptor $D_2$ (anti-D2R) in psychiatric disorders was studied by radioimmunoassay (RIA) [128]. 7.9% of the mood disorder patients including 33 MDD had anti-5HT1A and 9.5% had anti-D2R compared to healthy controls which were seronegative. Anti-D2R was significantly associated with the severity of guilt feeling and depressive mood. To our knowledge, no further experiments have been reported detecting or investigating the role of anti-5HT1A in psychiatric disorders.

IgG autoantibodies against D2R were identified by flow cytometry CBA with a cut-off at three standard deviations above the control mean using transfected HEK cells in a subgroup of children with basal ganglia encephalitis [15]. 12 of 17 children (aged 0.4–15 years, nine males) with basal ganglia encephalitis had anti-D2R, compared with 0 in 67 controls. The 12 anti-D2R positive patients had movement disorders and psychiatric disturbance characterized by Parkinsonism, dystonia, chorea, emotional lability, attention deficit, and psychosis. A later study showed a specific and significant reduction of D2R when transfected cells were incubated with anti-D2R and the extracellular N-terminus of D2R was revealed as the main immunogenic region [129]. 3 anti-D2R positive cases out of 43 were reported in the first episode of acute psychosis in children and the 17 controls studied were seronegative [130]. This is the first report of serum IgG autoantibodies to surface D2R in pediatric patients with isolated psychosis. And 3 of the patients were previously diagnosed with other types of mental disorders: one patient had attention-deficit/ hyperactivity disorder, behavior disorder, one had depression and anxiety, prematurity and one had anorexia nervosa [130].

**Anti-VGKC complex and associated proteins autoantibodies**

**Anti-LGI1, anti-Caspr2, and anti-DPPX**

Voltage-gated potassium channels (VGKC), typically formed by 4 different α subunits (there are 40 α subunits known), each associated with a β subunit (more than 12 β auxiliary proteins to α subunits), play a crucial role in returning the depolarized cell such as neurons to a resting state [26, 131]. Typically, they are tetramers of four certain α subunits arranged as a ring, each contributing to the wall of the transmembrane K+ pore. Additionally, there are other associated proteins like LGI1, Caspr2, Contactin2, ADAM22, and ADAM23, which can affect the function of VGKC and AMPAR (mentioned in the antibody effector function section) [132].

Autoantibodies to the VGKC complex (anti-VGKC complex) have been known for a long time and are involved in the pathogenesis of neuromyotonia, Morvan's syndrome, epilepsy and limbic encephalitis [26, 133, 134]. In recent years, researchers identified by CBA and IHC that the VGKC associated proteins LGI1 and Caspr2 are actually the main targets in autoimmune encephalitis. Kv4.2, a subtype of VGKC, is widely expressed in the CNS and autoantibodies directed against DPPX (an auxiliary subunit of Kv4.2 channels) (anti-DPPX) was also identified, yet in approximately 19% of the seropositive cases for the VGKC complex by RIA the antigen/s remain unknown [11, 14]. Epilepsy and limbic encephalitis are more frequently related to anti-LGI1, while peripheral nerve hyperexcitability disorders, like Morvan's syndrome, are more common in anti-Caspr2 positive cases [135]. Anti-LGI1 patients present a clinical spectrum of confusion, depression, paranoia, behavior disturbances, visual hallucinations, and dementia at onset of the disease [136-138]. 2 seropositive (one IgG type) anti-Caspr2 were found in 310 affective disorders, while in the same study, none anti-LGI1 and anti-DPPX seropositive cases were reported [101]. The largest described cohort of anti-DPPX (IgG) positive patients consists of 20 cases. Those sera or CSF positive cases were found in patients referred for evaluation of paraneoplastic neurologic autoimmunity (totally tested 83) and 41,812 samples submitted for evaluation of neural autoantibodies (0.02% positive anti-DPPX). Out of the 20 anti-DPPX positive patients, 20% showed depressive symptoms [14].

## Take-home message

Although an increasing number of studies have substantially improved our knowledge on autoimmunity in the CNS, still large controversy exists, especially due to the variation in the methodology used. Also, our knowledge is largely based on findings from autoimmune encephalitis cohorts. There are several methodological aspects which have to be considered when detecting NSAbs in psychiatric disorders, especially in depression or other mood disorders. Firstly, the antigens targeted by the autoantibodies can be composed of several subunits. Autoantibodies against each of the subunits can have different clinical significance and implications [1]. A good example is the detection of NMDA NR1 antibodies and N2A/B antibodies. Anti-NR1 is believed to be pathogenic in NMDAR encephalitis [96]. However, anti-N2A/B plays a role in NPSLE [107]. When autoantibodies target different subunits of other glutamate receptors or GABA receptors, they may cause different clinical symptoms. At the same time, most NSAbs target epitopes only if the antigens are expressed in their native conformation. Techniques like CBA, IHC of brain sections optimized to detect membrane proteins (rodent), and immunocytochemistry of cultures of rodent live hippocampal neurons fit this requirement. Thirdly, different concentrations of the same autoantibody might have different effects and biological relevance. For example, high titers of anti-$GABA_AR$ are specific for severe encephalitis and refractory seizures patients and low titers present in a broad range of neurology disorders and may lack specificity [8]. Another aspect which needs to be taken into account is the value of serum and CSF for detecting autoantibodies. The use of CSF for detecting NSAbs in depression has not been evaluated to date. Finally, NSAbs should be tested in a ‘‘panel’’ rather than a single one because of the overlap between symptoms and signs of different autoimmune encephalitis and psychotic disorders [139]. Also, the coexistence of several NSAbs occurs in the same individual and cause combine manifestations [9, 140, 141].

To summarize, NSAbs, targeting important neuronal receptors or interfering with ion channels and associated protein function, are responsible for psychiatric symptoms in autoimmune encephalitis cases. At the moment, several studies reported the presence of anti-NMDAR (NR1 and NR2B), anti-5-HT1A and D2R in depression cohorts. However, due to the heterogeneity of the methodology, variation in the samples used and the limited cohort size, there is insufficient evidence to support those NSAbs can cause depression without other obviously neurological symptoms. In the future, large cohorts, longitudinal studies need to be performed using sensitive, quantitative and reproducible methods without loss of antigen conformation. Finally, analysis of autoantibodies targeting neuronal surface antigens relevant to the pathology of depression should be performed.

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# Chapter 4

## Novel neuronal surface autoantibodies in plasma of patients with depression and anxiety

Shenghua Zong, Carolin Hoffmann, Marina Damas et.al., ***Submitted to JAMA Psychiatry***

## Abstract

**Background:** Certain autoantibodies targeting ion channels and membrane receptors cause autoimmune encephalitis and present with neuropsychiatric symptoms. Psychiatric symptoms are increasingly recognized as potentially of autoimmune origin. However, still little is known about the prevalence of neuronal surface autoantibodies in neuropsychiatric disorders especially in depression or anxiety.

**Objective:** We aimed to determine the prevalence of neuronal (surface) autoantibodies in plasma of patients with depression or anxiety as in sera of healthy control subjects and investigate if they correlate with certain subgroups.

**Methods:** Plasma from 2231 participants, including lifetime depression or anxiety patients (n=1739, 819 were currently diagnosed and 920 were in remission) and controls without these disorders (n=492), were tested by a combination of immunohistochemistry (IHC) on rat brain, cell-based assay (CBA) and staining on live neurons to investigate if they had neuronal autoantibodies especially neuronal surface autoantibodies (NSAbs). The prevalence of these autoantibodies between disorder groups (with a focus on current disorders) and controls was compared.

**Results:** Overall, 106 samples (4.8%) showed reactivity to brain tissue by IHC including 56 samples with a borderline score, 42 were weak positive and 8 were strong positive. There was no difference between disorder groups and controls. All the 106 samples were further tested by CBA and staining on live neurons. Only two had known neuronal autoantibodies by fixed CBA. 8 samples were positive in the live neuronal test targeting unidentified antigens, 7 from individuals with a current disorder (all had current anxiety, 2 also had current depression), 1 from the control group, and none from patients in remission (7/44 vs 1/25 vs 0/37, p=0.018).

**Conclusion:** The prevalence of known neuronal autoantibodies in patients with anxiety/depression is practically zero. However, novel autoantibodies might relate to patients with current disorders, which needs further investigation.

## Introduction:

Depression and anxiety disorders are among the most common illnesses in the community and in primary care, the economic cost of these which ranks among the top-five of all diseases [1, 2]. The diagnosis relies on symptomatology and questionnaires following the classification from the Diagnostic and Statistical Manual of Mental Disorders (DSM, currently version 5) and the International Statistical Classification of Diseases and Related Health Problems (ICD, currently version 11). However, the causes of these disorders are diverse and still poorly understood. The high prevalence of depression and anxiety in autoimmune diseases [3-6] suggests that those psychiatric disorders may be linked to autoimmunity [7]. Moreover, immune dysregulation has been observed directly in people with depression or anxiety disorders as well [8]. Previous studies on autoimmune encephalitis indicate that certain neuronal surface autoantibodies (NSAbs) relate to isolated symptoms of psychosis [9]. Those pathogenic autoantibodies mainly target neurotransmitter receptors, ion channels or associated proteins, which cause the disruption of the target antigens and lead to dysfunction of neural signal transduction in most of the cases [10]. It is well known that neurotransmitter transporters or receptors are involved in the pathology of depression and anxiety while they are also the targets of many anti-depressants [11, 12]. The question arises as to whether NSAbs can cause depression or anxiety when they target specific neuronal surface proteins that are probably involved in these disorders [13-15].

Previous studies in psychiatric diseases have mainly focused on NSAbs (especially anti-NMDA receptor antibodies) in psychosis or bipolar disorders [16-18] but the possible role of neuronal autoantibodies in depression and anxiety has received little attention. This is probably due to the fact that symptoms of depression and anxiety, are considered less severe or specific than psychotic symptoms which are commonly seen in the early stage of anti-NMDA receptor encephalitis [19]. A few studies included depression or anxiety cohorts but failed to reveal the specificity of the detected neuronal autoantibodies to these disorders [20, 21]. The limited cohort size or single autoantibody detection method used without further validation makes the results inconclusive.

In the clinic, neuronal autoantibodies to diagnose autoimmune encephalitis are usually detected by commercially available fixed CBA or tissue-based assays, which are used to detect autoantibodies to known, well-defined antigens. Live CBA, which in theory could preserve conformationally sensitive epitopes, is also used in some research laboratories and shows a higher chance to detect NSAbs in psychosis. Its specificity, however, remains debated [22]. When extending neuronal autoantibody detection from neurological disorders to new disorders (as in this case depression or anxiety), using a method that can cover novel neuronal antibodies would be preferable. Therefore, immunohistochemistry (IHC) using rat brain tissue optimized for the preservation of cell-surface antigens would be a good option [23].

In this study, we detected neuronal autoantibodies with a focus on NSAbs in the plasma of a large cohort of depression and anxiety as well as control individuals. We followed a procedure combining in tandem different methods including IHC on rat brain, live and fixed CBA and staining on live-cultured hippocampal neurons (Figure 1). We aimed to determine the prevalence of neuronal autoantibodies especially NSAbs in depression and anxiety and whether they correlate with certain disorder subgroups

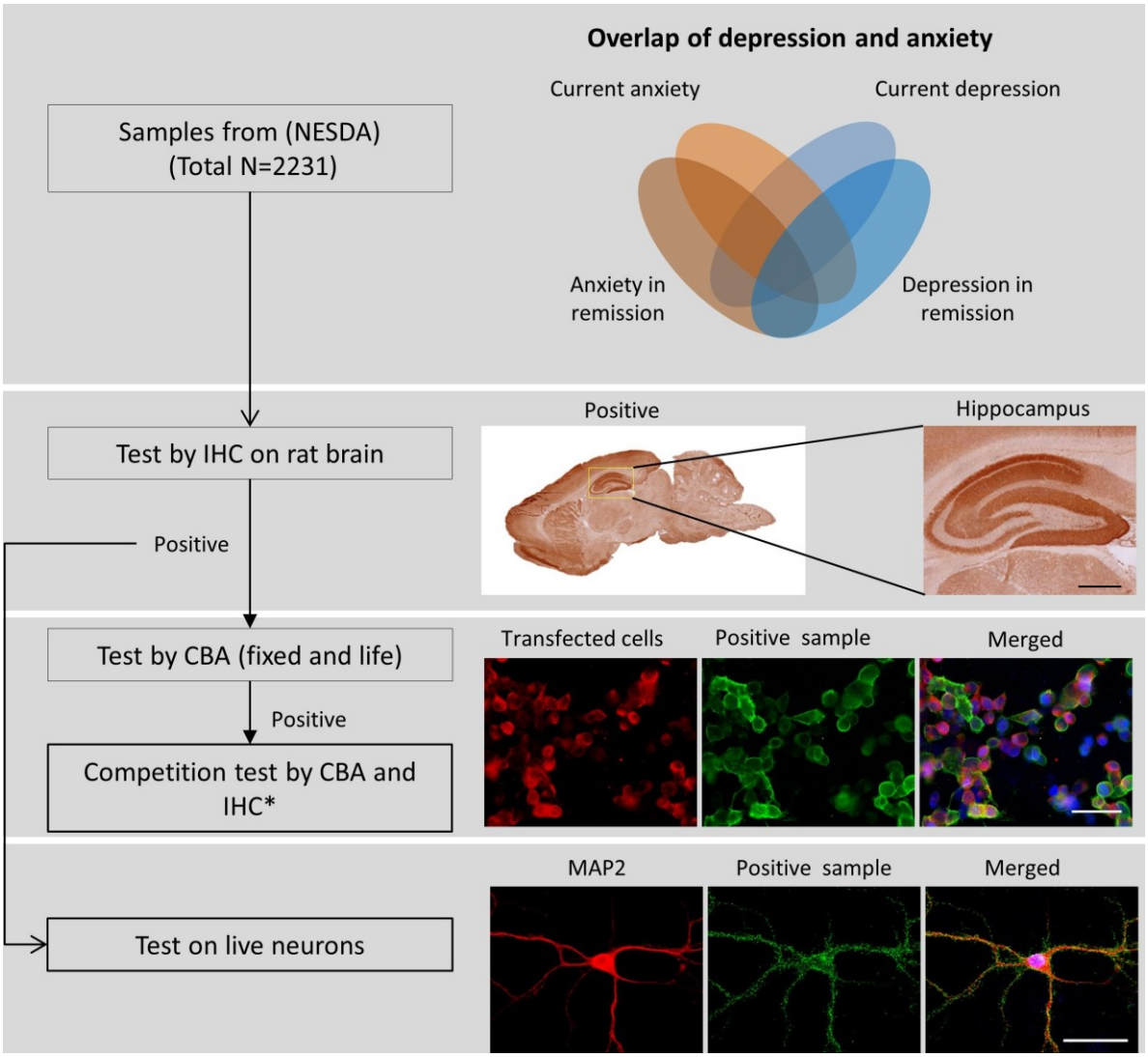



**Figure 1.** Working flow chart for neuronal surface autoantibody detection in plasma from the NESDA wave 3 (n=2231). Samples were first tested by immunohistochemistry (IHC) on rat brain. Then IHC positive samples were tested by cell-based assay (CBA) to 8 known neuronal antigens and live-cultured hippocampal neurons to check if they had known neuronal autoantibodies or novel neuronal surface autoantibodies. The image on the top right shows the basic overlap between depression and anxiety disorders among the individuals tested. The images below show examples of positive results for each method. The left image in row 2 is the IHC staining on whole rat brain (sagittal) given by an NMDAR autoantibody-positive sample from an encephalitis patient and the right image shows the details of a hippocampus staining pattern, scale bar = 500 µm. Images in row 3 show HEK cells transfected with GABAB receptors (red) and serum from an encephalitis patient giving strong staining on the transfected cells (green and merged), nuclei are stained in blue with DAPI, scale bar = 50 µm. Images in the last row show a neuronal marker MAP2 (red) and serum from an encephalitis patient with anti-DPPX autoantibodies giving strong staining on a neuron (Green and Merged). Nuclei were stained in blue with DAPI, scale bar = 50 µm.

## Material and methods

### Participants and samples

The Netherlands Study of Depression and Anxiety (NESDA) is an ongoing longitudinal cohort study designed to investigate the course of depression and anxiety disorders over a period of several years. From 2004-2007, 2981 participants aged 18 through 65 years were recruited with and without symptoms from primary care practices and specialized mental health institutions in the regions of Amsterdam and Leiden, and in the provinces of Groningen, Drenthe and Friesland of the Netherlands (https://www.nesda.nl). The whole cohort consists of persons with a current or remitted depression and/or anxiety disorder and controls without these disorders. The Composite Interview Diagnostic Instrument– lifetime version 2.1 – was used to diagnose depression and anxiety disorders according to Diagnostic and Statistical Manual of Mental Disorders – Fourth Edition (DSM-IV) algorithms. The focus is on Dysthymia, Major Depressive Disorder, Generalised Anxiety Disorder, Panic Disorder, Social Phobia, and Agoraphobia. To maintain a representative cohort, only two exclusion criteria existed: (1) a primary clinical diagnosis of a psychiatric disorder not subject of NESDA, which will largely affect course trajectory: psychotic disorder, obsessive-compulsive disorder, bipolar disorder or severe addiction disorder, and (2) not being fluent in Dutch since language problems would harm the validity and reliability of the collected data. A detailed description of the NESDA study design and sampling procedures can be found elsewhere [24]. The research protocol was approved by the ethical committee of participating universities and all respondents provided written informed consent.

2231 respondents' plasma samples from the two-year follow-up (the latest samples available when this sub-study was proposed) were tested initially for neuronal autoantibodies with a focus on NSAbs. From patients who had NSAbs. Plasma samples were stored at -80 ˚C. Aliquoted samples were kept at -20 ˚C during the tests. All the participant data was retrieved from NESDA after the tests (Table 1). In brief, of the 2231 respondents, 35% were male, 65% female. The mean age was 44.5 years (range from 19-68 years old). The group with current depression or anxiety disorder was defined as having a depressive and/or anxiety disorder in the past 6 months which was based on self-reporting, interview, and questionnaires. The remitted group consisted of patients in whom the last episode ended more than 6 months ago. 37% (819) of respondents had either a current depression or anxiety, or both in the past 6 months, including 211 had only current depression, 299 had current anxiety and 309 had both. 41% of respondents had a remitted depression and/or anxiety, and 22% of respondents were completely free of depression and anxiety.

### Serological Analyses

### Immunohistochemistry (IHC)

To test for the presence of autoantibodies to neuronal antigens, 2240 plasma samples from 2231 individuals were tested for the presence of autoantibodies against rat brain by IHC. All the information of those individuals including grouping and replicates was blinded during the test. Briefly, fresh adult rat brain (Lewis, male) were fixed in 4% paraformaldehyde for 1 hour, dehydrated in 40% sucrose for 48 hours, frozen in liquid nitrogen and cut into five to seven-micrometers-thick sagittal serial sections. These sections were serially incubated with 0.3% $H_2O_2$ for 15 minutes, 5% goat serum for 1 hour, 200 ul plasma from the NESDA cohort (1:200 diluted in 5% goat serum) overnight at 4 °C and with biotinylated goat anti-human IgG Fcγ (1:3200 in 5% goat serum, Jackson laboratory, #109-066-008) for 2 hours, each step was followed by washing 3 times with PBS. The reactivity was visualized using the avidin-

## Table 1. The demographic characteristics of the NESDA cohort (at the 2-year follow-up )

| | Current depression / anxiety[1] (n = 819 ) | Remitted depression / anxiety[2] (n = 920) | Control group (n = 492) | P value** |
|---|---|---|---|---|
| Mean age (SD) | 44.9 (12.3) | 44.5 (13.2) | 43.6 (14.6) | 0.218 |
| Age range (y) | 19-66 | 19-68 | 20-66 | |
| Female (%) | 552 (67.4%) | 606 (65.9%) | 291 (59.1%) | 0.008 |
| Subgroups | | | | |
| Depression | 520 (63.5%) | 758 (82.4%) | -- | |
| Major depressive disorder | 475 (91.3%) | 745 (98.2%) | -- | |
| Dysthymia | 198 (38.1%) | 210 (27.7%) | -- | |
| Anxiety | 608 (74.2%) | 612 (66.5% ) | -- | |
| Panic disorder with agoraphobia | 107 (17.6%) | 130 (21.2%) | -- | |
| Panic disorder without agoraphobia | 140 (23.0%) | 149 (24.3%) | -- | |
| Social Phobia | 306 (50.3%) | 266 (43.4%) | -- | |
| Generalized anxiety disorder | 166 (27.3%) | 259 (42.3%) | -- | |
| Agoraphobia without panic disorder | 125 (20.6%) | 109 (17.8%) | -- | |
| Using psychiatric medication in recent 2 years* | 324 (39.6%) | 195 (21.1%) | 23 (4.7%) | |
| Somatic diseases* | | | | |
| Diabetes | 52 (6.3%) | 45 (4.9%) | 17 (3.5%) | 0.066 |
| Stroke | 12 (1.6%) | 17 (1.8%) | 12 (2.4%) | 0.424 |
| Arthritis or arthrosis | 193 (23.6%) | 160 (17.4%) | 86 (17.4%) | 0.002 |
| Chronic none specific lung disease | 118 (14.4%) | 110 (12.0%) | 45 (9.1%) | 0.019 |
| Rheumatism (fibromyalgia, SLE, rheumatoid arthritis) | 87 (10.6%) | 53 (5.8%) | 14 (2.8%) | <0.0001 |
| Tumor | 50 (6.1%) | 64 (7.0%) | 27 (5.5%) | 0.546 |
| Ulcer | 16 (2.0%) | 10 (1.1%) | 2 (0.4%) | 0.047 |
| Intestinal disorders | 166 (20.3%) | 134 (14.6%) | 35 (7.1%) | <0.0001 |
| Allergies (Hay fever, Eczema) | 267 (32.6%) | 312 (33.9%) | 140 (28.5%) | 0.217 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Thyroid disease (Graves, hyperthyroid) | 35 | (4.3%) | 35 | (3.8%) | 17 | (3.5%) | 0.764 |
| Head injury | 20 | (2.4%) | 22 | (2.4%) | 5 | (1.0%) | 0.153 |
| Sickness one week prior to blood drawn* | | | | | | | |
| Fever | 38 | (4.6%) | 42 | (4.6%) | 18 | (3.7%) | 0.691 |
| Cold | 233 | (28.4%) | 252 | (27.4%) | 135 | (27.4%) | 0.873 |

1. Depression and /or anxiety present in the six months prior to assessment.
2. Lifetime depression and/or anxiety diagnosis, but not in the six-months prior to assessment.
*The number is depended on questionnaires when the answer is 'yes'.
** t-result is used for comparing the age difference between groups and chi-square test is used for comparison of gender, somatic diseases, and sickness prior to blood drawn between different groups.

biotin-peroxidase (Vector laboratory, Inc., # PK 6100) method. After dehydration, slides were mounted using DPX (Klinipath, #C933401) or Entallen (Millipore Sigma, #1.07961.0100). If tissue was damaged during the procedure, the staining was repeated and analyzed again. Each staining included a positive control serum known with anti-NMDAR or anti-AMPAR or Anti-DPPX autoantibodies from a patient with autoimmune encephalitis and a negative control from a healthy individual.
After the staining, slides were scanned by VENTANA iScan HT scanner at 20 times resolution and the images generated were scored from 0 to 3 and "inconclusive" by an experienced observer using Ventana Image Viewer (Vision 3.1.4) according to patterns and staining density on the hippocampus (nuclei staining was not taken into account). Generally, when there was an absence of staining in the hippocampus similar to healthy control, it was considered negative and a score 0 was given. When there was a clear pattern observed, it was considered positive and a score was given according to the staining intensity. Positive samples were scored from 1 to 3 depending on the intensity of the staining, from less to more intense, whereby a score 3 was given if the intensity was similar to the positive control. If the staining was blurry or quality was otherwise impaired, it would be considered 'inconclusive'; Plasma considered ≥ 1 or inconclusive at the first round of staining was repeated to validate the staining results and evaluated by 2 experienced observers independently. If two rounds of staining for one certain sample resulted in the same score, it was considered the final score. If the scores were different between experiments, the staining was repeated at least once more and a final score was given according to all the pictures available for this sample. Those with score 0 were considered negative, 1 was considered borderline, 2 was considered as weak positive and 3 was considered as strong positive.
Autoantibodies titers were tested by using diluted samples with dilution factor 2 (from 1:200 till 1:25600) staining on rat brain slices. The titer was determined when the diluted sample was still positive at the lowest dilutions. For example when a sample is positive at the dilution of 1: 3200 but become negative at 1:6400, then the autoantibody titer of this sample is defined as 3200. A similar strategy was used in the methods of CBA or staining on live cultured neurons.

**Cell-based assay**

**Fixed cell-based assay:**

To test autoantibodies to known neuronal antigens, including NMDAR, AMPAR, GABAAR, GABABR, LGI1, Caspr2, GAD65 and GAD67, HEK cells were transfected with plasmids carrying the recombinant cDNA of those proteins. The sources of the plasmids used were detailed as follows. Clones containing full-length human cDNA sequences coding for the GRIN1 (NM_000837.1) and GRIN2B (NM_000834.4) receptor were obtained from the Thermofisher EST collection (Thermofischer Scientific). GRIN1 was digested with Psil and GRIN2B was digested with EcoRI, and were cloned into pcDNA 3.1 digested with EcoRV and transformed in DH10B cells (NEB, C4040-03). Both plasmids where sequenced (GATC Biotech) and confirmed to correspond with the GRIN1 and GRIN2B reference sequences. For the live CBA we used human GRIN1 in pIRES-eGFP[25] that was kindly provided by Fabienne Brilot-Turville (University of Sydney). Human AMPAr was expressed from human GluR1 (pTriEx1backbone) and GluR2 (pDest-40 backbone) and human GABABR from GABBR1(pDest-40 backbone) and GABBR2 (pTriEx1backbone). Human LGI1 was cloned in frame with the transmembrane region of Caspr2 into pcDNA3 to generate membrane bound LGI1 and with mCherry for the live CBA (pIRES2-DsRed2 backbone). The cloning details were described previously [26]. These 6 plasmids were a kind gift from Patrick Waters (University of Oxford). GABAAR plasmids were obtained from Erdem Tuzun (Istanbul

University, Turkey)[27] and expressed the human alpha1, beta2, and gamma1 subunit. Human Caspr2 was received from Catherine Faivre-Sarrailh (CNRS, Marseille)[28] with a pcDNA3 backbone with a mCherry [29] tag for the live CBA and without the tag for fixed CBA. The GAD plasmids expressed human GAD65 and GAD67 from the pCMV6-XL5 plasmid which was a kind gift from Francesc Graus (IDIBAPS, Barcelona) [30].

HEK293 cells were plated on coverslips and transfected with 4 µg expression vectors of the according to antigens and expression allowed for 22-26 hours (h). Cells were fixed in 3.6% formaldehyde (TAAB, #F006,) for 10 minutes and permeabilized with 0.3% Triton-X-100 for 10 min. After blocking with 1% bovine serum albumin (BSA) for 1 h, cells were incubated with human sera diluted 1:40 in 1% BSA together with an antibody targeting the according antigen for 1 h at room temperature (20 °C). A complete overview of the antibodies used can be found in supplementary Table 1. Staining was visualized using the corresponding secondary antibodies. The screening always included a positive control from an autoantibody-positive patient and a negative control individual of human serum except for the anti-GABAAR staining in which no positive control was available. Cover glasses were mounted onto 7 µl DAPI mounting medium (Vector laboratories, #H-1200) and evaluated by two (of which one blinded) trained observers independently on a BX51 Olympus microscope for antibody reactivity.

**Supplementary Table 1: Antibodies used for cell-based assay**

| Antibody | source | dilution |
|---|---|---|
| anti-GluN1 | #PAB12310, Abnova | 1 : 500 |
| anti-LGI1 | #AB30868, Abcam | 1 : 1000 |
| anti-Caspr2 | # AB33994, Abcam | 1 : 1000 |
| anti-GABAAR | #75136, Antibodies Incorporated | 1 : 20000 |
| anti-GABABR | #sc14006, Santa Cruz Biotechnology | 1 : 500 |
| anti-GAD65 | 7309LB, Christina Hampe, (University of Washington) | 1 : 1000 |
| anti-GAD67 | 10266/20B, Christina Hampe, (University of Washington) | 1 : 1000 |
| goat-anti human-IgG-Alexa488* | # A11013, invitrogen | 1 : 1000 |
| donkey-anti-rabbit-Alexa594* | #A21207, invitrogen | 1 : 1000 |
| goat-anti-human-IgG Fcγ-Alexa488** | #109-546-170, Jackson | 1 : 1000 |
| goat-anti-rabbit-Alexa594 | #111-585-144, Jackson | 1 : 1000 |
| goat-anti-mouse-Alexa594*** | #A11005, invitrogen | 1 : 1000 |

* were used for the NMDAR screening. Due to the cross-reactivity of a new batch of antibody (lot 1495793), the secondary antibodies were changed to the other two secondaries.
** the dilution for live CBA is 1: 750.
***used only for GABAAR

## Live cell-based assay:

The live CBA was performed as described for the fixed CBA with small modifications. HEK293 cells were grown and transfected as described with the difference that antigens were expressed with fluorescent reporter proteins, if available (LGI1-GFP, GRIN1-GFP, and Caspr2-mCherry). Transfected HEK cells were incubated with human serum, diluted 1:50 in DMEM with 1% BSA and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic

acid (HEPES) at room temperature for 1h followed by fixation in 3.6% formaldehyde. Cells were then incubated, without additional permeabilization or blocking steps, in the presence of the secondary antibodies for visualization. Mounting and analysis were done in the same way as for the fixed CBA.

### Neuronal staining

Rat hippocampal neuronal staining was performed following a protocol from Dr. Dalmau's laboratory with small adaptations [31]. Briefly, cultured cells were incubated with patients' sera (1:50 in Neurobasal with 1% BSA and 25mM HEPES) for 1 hour at room temperature followed by fixation with 4% paraformaldehyde, bound antibodies were labeled with goat-anti-human-IgG Fcγ-Alexa488 (1:1000, #109-546-170, Jackson) and visualized in the BX51 Olympus microscope. Positive controls with known autoantibodies to neuronal surface antigens and negative controls from healthy individuals were included. The results were checked by 2 experienced observers and were scored as negative, weak positive and strong positive according to the fluorescent signal on the surface of neurons after staining.

### Statistical analysis

We used the Fisher exact test to compare the prevalence of IHC positives between groups and compare the prevalence of CBA and neuronal staining positives between subgroups. We performed a Chi-Square test for categorical values (sex) and ANOVA for continuous values (age). Autoantibody prevalence in patients only with currently anxiety or depression was separated for the comparison to avoid overlapping factors. All analyses were performed using IBM SPSS Statistics version 23.0.

## Results

### 2.2% (50) of the samples were found positive with 11 different unknown patterns in IHC

In total 2231 samples were tested by IHC on rat brain to check if they had autoantibodies targeting neuronal antigens in the hippocampus. Of the analyzed samples, 4.8% (106) had scores above 0 and 2.2% samples with scores above 1 were considered as positive, including 42 with score 2 (weak positive) and 8 with score 3 (strong positive) (Table 2). In the positive samples, 11 different staining patterns were found that were different from known patterns reported previously [10, 32] (Figure 2, Supplementary Figure 1). Overall, however, the percentage of positive samples in the lifetime depression or anxiety group and in controls is not different based on the cut off above 1 (39/1793 vs 11/492, p=0.99).

All the samples (n=8) with score 3 were exclusively found in disorder groups, but not at a significant level between current disorder group compared to remitted group or controls (6/819 vs 2/920 vs 0/492, p=0.071). In these 8 samples, five was later confirmed the autoantibodies targeted neuronal surface antigens (Table 3, case 1-5; Figure 1, pattern B and C), were from current anxiety patients (one also had current depression). The other three, later showed negative for NSAbs, were from patients with current depression or remitted disorders.

### Autoantibodies to known antigens were rarely found

All 106 samples that had IHC scores above 0 were tested for cell surface autoantibodies using both fixed and live CBA, and tested for anti-GAD65 and anti-GAD67 using fixed CBA only.

**Table 2: Prevalence of neuronal autoantibodies in the 2231 NESDA respondents**

| | Current depression or anxiety [1] | | Remitted depression or anxiety [2] | | Control group | | P value |
|---|---|---|---|---|---|---|---|
| **IHC (Tested: N = 2231)** | 819 | | 920 | | 492 | | |
| Borderline | 23 | (2.8%) | 19 | (2.1%) | 14 | (2.8%) | -- |
| Weak positive | 15 | (1.8%) | 16 | (1.7%) | 11 | (2.2%) | -- |
| Strong positive | 6 | (0.7%) | 2 | (0.2%) | 0 | | N / S*(0.071) |
| **CBA (Tested: N=106)** | 44 | | 37 | | 25 | | |
| Anti-NMDAR | 0 | | 0 | | 0 | | N / S |
| Anti-AMPAR | 0 | | 0 | | 0 | | N / S |
| Anti-Caspr2 | 1 | (2.3%) | 5 | (13.5%) | 2 | (8.0%) | N / S (0.12) |
| Anti-LGI1 | 0 | | 1 | (2.7%) | 0 | | N / S (0.58) |
| Anti-GABAAR | 0 | | 1 | (2.7%) | 0 | | N / S (0.58) |
| Anti-GABABR | 2 | (4.5%) | 1 | (2.7%) | 0 | | N / S (0.79) |
| Anti-GAD65/67 | 2 | (4.5%) | 0 | | 1 | (4.0%) | N / S (0.46) |
| **Neuronal staining (Tested: N=106)** | 44 | | 37 | | 25 | | |
| Strong positive | 7 | (15.9%) | 0 | | 1 | (4.0%) | 0.018 |

[1] Depression and /or anxiety present in the six months prior to assessment.
[2] Lifetime depression and/or anxiety diagnosis, but not in the six-months prior to assessment.
* N / S: no significant difference. α =0.05

2 samples were found positive by fixed CBA, one was positive for anti-Caspr2, one was positive for both anti-GAD65 and anti-GAD67, both with correlated IHC patterns (pictures not shown). 12.3% (13) samples were found positive by live CBA, eight positive for anti-Caspr2, one for anti-LGI1, one for anti-GABAAR and three for anti-GABABR. However, none of those neuronal surface autoantibodies detected by live CBA had the correlating antigen-binding pattern on rat brain slice by IHC. Additionally, a competition experiment was performed for all anti-Caspr2 positive samples identified by live CBA as described in a previous study [33], adding the plasma on Caspr2 transfected cells and then incubating the pre-absorbed sample on rat brain (Supplementary Figure 2). No competition effect/reduced staining on rat brain was observed, indicating that the IHC positive signal in the hippocampus is not caused by antibodies binding to Caspr2.

### 7 out of 8 individuals with unknown NSAbs had current disorders

All the 106 samples that had IHC score above 0 and also 40 samples randomly picked from IHC negative samples were tested by using live neuronal staining to see if they had NSAbs. 8 positives were found. 5 were from IHC score 3, 3 from score 2 and none was found in score 1 or negative samples. According to the immunofluorescent intensity of the staining, the identified samples were further categorized into 3 strong positives (Fig 3, Table 3 Case 1, 4, 6) and 5 weak positives (Table 3). All were from individuals with current depression or anxiety group (all had current anxiety, 2 had current depression) except one which was from

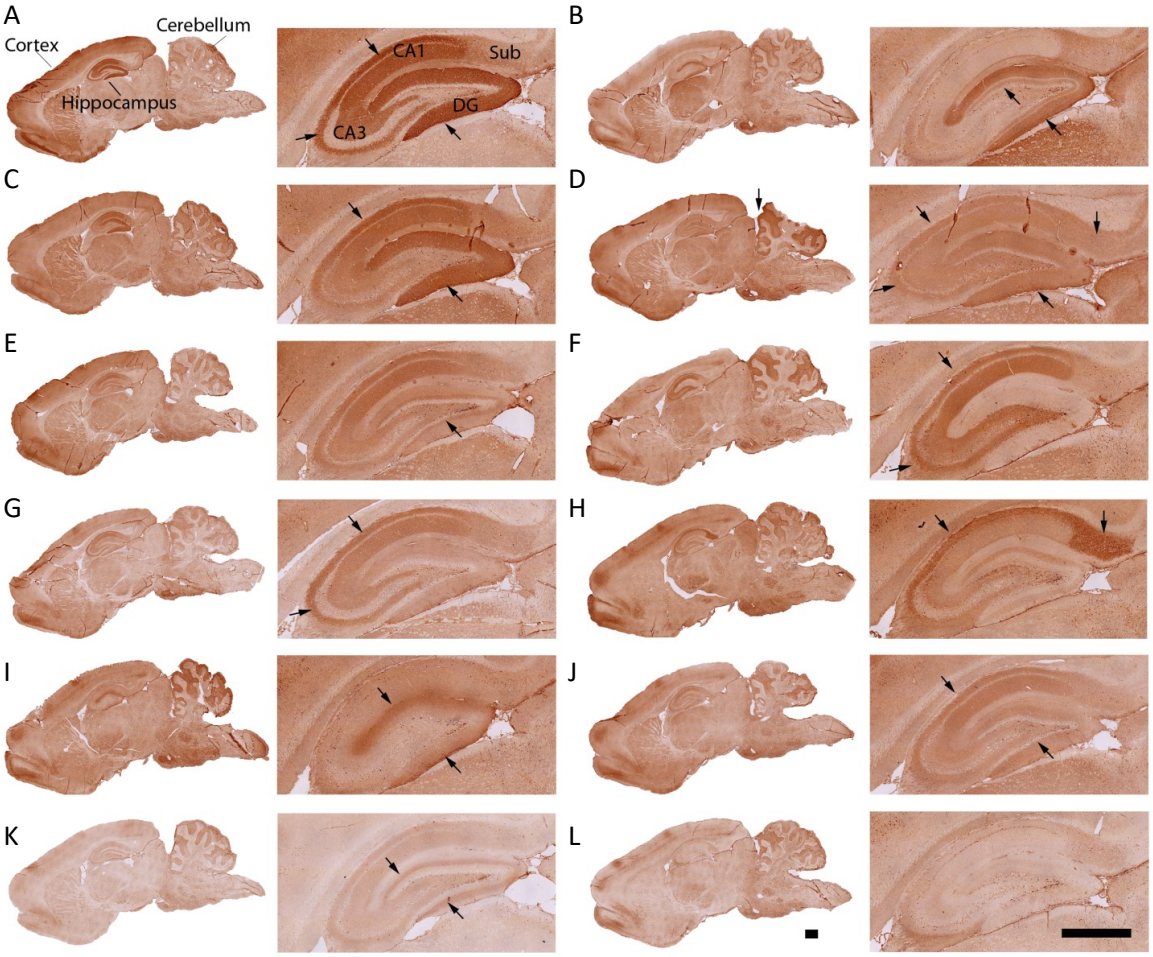
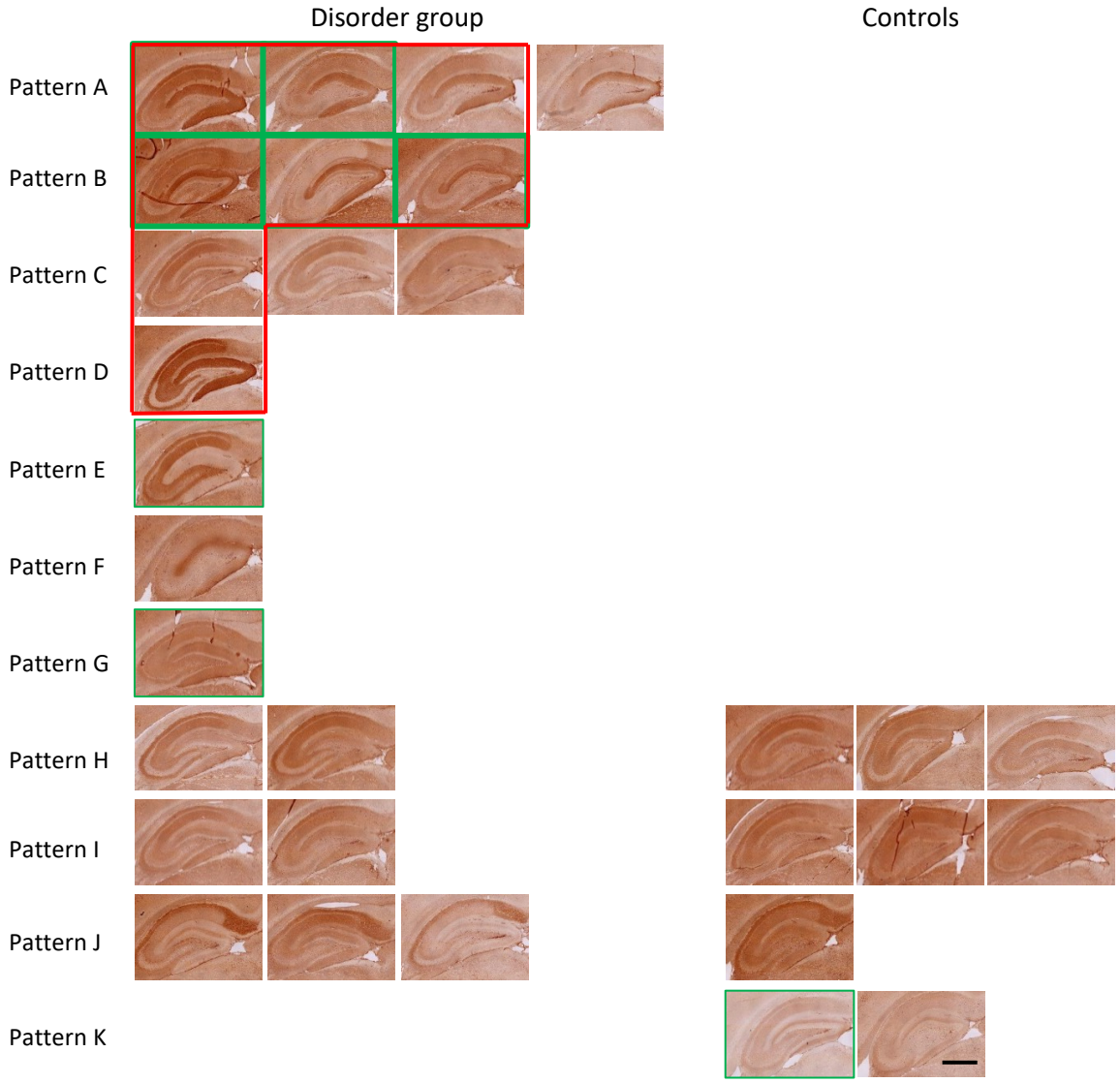



**Figure 2.** IHC staining of unidentified antigens. A-K represent 11 IHC staining patterns (whole brain and hippocampus) given by autoantibodies in human plasma without identification of the specific antigen. L was stained with plasma from a healthy control. Arrows show the strongest reactivity regions. Scale bar = 500 µm.




**Supplementary Figure 1.** IHC staining of unidentified antigens. A-K represent 11 IHC staining patterns (whole brain and hippocampus) given by autoantibodies in human plasma without identification of the specific antigen. L was stained with plasma from a healthy control. Arrows show the strongest reactivity regions. Scale bar = 500 µm.

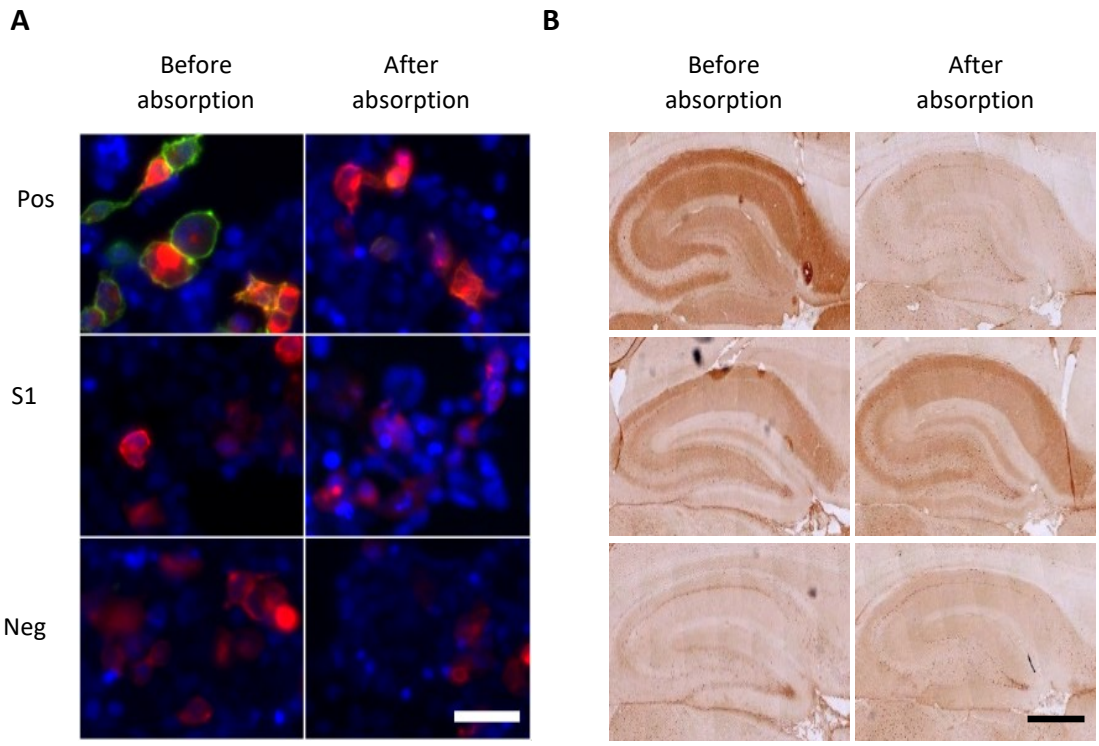
**Table 3. Characteristics of the 8 individuals with autoantibodies to unidentified neuronal surface antigens at the 2-year follow-up**

| No. | Gender | Age (year) | IHC score/ pattern[1] | Titre by IHC | Diagnosis[2] | Anxiety/Depression onset age (year) | Antidepressants usage during last 2 years | Fever/cold[3] | Chronic comorbidities |
|---|---|---|---|---|---|---|---|---|---|
| Case 1 | Female | 46 | 3/B | 12800 | Current anxiety | 8 / -- | No | Cold | Hay Fever |
| Case 2 | Male | 55 | 3/B | 6400 | Current anxiety, remitted depression (single episode) | 53/14 | No | Cold | Hypertension |
| Case 3 | Female | 58 | 3/B | 800 | Current anxiety and depression | 56/56 | No | Cold | Chronic bronchitis, diarrhea, eczema, psoriasis |
| Case 4 | Female | 57 | 3/C | 3200 | Current anxiety | 55/ -- | No | Cold | Hypertension; breast cancer eczema; arthritis; Renal pelvic inflammation with encapsulated kidney stone; chronic heat conditions (unspecified); |
| Case 5 | Female | 31 | 3/C | 3200 | Current anxiety, remitted depression (single episode) | 29/20 | No | No | No |
| Case 6 | Male | 56 | 2/D | 1600 | Current anxiety, remitted depression (single episode) | 48/28 | No | No | Eczema |
| Case 7 | Female | 36 | 2/F | 1600 | Current anxiety and depression | 35/34 | No | No | chronic heart conditions (unspecified), ligament injury |
| Control 1 | Male | 20 | 2/K | 400 | None (Control group) | -- / -- | No | No | Injury (overloading of the knee) |

1. IHC pattern: there are 11 different IHC patterns found this study (seen in figure 1, A-K)
2. Current: Diagnosed with depression and /or anxiety within six-month when blood samples were collected

Remitted: Diagnoses with depression and/or anxiety earlier in life but no diagnoses with depression or anxiety within six-month when blood samples were collected.

3. Had a fever or a cold in the past week before the blood sample was drawn based on questionnaires




**Supplementary Figure 2**. CBA (A) and IHC (B) results before and after the absorption. A. HEK cells were transfected with Caspr2-mCherry (red). Human IgGs from blood samples were labeled in green and nuclei were labeled in blue. B. Rat brain slices were used for IHC staining and human IgG from the samples was labeled by DAB (brown). "Pos'' was positive serum control from an encephalitis patient with anti-Caspr2 autoantibodies. The staining intensity was negligible after pre-absorption. S1 is a plasma sample from the NESDA cohort which was weakly positive for Caspr2 by live CBA but its' IHC pattern was not depleted by pre-absorption. ''Neg'' is the negative plasma control from a healthy individual. CBA Scar bar = 20 µm. IHC Scale bar = 500 µm.

the control group, while none was from individuals in remission (7/44 vs 1/25 vs 0/37, p=0.018; the significance was contributed by current disorders vs remitted, 7/44 vs 0/37, p=0.014 (adjusted α=0.017)).

Clinical information for those 8 individuals is shown in table 3. Except the one from control had relatively low titer (400), the other 7 were all had current anxiety of which 2 also had current depression. Interestingly, none of those patients with disorders had used anti-depressant drugs in the last 2 years before blood was tested. The age, gender or chronic comorbidities were not similar in those NSAbs positive cases. The anxiety sub-diagnoses of the 7 NSAbs positive cases were diverse too (3 had agoraphobia, 2 had generalized anxiety disorders, 1 had social phobia and 1 had panic without agoraphobia). The BAI (Beck anxiety index) or IDS (inventory of depressive score) was not different compared to the patients without NSAbs (data not shown).

To better characterize these NSAbs, positive individuals, we further retrieved the diagnoses data (depression or anxiety present or not) and tested the plasma samples of these 8 cases at the baseline (the first plasma collected time) and 6-year follow-up (the next plasma collected

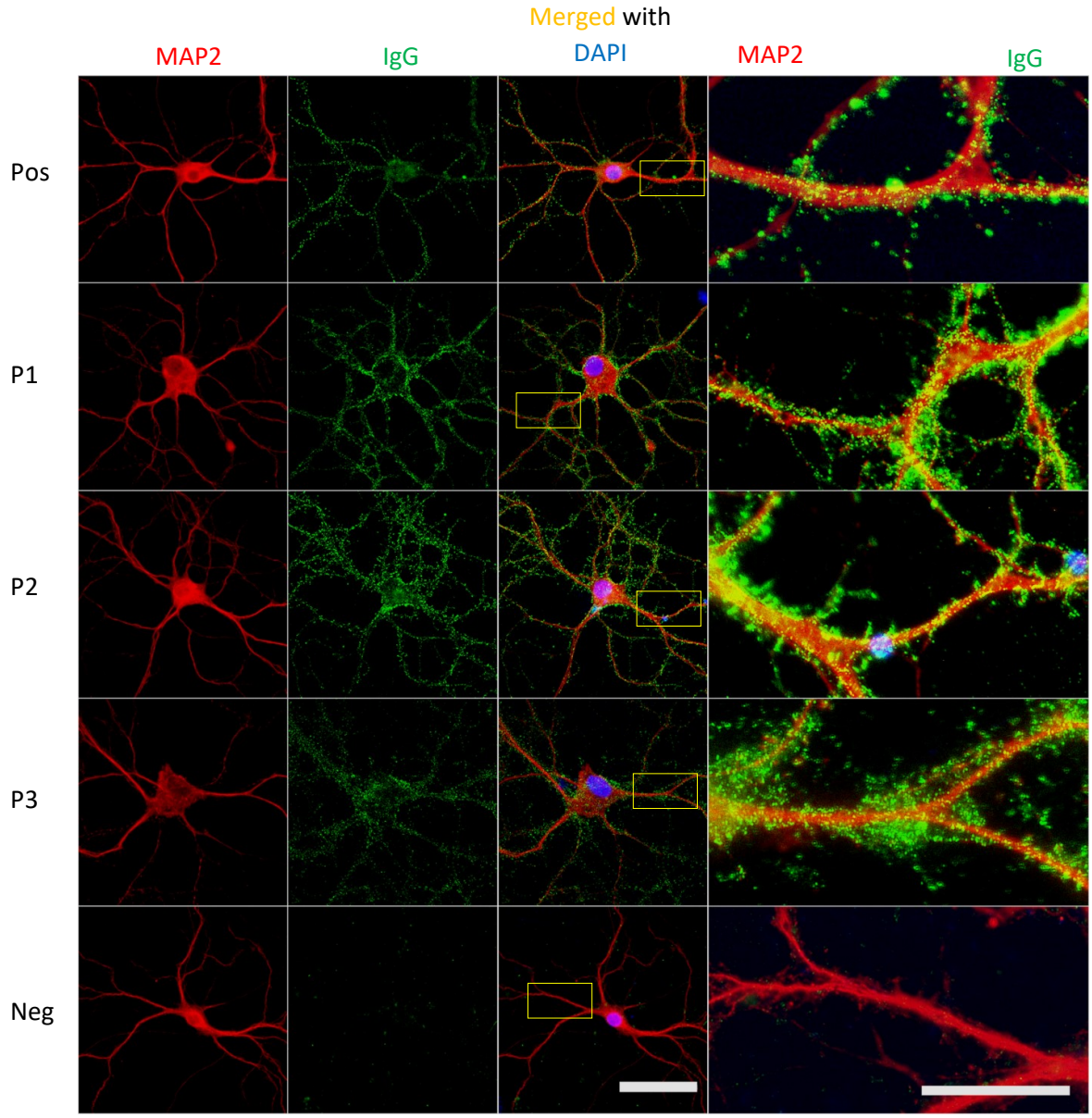
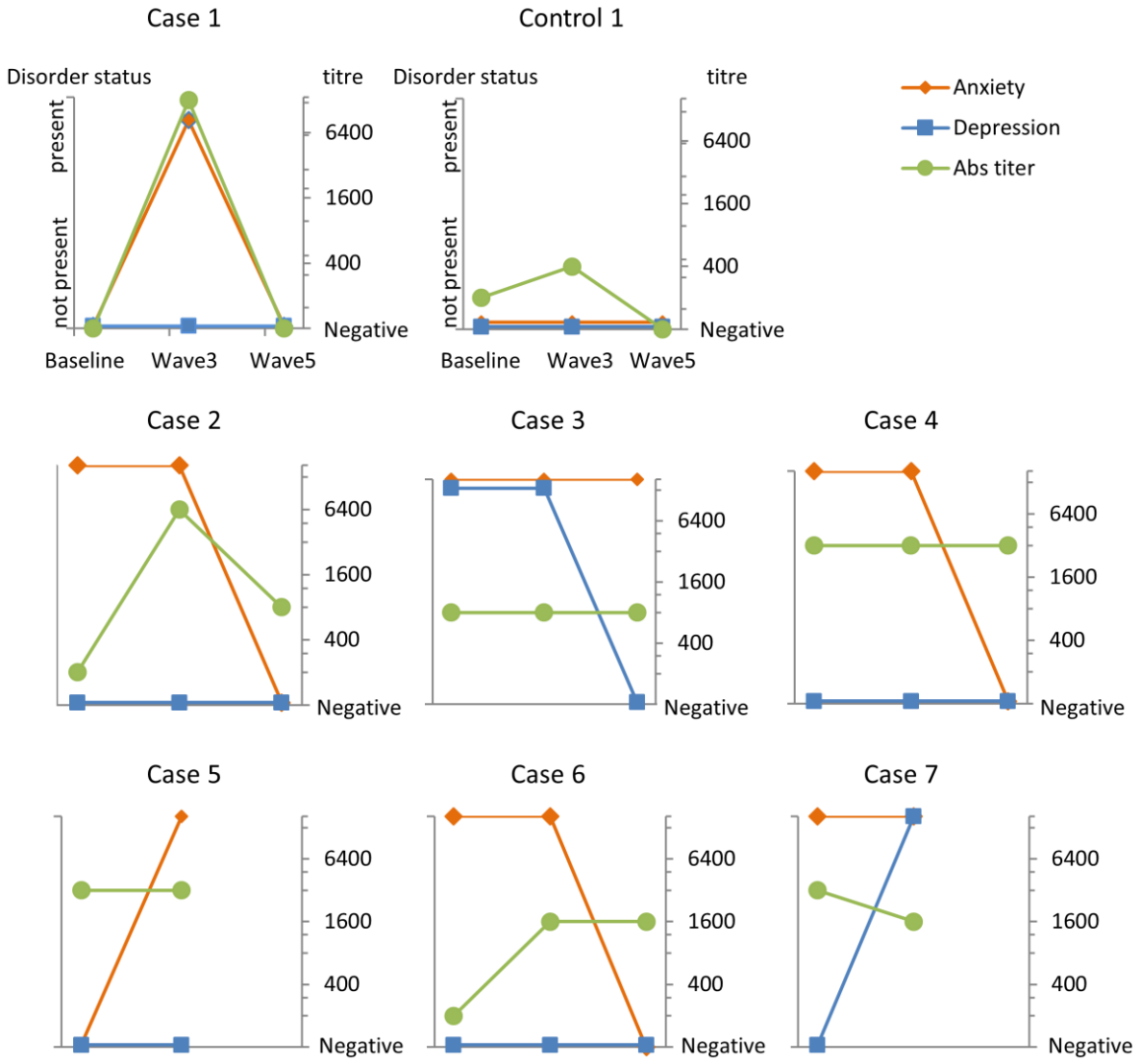



**Figure 3.** Representative immunofluorescence staining on primary hippocampal live neurons using strong IHC positive plasma samples. Plasma samples from patient 1 (P1), patient 2 (P2) and patient 3 (P3) showed clear reactivity on neurons. "Pos" is a positive control staining using an anti-NMDAR positive serum, ''Neg'' is a negative control from a healthy individual. Scale bar= 50 µm. The zoom regions showed the speckled staining along the dendrite (last column). Scale bar= 20 µm. Map2 is stained in red, human IgG in green and nuclei in blue.

time). The result was inconclusive as the changes of NSAbs during the period of 6 years were divers from one each other and only in only 3 cases, the changes of autoantibodies were positively correlated with the changes of anxiety diagnoses (the details were seen in supplementary figure 3).




**Supplementary figure 3.** NSAbs titers and disorder status changing over time in the 7 NSAbs positive cases from disorder group and I case from controls. Antibody titers in the 8 cases' samples from different time points (baseline, wave 3 (2-year follow-up) and wave 5 (6-year follow-up)) were tested by immunohistochemistry (right axis, showed with dilution factors, starting dilution was 1 in 200) and their disorder status were also tracked (right axis, present or not present). In case 1, case 3 and case 7 the presence of autoantibodies and anxiety status were well matched. Control1 was a case without lifetime depression or anxiety.

## Validation of autoantibody detection

The 20 samples which gave the strongest staining intensity by IHC (including the 8 positive samples on live neurons) were sent to Dr. Dalmau's laboratory in Barcelona for further analysis. Expectedly, they did not target known neuronal antigens by CBA. 3 strong positive samples on live neurons (Figure 3) were confirmed in their assay. The additional 5 samples were judged as negative. The main difference is in Barcelona they used a dilution of 1 in 200 compared to a

dilution 1 in 50 in our laboratory. When further diluted the samples to 1 in 200 in our laboratory, they became negative as well. Thus, these samples were still treated as NSAbs positive but just at a lower titer.

**Discussion**

To our knowledge, this is the largest study on the prevalence of neuronal antibodies in plasma samples of patients with depression or anxiety. Overall 2.2% positive samples with 11 unknown distinguishable staining patterns on brain tissue were found. The prevalence of known neuronal autoantibodies detected by IHC and fixed CBA was practically zero, while the positive samples found by live CBA were not different between groups. We found only a small number of samples with novel NSAbs, more positive samples belonged to individuals who were suffering from anxiety or depression compared to patients in remission or controls. Those NSAbs positive patients all had current anxiety and had not used anti-depressant drugs in the last 2 years before antibody detection.

In recent years, studies showed that NSAbs could cause psychiatric symptoms in autoimmune encephalitis and it has been hypothesized that they might be causative of the psychiatric symptoms in a subgroup of psychiatric disorder patients [10, 17, 34, 35]. It has been reported that autoantibodies to known NSAbs can be detected in first-episode psychosis, bipolar disorders or major depression [22, 39, 40]. We did not find that NSAbs could relate to depression or anxiety which corroborate with earlier reports [36-38]. However, none of the previous studies checked for the presence of potentially novel NSAbs here our findings showed a potential research interest in searching for novel NSAbs in patients with current depression or anxiety. While by using the same antibody screening strategy, another study from us did not find in number of novel NSAbs in patients with psychotic disorders (unpublished data, seen in chapter 6).

As shown in this manuscript, the novel NSAbs positive patients diagnose subtypes are rather heterogeneous although they all had current anxiety. One possible explanation is that the 8 NSAbs positive samples showed 5 different staining patterns on the rat brain. It indicates that those NSAbs target different proteins on the surface of neurons and thus could lead to different clinical manifestations if they are pathogenic [15]. The identification of the autoantigens and autoantibody mechanism studies may further clarify if these NSAbs are pathogenic or not.

Besides anxiety, another common factor these NSAbs positive patients shared is none antidepressant medication history. A previous review has summarised the immunomodulatory effects of anti-depressants which mainly focus on anti-depressants' relation to T cells and cytokines [41], little is known if those drugs may also affect the function of B cells and change the level of autoantibodies secretion.

We found a series of weakly positive samples with know NSAbs only by live CBA, of which no difference was found between disorder group and controls. Similar results were reported by a recent study which investigated the value of known NSAbs in first-episode psychosis expect that we did not find any anti-NMDAR positive cases, which might due to a different cohort was screened. Those antibodies found by only live CBA might target conformational epitopes that lose immunoreactivity by paraformaldehyde or triton-X100 during the step of fixation/permeabilization and their clinical value should be considered differently compared to those that could be confirmed by fixed CBA or IHC.

There are several explanations about why we find these anti-brain antibodies in all groups. Firstly, the control groups are not completely free from somatic diseases. Some autoimmunity conditions may contribute to the existence of the autoantibodies. Secondly, it is known that anti-brain antibodies may occur in the general population and by no means need to be relevant or causative for the disease [38, 42] which leads to a false-positive result. Lastly, it is uncertain that those circulating antibodies could pass the blood-brain-barrier. Former studies have revealed the autoantibodies would be disease-related (pathogenic) when disruption of the blood-brain barrier integrity, which may also explain part of the cases with neuronal surface autoantibodies but showed no symptoms [21, 42].

This cohort study has several limitations: 1) although we reported novel NSAbs were found, not all the known antigens that have been reported were excluded by using CBA in this study (including D2R, mGlu5, mGlu1, neurexin-3α, IgLON5, DNER (Tr), Glycine receptor and amphiphysin) but were excluded according to the IHC patterns empirically; 2) we only analyzed peripheral blood samples, while no cerebrospinal fluid material was available, so the question as to whether those antibodies pass the blood-brain-barrier still remains to be investigated.

To conclude, there is no difference in the prevalence of known neuronal autoantibodies between depression or anxiety groups and the controls without a mental disorder. Novel NSAbs may exist in a subgroup of patients with current anxiety or depression; a cohort of with solely current anxiety or depression patients should be selected and tested for these novel NSAbs in both blood and cerebrospinal fluid samples to validate this finding. Considering the rareness of the novel NSAbs found in this study, another more reasonable way to prove their clinical relevance is to identify the autoantigens and to further study the possible role in the pathophysiology of mental disorders.

**Conflict of Interest Statement**

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.

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# Chapter 5

## Low prevalence of autoantibodies related to neuronal and rheumatic autoimmune disease in sera of patients with psychotic disorders

**Corresponding manuscripts (this chapter was divided into 2 manuscripts when submitted for publication):**

1. Carolin Hoffmann*, Shenghua Zong*, Marina Mané-Damas, et al., JAMA Psychiatry, 2019, ***accepted.*** **(*** authors **contributed equally** to this manuscript)
2. Carolin Hoffmann, Shenghua Zong, Marina Mané-Damas, et al., ***in preparation***

## Abstract

Psychiatric symptoms are increasingly recognized as potentially of autoimmune origin, either in the range of newly discovered encephalitis-related antibodies or related to systemic autoimmune diseases like systemic lupus erythematosus (SLE). We aimed to determine the prevalence of neuronal- and systemic autoimmune rheumatic disease (SARD) related-antibodies in sera of patients with the psychotic or affective disorder as well as in sera of healthy control subjects. The included cohort comprised 621 individuals diagnosed with psychotic disorders (first episode and chronic), 70 individuals with affective disorders, 41 with other mental disorders, and 257 controls. Overall, 4.1% of all sera showed hippocampal autoantibody binding as detected by reactivity on rat brain tissue using immunohistochemistry (IHC) with no difference between groups. Further characterization by live and fixed cell-based assays (CBA) for detecting specific neuronal surface antibodies (NSAbs), and antibodies against glutamic acid decarboxylase (GAD) revealed low prevalence (1.2%) in all groups and was observed for Caspr2, GAD65 and GAD67 autoantibodies. We identified brain-reactive autoantibodies (in all groups) that target unknown antigens. Two sera (from one individual with schizophrenia and one healthy participant) reacted with live hippocampal neurons. Lastly, SARD-related antibodies, tested by immunofluorescence on HEp-2 substrate were increased in psychotic disorders, but only in 3 patients did antibody testing hint at a possible diagnosis of SLE when analyzing additional enzyme-linked-immuno-assay. Overall, the prevalence of neuronal autoantibodies was very low with no significant difference between healthy controls and patients with mental disorders. Further research into the identification of possible novel antigens and their pathological involvement is warranted.

## Introduction

Increasing evidence indicates that psychiatric symptoms can be caused by autoimmune conditions [1, 2]. The occurrence of several neuronal surface and synaptic autoantibodies (NSAbs) seems associated with different neuropsychiatric phenotypes including isolated symptoms of psychosis [3, 4]. Importantly, many previously idiopathic syndromes now known as autoimmune encephalitis, respond very well to immunosuppressive treatment [5-7]. Psychiatric symptoms can also be related to systemic autoimmune rheumatic disease (SARD), such as systemic lupus erythematosus (SLE) [8]. Antibodies may target ribosomal P protein (RibP), thought to predict neuropsychiatric manifestations in SLE [9]. Most studies have focused on neuronal autoantibodies targeting the N-methyl-D-aspartate-receptor (NMDAR) and the voltage-gated potassium channel (VGKC) complex [10-16] because these antibodies are known to cause autoimmune encephalitis with psychosis [3, 17]. However, the question of whether NSAbs play a role in psychosis is still debated because study results vary greatly, e.g. the reported prevalence of NMDAR autoantibodies ranges from 0 to 11.6% [16, 18-23]. Discrepancies might be explained by several factors of which an important one is the choice of the cohort within the very broad syndromes of psychosis. For instance, in first-episode psychosis (FEP) and in post-partum psychosis, autoimmunity might be more common [10, 21, 22]. The choice of test methods seems to influence results [21, 22, 24]. Different groups use either live or fixed cell-based assays (CBA) and additional methods such as immunohistochemistry (IHC) on rat brain and live neurons. The use of only one screening method, while omitting the analysis of control cohorts, makes the comparison of the results a daunting task.

For an extensive analysis, we thus chose to screen sera from a large cohort of patients with various methods to answer the question of whether NSAbs or SARD-related antibodies are more common in patients with psychotic disorders.

## Methods

### Study population

Samples and patient data were collected with written informed consent according to national and institutional ethical guidelines and the Helsinki Declaration, with additional informed consent by legal representatives for patients under age 18. The ability to provide written informed consent was evaluated by a psychiatrist by a face-to-face interview using a series of open-ended questions evaluating comprehension, reasoning, choice-making and appreciation skills of the patient. The study represents a wide cohort of psychotic disorders and covers potential differences in diagnosis (Table1). Cohort 1 from Belgium includes 203 patients with a DSM-IV diagnosis of psychotic disorders and 45 with affective disorders; samples had been collected between 2003 and 2007, previously screened for metabolic disturbances [25]. It also includes 13 healthy individuals without psychiatric antecedents and 23 with other mental disorders (OMD) without psychotic symptoms. Cohort 2 consists of 40 patients with psychotic disorders or affective disorders with psychotic features according to DSM IV that were recruited at the Université Paris-Est Créteil. Cohort 3 includes 300 patients at the Istanbul University with the DSM-IV diagnosis of schizophrenia; samples were collected between 2011-2012. Cohort 4 was recruited at the Erasmus Medical Center (EMC) Rotterdam. It included samples from 95 patients with psychotic disorders, 8 with affective disorders as well as from 18 patients with a range of OMD. Samples from this project have previously been used

by Schwarz et al [26]. The cohorts from Rotterdam and France are part of the European network of national schizophrenia networks studying Gene-Environment Interactions (EU-GEI, http://www.eu-gei.eu).
Cohort 5 consisted of 44 healthy controls recruited by the Hospital General Universitario Gregorio Marañon in Madrid among patients' friends, colleagues, and neighbors of patients [27]. Cohort 6 consists of 200 controls, that are anonymized blood donors from Sanquin Maastricht. In a pre-screening process, the donors were confirmed to be healthy by general indicators, tested by interviews, hemoglobin and other blood parameters, blood pressure, pulse, and body temperature as well as the absence of infectious diseases but without specific mental health screening.

**Table 1: Demographic description of cohorts**

| | Cohort 1 | Cohort 2 | Cohort 3 | Cohort 4 | Cohort 5 | Cohort 6 |
|---|---|---|---|---|---|---|
| **Healthy, n** | 13 | - | - | - | 44 | 200 |
| **OMD[1], n** | 23 | - | - | 18 | - | - |
| **Affective disorders, n** | 45 | 17 | - | 8 | - | - |
| **Psychotic disorders, n** | 203 | 23 | 300 | 95 | - | - |
| **Source** | University Psychiatric Center Catholic University Leuven in Kortenberg | Public services (emergency wards, in- and out- patient clinics) and private clinics in the Paris region (Créteil) | Istanbul University, Aziz Sancar Institute of Experimental Medicine | Erasmus Medical Center (EMC) Rotterdam. | Seven sites from the Spanish Psychiatric Research Network (CIBERSAM) | Sanquin Maastricht |
| **Criteria** | Patients:<br>DSM-IV diagnosis of schizophrenia, schizoaffective disorder, or bipolar disorder<br>Healthy:<br>No psychiatric antecedents or medication | DSM-IV diagnosis of psychotic disorder or mood disorders with psychotic features; substance-induced psychosis were excluded | DSM-IV diagnosis of schizophrenia | Consecutively admitted patients which initially presented with psychosis, and were finally diagnosed with schizophrenia, as well with a range of other mental disorders | Absence of any psychiatric diagnosis according to DSM-IV criteria<br>No presence of a severe medical condition, and no current or past treatment with any antipsychotic drug | Blood Donors, confirmed to be healthy by general indicators, tested by interviews, hemoglobin and other blood parameters, blood pressure, pulse, and body temperature as well as the absence of infectious diseases |
| **Time-span** | November 2003 to July 2007 | June 2010 to May 2014 | 2011 to 2012. | | January 2007 to December 2010 | March 2014 |
| **Reference** | [25, 28] | [29] | - | [26] | [27] | - |

[1] OMD= other mental disorder

## Psychiatric diagnosis

The diagnosis was established by the treating psychiatrists based on the DSM-IV. We grouped the patients into affective disorders (bipolar and major depressive disorder), psychotic disorders (schizophrenia, schizoaffective, brief psychotic disorder, first-episode psychosis (FEP), and other psychotic diagnoses i.e; psychosis not otherwise specified, delusional disorder, substance-induced psychosis, paranoid, schizophreniform, and schizoid personality disorder) and "other" mental disorders (OMD) i.e. non-psychotic, non-affective disorders.

## Antibody screening strategy

Serum samples were screened by rat brain IHC for hippocampal antibody reactivity, and, if positive, analyzed by CBA for 8 different antigens and staining of live primary rat hippocampal neurons. The CBA was used as a fixed and live-cell method for the 6 NSAbs to account for differences in antigen-antibody reactivity. An overview of our screening strategy is given in Fig 1A. Additional sub-cohorts (independent of neuropil reactivity on rat brain) were tested for the most reported antigens NMDAR (by CBA) and VGKC complex (by RIA). Sera tested positive in one of these methods were re-tested in the laboratory of Prof. Dalmau according to its standard diagnostic procedures (IDIBAPS, Barcelona) so as to compare our grading with the cut-off for autoimmune encephalitis. Lastly, bystander/systemic autoimmunity was tested by screening sub-cohorts for antibodies against antinuclear antigens (ANA), and antibodies against double-stranded DNA (dsDNA), ribosomal P (RPP), and cardiolipin (aCL).

## Analysis of neuronal autoantibodies on rat brain immunohistochemistry as the first screening step

Procedures were approved by the animal experiment committee at Maastricht University as well as the central committee of the animal experiment (CCD) (WP 2016-005-001). Neuronal autoantibodies were identified by IHC on rat brain tissue following standard methods [30, 31]. In brief, Lewis rat brains were fixed for one hour in 4% paraformaldehyde and cryoprotected by 30% sucrose solution. After blocking with 0.3% $H_2O_2$ and 5% goat serum, sections were incubated with human serum diluted 1:200 in 5% goat serum overnight at 4 °C. After incubating with biotinylated goat anti-human IgG Fcγ (1:3200, 109-066-008, Jackson laboratory) for 2 h at 20 °C, tissue was incubated with VECTASTAIN Elite ABC kit (Vector lab., # PK 6100) during 1 h at 20 °C and the reactivity developed using diaminobenzidine. Staining included negative controls of "healthy" serum and seropositive controls from autoimmune encephalitis patients (against various autoantigens). Images were taken by the VENTANA iScan HT slide scanner (20x objective) and graded 0 to 3 on the screen (Ventana Image Viewer) for the hippocampal reactivity of sera based on the intensity and contrast of the staining (see in figure 1 B). All stainings that were scored 1-3 and inconclusive cases were repeated and validated by two independent observers. Those with inconsistent results were repeated at least once more and a final score (positive if grade >1) was given according to all images of one sample.

## Measuring specific neuronal autoantibodies (cell-based assay)

Specific antibody screening detection was performed using an in-house CBA for the following antigens: NMDAR (GluN1 alone and GluN1/GluN2B), leucine-rich glioma-inactivated 1 (LGI1), contactin-associated protein-like 2 (Caspr2), α-amino-3-hydroxy-5-methyl-4-

isoxazolepropionic acid receptor (AMPAR), γ-aminobutyric acid receptor subunit A and B (GABAAR, GABABR), and glutamic acid decarboxylase isotypes 65 kDa and 67 kDa

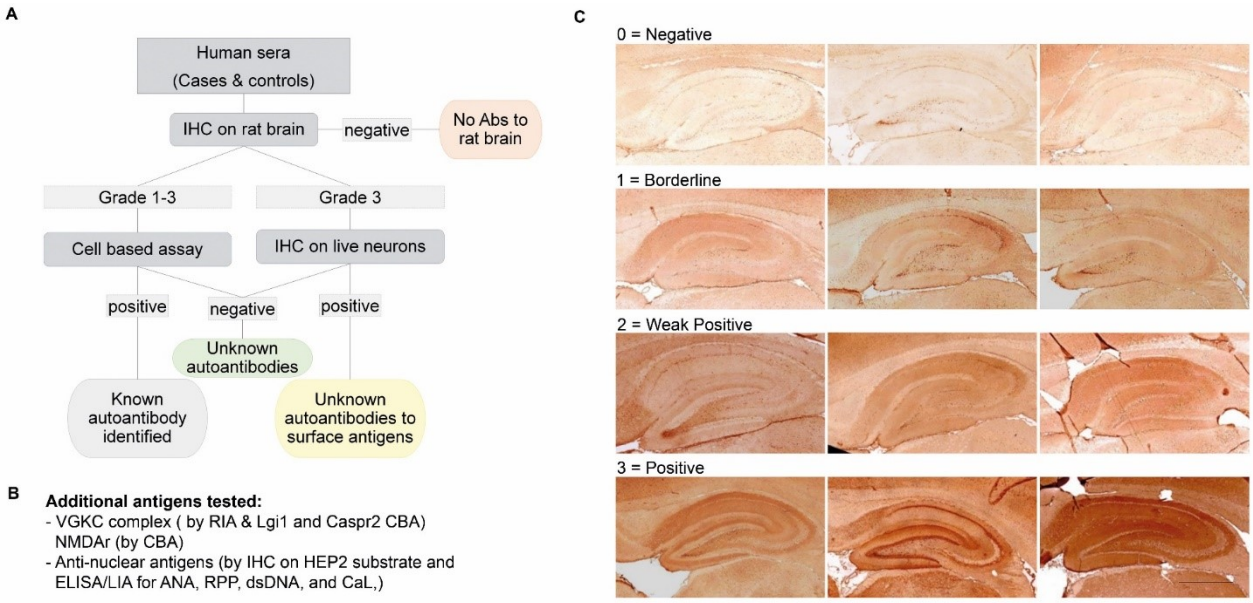


**Figure 1.** Screening strategy for autoantibody detection using rat brain immunohistochemistry (IHC), cell based assay (CBA), and staining on rat hippocampal primary live neurons. A) The flow-chart illustrates the autoantibody testing strategy with rat brain IHC as initial screening step. First sera samples were incubated on rat brain tissue, antibody reactivity was shown by using an anti-human IgG specific secondary. Stainings were graded as indicated in **B** from 0 to 3. If the staining was found negative no further steps were undertaken, if the staining was graded 1-3 it was tested by CBA and if it was tested 2 or 3 it was also tested by live neuronal staining. **B)** The grading of rat brain IHC based on four staining intensities of the hippocampus from negative to strong positive is shown by 3 representative examples of the according grade. Scale bar = 500 µM **C)** Reactivity to other antigens was tested independently of the IHC results on rat brain and included screening for antibodies against the voltage-gated-potassium-channel complex antigens (LGI1 and Caspr2) by radioimmunoassay (RIA) followed by IHC, CBA and neuronal staining, N-mehtyl-D-aspartate receptor (NMDAr) autoantibodies by CBA, and anti-nuclear-antigen (ANA) screen by IHC on HEP2 substrate, enzyme-linked immunosorbent assay (ELISA) and line immunoassay (LIA).

(GAD65, GAD67). HEK293 cells were plated on coverslips and transfected with 4 µg expression vectors of the respective human antigens and expression allowed for 22-26 h (source of plasmids described below). Cells were fixed in 3.6% formaldehyde (#F006, TAAB) for 10 min and permeabilized with 0.3% Triton-X-100 for 10 min. After blocking with 1% bovine serum albumin (BSA) for 1 h, cells were incubated with human sera diluted 1:40 in 1% BSA together with an antibody targeting the according antigen for 1 h at 20 °C. For an overview of antibodies used and staining with secondary antibodies see supplementary Table 1. Screenings always included a positive control from an autoantibody-positive patient and a negative human serum control. Cover glasses were mounted onto 7 µl DAPI mounting medium (#H-1200, Vector Laboratories) and evaluated by two (of which one blinded) observers independently on the BX51 Olympus microscope for antibody reactivity. When positive, the staining was repeated with serial dilution (1:50 up to 1:3200).

Live CBA's were performed for all 6 NSAbs as described for the fixed CBA with small modifications. HEK293 cells were grown and transfected as described with the difference that

antigens were expressed with fluorescent reporter proteins, if available (LGI1-GFP, GRIN1-GFP, and Caspr2-mCherry). Human serum was incubated, diluted 1:50 in DMEM with 1%

**Supplementary Table 1: Antibodies used for cell-based assay**

| Antibody | source | dilution |
|---|---|---|
| anti-GluN1 | #PAB12310, Abnova | 1 : 500 |
| anti-LGI1 | #AB30868, Abcam | 1 : 1000 |
| anti-Caspr2 | # AB33994, Abcam | 1 : 1000 |
| anti-GABAAR | #75136, Antibodies Incorporated | 1 : 20000 |
| anti-GABABR | #sc14006, Santa Cruz Biotechnology | 1 : 500 |
| anti-GAD65 | 7309LB, Christina Hampe, (University of Washington) | 1 : 1000 |
| anti-GAD67 | 10266/20B, Christina Hampe, (University of Washington) | 1 : 1000 |
| goat-anti human-IgG-Alexa488* | # A11013, invitrogen | 1 : 1000 |
| donkey-anti-rabbit-Alexa594* | #A21207, invitrogen | 1 : 1000 |
| goat-anti-human-IgG Fcγ-Alexa488** | #109-546-170, Jackson | 1 : 1000 |
| goat-anti-rabbit-Alexa594 | #111-585-144, Jackson | 1 : 1000 |
| goat-anti-mouse-Alexa594*** | #A11005, invitrogen | 1 : 1000 |

* were used for the NMDAR screening. Due to the cross-reactivity of a new batch of antibody (lot 1495793), the secondary antibodies were changed to the other two secondaries.
** the dilution for live CBA is 1: 750.
***used only for GABAAR

BSA and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at 20 °C for 1h followed by fixation in 3.6% formaldehyde. The secondary antibodies were incubated without additional permeabilization or blocking steps. Mounting and analysis were done as for the fixed CBA.

**Sources of plasmids:** Clones containing full-length human cDNA sequences coding for the GRIN1 (NM_000837.1) and GRIN2B (NM_000834.4) receptor were obtained from the Thermofisher EST collection (Thermofischer Scientific). GRIN1 was digested with Psil and GRIN2B was digested with EcoRI, and were cloned into pcDNA 3.1 digested with EcoRV and transformed in DH10B cells (NEB, C4040-03). Both plasmids where sequenced (GATC Biotech) and confirmed to correspond with the GRIN1 and GRIN2B reference sequences. For the live CBA we used human GRIN1 in pIRES-eGFP[22] that was kindly provided by Fabienne Brilot-Turville (University of Sydney). Human AMPAr was expressed from human GluR1 (pTriEx1backbone) and GluR2 (pDest-40 backbone) and human GABABR from GABBR1(pDest-40 backbone) and GABBR2 (pTriEx1backbone). Human LGI-1 was cloned in frame with the transmembrane region of Caspr2 into pcDNA3 to generate membrane bound LGI-1 and with mCherry for the live CBA (pIRES2-DsRed2 backbone). The cloning details were described previously [32]. These 6 plasmids were a kind gift from Patrick Waters (University of Oxford). GABAAR plasmids were obtained from Erdem Tuzun (Istanbul University, Turkey)[33] and expressed the human alpha1, beta2, and gamma1 subunit. Human Caspr2 were received from Catherine Faivre-Sarrailh (CNRS, Marseille)[34] with a pcDNA3 backbone with a mCherry [35] tag for the live CBA and without the tag for fixed CBA . The GAD plasmids expressed human GAD65 and GAD67 from the pCMV6-XL5 plasmid which was a kind gift from Francesc Graus (IDIBAPS, Barcelona) [36].

### Measuring VGKC complex antibodies by RIA

The presence of VGKC complex autoantibodies was determined using a radioimmunoassay (RIA) according to the manufacturer's instruction (DLD Diagnostika GmbH, Hamburg, Germany) [37]. In short, it uses $^{125}$I-a-dendrotoxin-labeled VGKC extracts of the mammalian brain. Samples containing antibodies less than 50 pM were considered negative, level of 50 to 100 pM inconclusive, and levels >100 pM positive. Positive RIA results were retested with rat brain IHC and CBA of transfected HEK293 cells for the VGKC complex proteins LGI1 and Caspr2 as well as on primary neuronal cell culture for confirmation of the antigen specificity [37].

### Primary neuronal cell culture

Rat hippocampal neuronal staining was performed as previously described [38]. Neurons were cultured in vitro and were then incubated with sera (1:200 in Neurobasal with 1% BSA and 25mM HEPES) for 1 h at 20 °C followed by fixation with 4% paraformaldehyde. Bound antibodies were labeled with goat-anti-human-IgG Fcγ-Alexa488 (1:1000, #109-546-170, Jackson) and visualized with a BX51 Olympus microscope.

### Measuring SARD-related autoantibodies

Screening for SARD-related autoantibodies was performed in collaboration with IMMCO Diagnostics (Buffalo, New York, USA). Immunofluorescent analysis (IFA) for ANAs was performed using ImmuGlo™ ANA HEp-2 kit (#1103, Immco Diagnostics) according to the manufacturer's instructions. Enzyme-Linked Immunosorbent Assay (ELISA) was performed to test for the presence of ANAs (using ImmuLisa™ Enhanced ANA Screen ELISA (# 5175), ImmuLisa™ Double-stranded DNA antibody Enhanced ELISA (#5120), IMMULisa Ribosomal P (# 4133) and ImmuLisa™ Cardiolipin IgG, IgA and IgM antibody (ACA) Enhanced ELISAs (#5118G, #5118A and #5118M). Results are expressed in ELISA Units per milliliter (EU/ml) and reported as positive or negative. The threshold for positivity was >50 EU/ml for dsDNA and >20 EU/ml for all other antigens.

### Statistics

To test for the difference of rat brain IHC and ANA indirect immunofluorescence scores between the groups, we performed a non-parametric Kruskal-Wallis test. All tests were done in IBM SPSS Statistics version 23.0 for Windows.

## Results

### Autoantibodies against known neuronal surface antigens are rare and do not differ between healthy controls and disease groups

Patients diagnosed with psychotic disorder (621), with affective disorders (70), with OMD (41) and healthy controls (257) were tested for reactivity on rat brain IHC to select a cohort of individuals with potential anti-brain autoimmunity (Table 2). The reactivity did not significantly differ between groups (Kruskal-Wallis, p=0.116), whereby 27 individuals (4.4%) diagnosed with psychotic disorders, 1 (1.7%) with affective disorders disorder, none with OMD, and 13 (5.1%) healthy controls had autoantibodies binding to rat hippocampus (grade > 1). Also, when analyzing differences in stainings of higher intensity, there was no statistical difference between groups. In our laboratory, 9.8% of all sera were graded 1-3 and thus

included for CBA testing. Out of these, 1.2% sera identified positive for known antigens by CBA (see Table 2). Identified autoantibodies included GAD65 and GAD67. However, sera positive for only GAD67 (without coexisting GAD65 antibodies) had each a different hippocampal binding pattern and were not GAD67 antibody positive in the IDIBAPS laboratory and therefore were considered not to contain neuronal antibodies or antibodies against unknown antigens. With the live, but not fixed CBA, 6 Caspr2 positive sera were identified, but only one was consistently Caspr2 positive across different methods. No sera had antibody reactivity against live neurons at a dilution 1:200, but sera from one healthy and one individual with schizophrenia were positive when decreasing the dilution to 1:50. Sera graded 3 by IHC or positive by any CBA (n=25) were retested in Prof. Dalmau's laboratory (IDIBAPS, Barcelona) where no additional antibodies were identified. In conclusion, we only consider 2 sera reactive to GAD65/67 and one weak reactive to Caspr2 (Table 3).

**Novel hippocampal patterns of rat brain IHC are not specific for mental disorder**

Several sera gave unknown patterns on the hippocampus suggesting that they target novel antigens. Hippocampal stainings with grading 3 (excluding one with GAD65 antibodies, thus 19 in total) could be grouped into eight patterns (Figure 2), of which five were visible in several sera and another three had unique staining patterns (Figure 2). Two of these sera were also reactive on live neurons (indicated with * in Figure 2). Pattern A was prominent and seen with five sera (four from patients with schizophrenia and one from a control individual). This pattern gave a gradient in the dentate gyrus and synaptic areas of the cornu ammonus (CA). Pattern D was similar to that in a previously published staining by Bergink et al.[38] with a serum from a patient with postpartum psychosis. As seen in Figure 2 these sera originated from a schizophrenic patient and a control individual.

**No NMDAR autoantibodies are detected by CBA**

A subgroup of the here presented cohort of schizophrenia spectrum disorders was previously shown to be NMDAR antibody-negative in fixed CBA [16]. Because other studies of similar cohorts found in some cases a higher prevalence of anti-NMDAR IgG, here we extended the screening with randomly selected 101 patients (total of 239 patients with psychotic disorders, 65 with affective disorders, and 37 with OMD) and 214 healthy controls by fixed CBA (without prescreening by rat brain IHC). All additionally tested sera were found negative for NMDAR autoantibodies (Table 2).

**Antibodies against the VGKC complex cannot be confirmed as LGI1 or Caspr2 specific**

Some previous studies have reported VGKC complex autoantibodies to be increased in cohorts of patients with psychosis, with RIA the most used method. Thus, we additionally tested with this RIA a cohort of 101 schizophrenia patients from cohort 1 that had either a diagnosis of FEP, were treated with clozapine or were aged ≤ 30. Two patients were found positive (>100 pM) and five inconclusive (50 to 100 pM). However, none could be confirmed as positive on CBA for LGI1 and Caspr2, rat brain IHC or live neurons.

## Table 2: Summary of antibody screening results

| | | | | | | sub-diagnoses psychotic disorders | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Controls[1] | Other mental disorders | Affective Disorder | Psychotic disorders | Schizophrenia | Schizoaffective | Brief psychotic disorder | FEP | Other psychotic diagnoses |
| | **No.** | **257** | **41** | **70** | **621** | **476** | **45** | **38** | **45** | **25** |
| | **av. age** | 44.1 | 27.3 | 31.6 | 34.4 | 36.6 | 31.3 | 26.7 | 22.2 | 34.1 |
| | **% female** | 47.5 | 24.4 | 51.4 | 39.9 | 39.5 | 62.2 | 34.2 | 35.6 | 40 |
| **Methods:** | | | | | | | | | | |
| **IHC rat brain** | **tested, No.** | **257** | **41** | **70** | **621** | **476** | **45** | **38** | **45** | **25** |
| | **grade=1 No. (%)** | ’14 (5.4) | 1(2.4) | 2 (2.9) | 40 (6.4) | 29 (6.1) | 4 (9.3) | 2 (5.3) | 2 (4.4) | 3 (12) |
| | **grade=2 No. (%)** | ’8 (3.1) | 0 (0) | 0 (0) | 13 (2.1) | 10 (2.1) | 2 (4.7) | 1 (2.6) | 0 (0) | 0 (0) |
| | **grade=3 No. (%)** | ’5 (1.9) | 0 (0) | 1 (1.7) | 14 (2.3) | 10 (2.1) | 1 (2.3) | 2 (5.3) | 1 (2.2) | 0 (0) |
| **CBA (IHC+cohort)** | **tested, No.** | **27** | **1** | **3** | **67** | **49** | **7** | **5** | **3** | **3** |
| | identified antigen | 1x GAD65; 2x GAD67; 3x Caspr2* | | | 1x GAD65; 2xGAD67; 3x Caspr2 | 1xGAD65; 1x GAD67; 2x Caspr2* | | 1x Caspr2* | | |
| **Live Neurons** | **tested, No.** | **5** | **0** | **1** | **14** | **10** | **1** | **2** | **1** | **0** |
| | **positive, No.** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **NMDAr CBA** | **tested, No.** | **214** | **37** | **65** | **239** | **123** | **22** | **36** | **40** | **25** |
| | **positive, No. (%)** | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| **VGKC RIA** | **tested, No.** | **0** | **0** | **2** | **106** | **55** | **19** | **0** | **32** | **0** |
| | **borderline, No. (%)** | | | 0 (0) | 3 (2.8) | 2 (3.7) | 0 (0) | | 1 (3.1) | |
| | **positive, No. (%)** | | | 0 (0) | 4 (3.7) | 4 (7.2) | 0 (0) | | 0 (0) | |
| **SARD-related antibodies** | **tested, No.** | **152** | **23** | **45** | **199** | **114** | **41** | **0** | **43** | **0** |
| **ANA IFA** | **borderline, No. (%)** | 22 (14) | 3 (9.1) | 5 (11.6) | 20 (10) | 10 (9.1) | 7 (17.9) | | 3 (7.0) | |
| | **positive, No. (%)** | 2 (1.3) | 1 (4.3) | 5 (11.6) | 23 (19.3) | 13 (11.4) | 8 (20.5) | | 2 (4.7) | |
| **ANA ELISA** | **positive, No. (%)** | 2 (1.3) | 1 (3) | 1 (2.3) | 4 (2) | 2 (1.8) | 2 (5.1) | | 0 (0) | |
| **dsDNA ELISA** | **positive, No. (%)** | 1 (0.7) | 1 (4.3) | 0 (0) | 7 (3.5) | 4 (3.5) | 3 (7.7) | | 0 (0) | |
| **RPP ELISA/LIA** | **positive, No. (%)** | 9 (5.9) | 0 (0) | 0 (0) | 7 (3.5) | 5 (4.5) | 2 (5.1) | | 0 (0) | |
| **aCL ELISA** | **positive, No. (%)** | 12 (7.9) | 1 (3) | 1 (2.3) | 9 (4.5) | 6 (5.5) | 2 (5.1) | | 1 (2.3) | |
| | No. (IgA/IgG/IgM) | (1/5/0) | (0/0/0) | (1/1/0) | (3/1/1) | (1/1/1) | (1/0/0) | | (1/0/0) | |

[1]Controls consist of 200 blood donors and 57 individuals without a psychiatric diagnosis.

*Positive by live CBA

**Table 3: Characteristics of patients with positive CBA results**

| | CBA result[1] | Conc. | Diagnoses | Age | Sex | IHC grade[2] (UM/IDIBAPS) | correlating pattern[3] | Live neurons | Combined conclusion[4] |
|---|---|---|---|---|---|---|---|---|---|
| Case 1 | Caspr2 | 1:50 | schizophrenia | 41 | m | 1/neg. | Yes | neg. | ? |
| Case 2 | Caspr2 | 1:50 | brief psychotic | 39 | f | 2/pos. | No | neg. | ? |
| Case 3 | Caspr2 | 1:100 | control[5] | 67 | f | 2/pos. | Yes | neg. | Caspr2+ |
| Case 4 | Caspr2 | 1:200 | control[5] | 23 | f | 1/neg. | Yes | neg. | ? |
| Case 5 | Caspr2 | 1:100 | schizophrenia | 28 | f | 1/neg. | No | neg. | ? |
| Case 6 | Caspr2 | 1:100 | control[5] | 47 | f | 1/neg. | No | neg. | ? |
| Case 7 | GAD65/67 | 1:6400 | schizophrenia | 43 | f | 3/pos. | Yes | neg. | GAD65/67 |
| Case 8 | GAD65/67 | 1:3200 | control[5] | 59 | m | 2/NA | Yes | neg. | GAD65/67 |
| Case 9 | GAD67 | 1:100 | schizophrenia | 31 | f | 1/neg. | No | neg. | ? |
| Case 10 | GAD67 | 1:100 | control[5] | 55 | f | 1/neg. | No | neg. | ? |
| Case 11 | GAD67 | 1:200 | psychosis NOS | 32 | m | 1/neg. | No | neg. | ? |
| Case 12 | GAD67 | 1:100 | control[5] | 68 | m | 2/pos. | No | neg. | ? |

CBA = cell-based assay, IHC = Immunohistochemistry, NOS = not otherwise specified, Conc. = highest still positive serum concentration, UM= Maastricht University, IDIBAPS=Institut d'investigations Biomèdiques August Pi i Sunyer

[1] CBA results for Caspr2 are from live cells, CBA for GAD was performed on fixed cells

[2] IHC at UM was graded 0-3 and at IDIBAPS positive/negative

[3] indicates whether the IHC hippocampal pattern resembles the typical pattern of the antigen identified by CBA

[4] A conclusion is drawn based on the combination of different methods. Only, if a serum is tested positive by CBA and the IHC pattern correlated with the CBA results, it is considered positive

[5] Controls consist of blood donors and individuals without psychiatric diagnoses

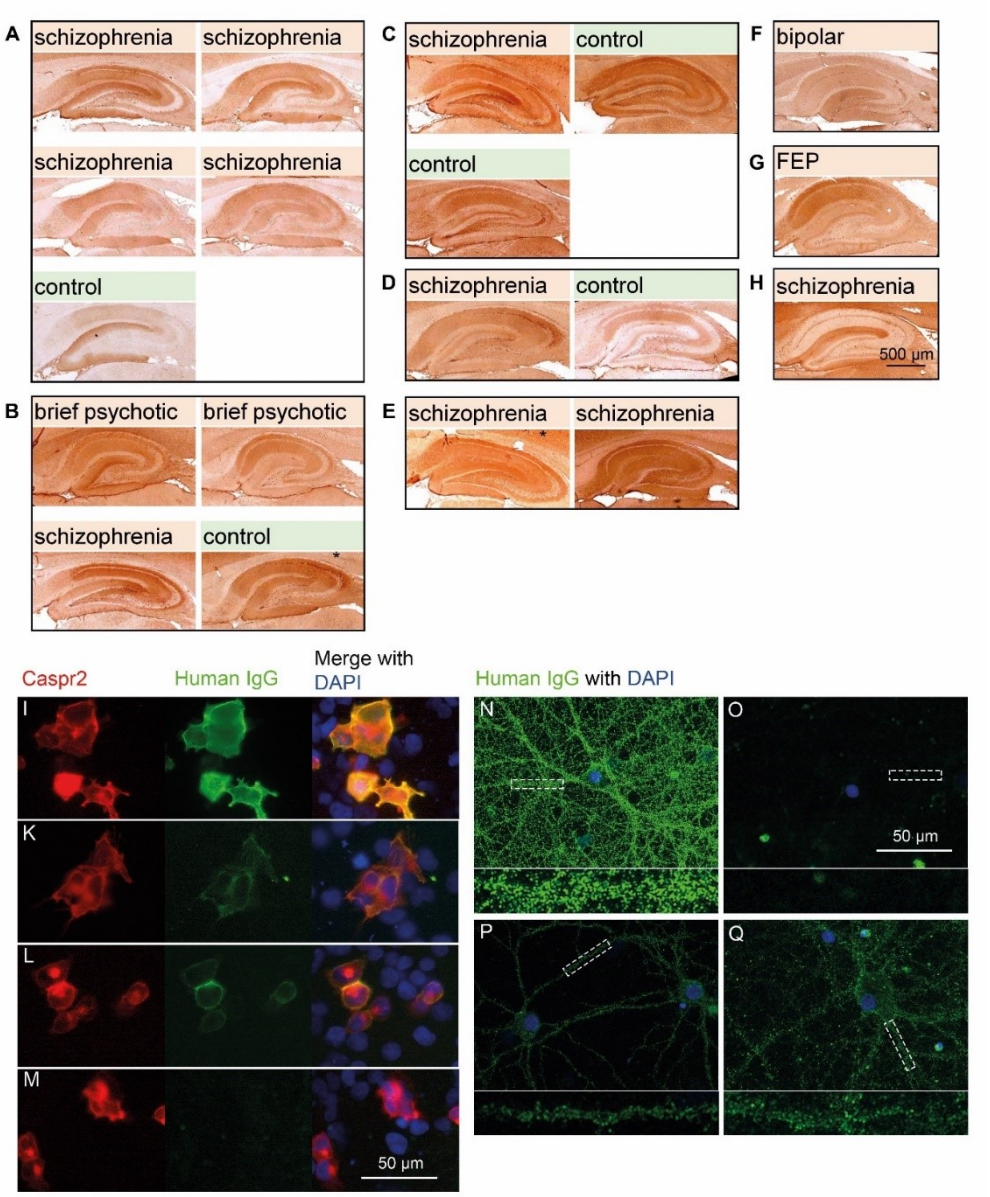


**Figure 2.** Images from sera positive by rat brain immunohistochemistry (IHC), for Caspr2 or neuronal cells. Hippocampal IHC patterns graded 3 and unknown for the specific antigen were sorted according to similarity into eight groups. Each image represents reactivity of one single serum. Sera from control individuals are labelled in green. Each box represents sera with similar hippocampal pattern. Images labelled with * are from sera that were also reactive on live hippocampal neurons. I-M) Human sera were incubated on live HEK293 cells transfected with Caspr2 and stained with anti-human Alexa-488 (green). The red panel indicates the commercial antibody staining for the antigen Caspr2 (1:1000) followed by goat-anti-rabbit Alexa594 (1:1000). The presented results are from A) positive control serum (encephalitis). B+C) positively tested individuals and D) negative control. N-Q) Live hippocampal neurons were incubated with human serum and detected with anti-IgG fluorescent labelled secondary antibodies. Each image shows the full image neuron and underneath the zoom of the white stipulated area. Sera were diluted starting at a maximum concentration of 1:50 and included N) a positive control serum (encephalitis with DPPX antibodies), O) negative control serum, P) serum from a healthy individual with low reactivity on neurons, and Q) serum from an individual with schizophrenia and low reactivity to live neurons.

FEP= first-episode psychosis.

**SARD-related autoantibodies against ANA, dsDNA, RPP, and aCL are not increased in patients with psychotic disorders**

We investigated the prevalence of SARD-related antibodies in sera of a sub-cohort consisting of 199 patients with psychotic disorders, 45 with affective disorders, 23 patients with other mental disorders and 152 healthy individuals. The ANA reactivity of sera to ANAs on HEp-2 cells was increased in schizoaffective individuals compared to healthy (p= 0.032). However, the number of sera tested positive for specific antigens here was too low for relevant statistical analysis. We found that 3 sera (2 schizophrenia, 1 schizoaffective disorder) were consistently positive in different diagnostic assays ( Supplementary Table 2), so it might be relevant to test for clinical signs of SLE.

**Supplementary Table 2: Three cases with comorbid anti-nuclear autoantibodies specific to systemic lupus erythematosus**

| | | | | | ELISA | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Diagnose** | **age** | **sex** | **Illness duration (yrs.)** | **ANA IFA** | **ANA** | **dsDNA** | **RPP** | **aCL** | |
| Schizophrenia, (Paranoid Type) | 39 | Male | 0.5 | + | + | - | + | + | (IgG) |
| Schizoaffective Disorder | 23 | Female | 8 | + | + | + | - | + | |
| Schizophrenia, (Paranoid Type) | 21 | Female | 4.9 | + | - | - | + | + | (IgA) |

ELISA=enzyme-linked immunosorbent assay, RPP=ribosomal protein P, dsDNA=double-stranded Deoxyribonucleic acid, ANA = anti-nuclear antibodies, aCL= cardiolipin, ANA IFA= immunofluorescence on HEp-2 substrate for detection of antinuclear antibodies.

## Discussion

In the last 10 years, 16 novel autoimmune diseases of the CNS have been identified, leading to new treatment strategies for neurological and psychiatric syndromes. [39] However, our study indicates that the prevalence of NSAbs is low in patients with psychotic disorders. Moreover, it is not significantly different from prevalence in healthy populations. These findings are in line with previous reports [19, 40] but other studies found 1-11% of patients positive for NMDAR autoantibodies [18, 21, 22]. Reported discrepancies in the literature are likely caused by inter-laboratory variations of methodology, such as differences in used antigen species or splice-variants, fixation of the cell, etc. as well as differences in cohort selection. [21]. To confirm the low prevalence of NSAbs, CSF should be included. The neurological assessment could further help characterize cases with a suspected autoimmune condition.

The disadvantage of rat brain IHC and rat neuronal cultures is that autoantibodies against certain antigens might not be detected due to, for example, low antigen expression (e.g. D2DR) or interspecies difference in the amino acid sequence of antigen. Also, antibodies targeting yet unsuspected antigens on other cell types than neurons, e.g. microglia or astrocytes are likely not detected in neuronal cultures.

Concerning the occurrence of autoantibodies in healthy individuals, it should be noted that similar patterns in the IHC's of rat brain do not necessarily imply that antibodies bind to the identical antigen. In addition, in this study, most individuals in the control group were not specifically tested for symptoms of a mental disorder. Thus, it cannot be ruled out that these

individuals had undisclosed psychiatric symptoms or were developing them. However, it should be kept in mind that the presence of autoantibodies does not necessarily lead to disease [37, 41, 42] and it will thus be a difficult task to eventually distinguish pathogenic immunity from anti-brain autoimmunity as defined by protein binding.

SARD-related antibodies have been observed earlier in connection with mental illness. However, antibodies to specific antigens were only detected in 3 patients. Further clinical assessment of these patients would be necessary to determine a possible diagnosis of SLE. In view of the intracellular location of these antigens, perhaps more focus should be given to T-cell functioning in psychotic disorders. Several CNS autoimmune diseases are known to be largely T-cell mediated [43, 44],[45, 46],[47] and dysregulation of T-cells has also been observed in antibody-associated disorders such as NMDAr encephalitis, neuromyelitis optica and stiff person syndrome [48]

The role of autoimmunity in a subgroup of patients with psychotic disorders is undeniable. Yet, autoantibodies targeting neuronal surface antigens are rare and therefore challenging to identify and characterize, especially in high-throughput screening with a low pre-test probability. By now there is no doubt that autoantibodies against a number of neuronal surface antigens, besides neurological signs, do cause psychiatric symptoms including psychosis which can be treated by immunosuppression. This article shows, however, that the reverse is not true, namely that in psychiatric patients without neurological symptoms the presence of such antibodies is, at best, extremely rare as judged from the plasma assays. The prevalence of autoantibodies in psychosis has likely been overestimated in some initial studies. Future challenges are now to include the description of neurological symptoms [49] and develop new screening methods (for new and already identified antigens) that could help to identify a subgroup with general indication of an autoimmune condition including T-cell autoimmunity and CSF analysis.

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**Conflict of interest**

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grant from Euroimmun AG. MT has filed a patent for methods for typing neurological disorders and cancer, and devices for use therein. Celso Arango has been a consultant to or has received honoraria or grants from Acadia, Ambrosseti, Gedeon Richter, Janssen Cilag, Lundbeck, Merck, Otsuka, Roche, Servier, Shire, Schering Plough, Sumitomo Dainippon Pharma, Sunovion and Takeda.

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# Chapter 6

## Autoantibodies to glutamic acid decarboxylase 65 (GAD65-Abs) and other neuronal antigens in a cohort suspected of GAD-Ab related disorders: comparison of diagnostic methods and suggestions for clinical practice

**Corresponding manuscripts:**

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**Abstract**

**Background:** Autoantibodies against GAD65 (GAD65-Abs) can be detected by various methods and are associated with diverse autoimmune and paraneoplastic conditions. However, the value of these methods has not been clearly evaluated. Meanwhile, pathogenic autoantibodies other than GAD65 may co-occur in patients suspected with GAD-related disorders.

**Objective**: To compare GAD65-Ab detection methods and to search for other neuronal autoantibodies in a cohort of patients with suspected GAD-Ab related disorders.

**Methods:** Ninety-six consecutive sera previously assessed for GAD65-Ab level in routine clinical practice by ELISA were studied. Clinical indications for testing were suspected autoimmune encephalitis, epilepsy, diabetes mellitus type 1 or latent autoimmune diabetes in adults (DM1/LADA). Sera were re-tested for GAD65-Ab by ELISA, cell-based assay (CBA) and immunohistochemistry (IHC) on rat brain tissue. Moreover, the presence of other neuronal autoantibodies was tested by CBA for GAD67-Abs and IHC. Samples that were IHC positive to unknown antigens were further tested by immunofluorescent staining on cultured rat hippocampal live neurons and/or selected CBAs.

**Results:** Results from clinical tests (ELISA) were confirmed in 93 patients; 3 patients previously tested with low GAD65-Ab were tested negatively upon re-analysis by ELISA. From the 47 ELISA positive samples, 23% (11) were positive by GAD65-CBA and 17% (8) were positive by IHC. 21% (10) sera were also positive for GAD67-Ab by CBA. In the ELISA positive cases, the average GAD65-Ab levels in patients diagnosed as GAD-related encephalitis/epilepsy is higher than in DM1/LADA patients ($p=0.04$, t-test). All samples with GAD65-Ab levels above 10000 U/mL tested positive by both IHC and CBA, of which 3 cases were from GAD-related encephalitis/epilepsy and 4 from DM/LADA. None of the negative samples by ELISA was positive by GAD65 or GAD67-CBA. Two patients from the GAD-related autoimmune encephalitis/epilepsy cohort were positive by IHC for autoantibodies to antigens other than GAD65, one had Hu-Ab (consistent with the clinical test result) and another had anti-mGluR1-Ab according to the IHC staining patterns, which was confirmed by CBA. Two samples from the DM1 cohort were positive by IHC with unknown patterns but negative on live neurons.

**Conclusion:** Serum autoantibody levels in patients with GAD related autoimmune encephalitis/epilepsy were higher than in patients with DM1/LADA although high levels of GAD65-Ab (>10000 U/mL) that could be detected by CBA and IHC existed in both groups. Therefore, the clinical relevance, even of these high levels, remains to be elucidated. Only a small portion of patients suspected of GAD-related autoimmune disorders had other neuronal autoantibodies and their clinical significance should be studied individually.

## Introduction

Autoantibodies to GAD65 (GAD65-Ab) have been associated with a range of neurological conditions, such as stiff-person syndrome (SPS), epilepsy, limbic encephalitis, cerebellar ataxia and paraneoplastic neurological syndromes [1]. GAD65-Abs are also a predictive marker for the diagnosis of type-1 diabetes mellitus (DM1), as the presence of GAD65-Ab suggests autoimmune destruction of the insulin-producing beta cells in the pancreas [2]. However, the intracellular location of the epitope creates doubts about the pathogenicity of GAD65-Ab.

Normally, GAD65-Ab levels are considered to be much higher in neurological disorders related to GAD65-Ab than in DM1 [3-5]. These levels derive from enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA) tests, which is mainly established for detecting GAD65-Abs in DM1. As the spectrum of GAD-Ab related disorders expanding, the antibody levels between different diseases groups are needed to re-evaluate. Recently, thanks to the knowledge gathered from diagnostic methods for autoimmune encephalitis, cell-based assay (CBA) and rat brain based immunohistochemistry (IHC) have also been used for GAD65-Ab detection [12]. CBA is able to identify the binding of autoantibodies to their specific antigen which is expressed in their native conformation, which is especially useful to detect autoantibodies targeting conformational epitopes. IHC can detect autoantibodies to a broad range of proteins present in the rat brain (particularly in hippocampus or cerebellum) with high homology to human neuronal proteins. However, until now, a screening set-up for GAD65-Ab by IHC based on hippocampal pattern has not been reported.

Additionally, GAD65-Ab could have merely a bystander function especially in lower concentrations, and other pathogenic autoantibodies could be present in the same individual such as autoantibodies against γ-aminobutyric acid receptors (GABA-R) [6, 7], glycine receptor (GlyR) [8, 9] and other unidentified neuronal autoantibodies [10, 11], or even autoantibodies to GAD67, also in the absence of GAD65-Ab [12-14]. However, it is not clear from the clinical phenotype which patients could have autoantibodies against GABA-R, GlyR [5] or GAD67. Thus, searching for other neuronal autoantibodies in patients with GAD-Ab autoimmune-related neurological disorders may help to further explain the diversity of clinical manifestations.

Therefore, in the current study, we used sera from suspected autoimmune encephalitis/epilepsy and DM1 patients screened by ELISA for GAD65-Abs for clinical diagnosis and compared sera reactivity to GAD65 by ELISA, CBA, and IHC with focus on the hippocampal staining pattern. We intended to better illustrate the gap between these methods in GAD65-Ab detection, and additionally, we aimed to test for the presence of other neuronal autoantibodies (especially GAD67-Abs).

## Methods

### Cohort

We included sera that were screened for GAD65-Ab by ELISA at Maastricht University Medical Centre (MUMC+) and Kempenhaeghe Epilepsy Centre between 2010 and 2014 (reported previously)[15], and patient sera from patients with DM1 or Latent Autoimmune Diabetes of Adulthood (DM1/LADA) available at the clinical diagnostic laboratory at MUMC+. This cohort consisted of 119 patients with suspected autoimmune neurological disease and 117 patients with DM1/LADA [15]. From the samples that were initially tested ,

for this retrospective study serum was retrieved from 56 patients with suspected autoimmune encephalitis/epilepsy (10 positive for GAD65-Abs, 7 were diagnosed as GAD related encephalitis/epilepsy, and 3 with encephalitis/epilepsy), 46 negatives for GAD65-Ab and 40 patients diagnosed as DM1/LADA positive for GAD65-Ab. The clinical characteristics of the total study population are shown in Table 1. The clinical data including age, sex, clinical indication for the GAD-Ab test, comorbidity of tumors, and history of autoimmune disorders or cancer as well as the presenting symptoms [epilepsy, cognitive complaints (attention or memory deficits), psychiatric symptoms (anxiety, depression, and psychosis), encephalopathy (altered consciousness, confusion), extrapyramidal symptoms, ataxia, or paresis, as reported by the patient and/or documented by the treating physician] were obtained from the clinical records [16]. We also (re-)assessed the clinical diagnosis of GAD65-Ab related encephalitis/epilepsy (AV, RR).

**Table 1: Characteristics of the study population**

| | **DM1/LADA** | **Suspected autoimmune encephalitis/epilepsy** | | **p-value** |
|---|---|---|---|---|
| Previous GAD65-Ab ELISA result | 40 positive | 10 positive | 46 negative | |
| Age (Mean/range) | 38/4-68 | 36/15-66 | 42/9-70 | NS |
| Sex | | | | |
| female | 18 (46.2%) | 6 (60.0%) | 24 (52.2%) | NS |
| male | 21 (53.8%) | 4 (40.0%) | 22 (47.8%) | NS |
| children (< 18 years) | 9 (23.1%) | 3 (30.0%) | 1 (9.1%) | NS |
| Indication for anti-GAD test request | | | | |
| Autoimmune encephalitis | 0 | 4 (40.0%) | 27 (58.7%) | NS |
| Refractory epilepsy | 0 | 6 (60.0%) | 19 (41.3%) | NS |
| Diabetes | | | | |
| DM1/LADA | 100% | 6 (60.0%) | 0 | 0.0001* |
| Symptoms | | | | |
| Epilepsy | 0 | 10 (100%) | 42 (91.3%) | NS |
| Cognitive impairment | 0 | 5 (50%) | 14 (30.4%) | NS |
| Psychiatric | 10 (25.6%) | 2 (20%) | 16 (34.8%) | NS |
| Encephalopathy | 0 | 0 | 1 (2.2%) | NS |
| Extrapyramidal | 0 | 0 | 1 (2.2%) | NS |
| Ataxia | 0 | 0 | 1 (2.2%) | NS |
| Paresis | 0 | 1 (10%) | 2 (4.3%) | NS |
| Other autoimmune disorders | 0 | 0 | 3 (6.5%) | NS |
| Tumor | 0 | 1 (10%) | 2 (4.3%) | NS |
| Diagnosed as anti-GAD encephalitis/epilepsy | 0 | 7 | 0 | |
| Treated with immunotherapy | 0 | 4 | 0 | |
| Response to immunotherapy | 0 | 4 | 0 | |
| Diagnosed as other types of autoimmune encephalitis | 0 | 1 (Anti-Hu) | 1 (Anti-VGKC) | |

*Fisher exact test between positive and negative cases in the suspected autoimmune brain disease group. Abbreviations: GAD65-Ab: glutamate decarboxylase 65 autoantibodies; DM1/LADA: diabetes mellitus type 1 or latent autoimmune diabetes in adults; Anti-Hu: anti-Hu autoantibodies; Anti-VGKC: antibodies against the voltage-gated potassium channel-complex; NS: no significant difference.

Besides the GAD-Ab test, other neuronal autoantibodies were previously tested in routine clinical practice in seven patients from the suspected autoimmune encephalitis/epilepsy group as well. Three patients showed positive results (one case had autoantibodies to VGKC and GAD, one case had autoantibodies to Hu and GAD, and one case had VGKC autoantibodies only).

Ethical approval was obtained from the medical ethical committees of the two participating centers, MUMC+ and Kempenhaeghe (METC 15-4-002).

**Autoantibody detection methods:**

**ELISA**

During routine clinical diagnosis, an ELISA for GAD65-Ab detection was performed at several different (inter)national reference laboratories (all accredited according to national standards) using commercial ELISA kits following manufacturers' instructions. Measurements were expressed in units/mL, result values below 5 units/mL (U/mL) were considered negative. Due to inter-laboratory differences, the exact titers could not be compared. To compare antibody levels, all the samples were retested using the commercial ELISA kit (RSR Limited, Cardiff, UK) in our laboratory according to manufacturers' instructions.

**Cell-based assay for GAD65/67-Ab**

Antigen-specific screening for GAD65 and GAD67 was performed using sera and incubated it onto HEK293 cells transfected with plasmids pCMV6-XL5 containing human GAD65 and GAD67 (obtained from Dr. Francesc Graus as a kind gift). Generally, HEK293 cells were plated on coverslips coated with poly-D-lysine (#P7280, Sigma) in 60 mm-culture plates (#628160, Greiner Bio-One) in Dulbecco's Modified Eagle Medium with 10% fetal calf serum, 4 mM L-glutamine and 100 units/ml penicillin-streptomycin and incubated overnight to attach. Cells were transfected with polyethylenimine (#23966, Polysciences Inc.) and 4 µg expression vectors encoding the according antigen and expression allowed for 22-26 hrs. Cells were fixed in 3.5% formaldehyde (#87837.180, VWR) for 10 minutes and permeabilized with 0.3% Triton-X-100 for 10 minutes. After blocking with 1% bovine serum albumin (BSA) for 1 h, cells were incubated with human sera diluted 1:40 in 1% BSA together with an antibody targeting the according antigen for 1 h at RT. The two commercial rabbit anti-human GAD65 (1:1000, 7309LB) and GAD67 antibodies (1:1000, 10266/20B) were kind gifts of Christiane Hampe (University of Washington). Goat-anti-human-IgG-Alexa488 (1:1000, # A11013, invitrogen) and donkey-anti-rabbit-Alexa594 (1:1000, #A21207, invitrogen) were used as secondary antibodies for visualization of the staining. Cover glasses were mounted onto 7 µl DAPI mounting medium (#H-1200, vector laboratories) and evaluated by two observers of which one was blinded of the sample's information. Samples were analyzed on a BX51 Olympus microscope for antibody reactivity. A serum sample positive for GAD65-abs and GAD67-Abs from a refractory epilepsy patient who was retested several times during the disease progress in the clinic was used as positive control and another sample from a healthy individual was used as negative control. Results were graded as strong positive, positive, weak positive and negative. All the samples were tested once and positive samples were repeated at least once more for verification.

## Immunohistochemistry (IHC) on rat brain

Neuronal autoantibodies were identified by IHC on rat brain tissue following standard procedures with minor adaptation [17]. In brief, rat brains were fixed for 1 h in 4% paraformaldehyde and cryoprotected by incubating in 30% sucrose solution. Frozen brains were cut into 7-µm thick tissue sections using a Leica CM3050S cryostat and stored at -80 ºC. Sections were subsequently blocked with 0.3% $H_2O_2$ for 10 minutes followed by incubation with 5% goat serum for 15 minutes at RT. Next, sections were incubated with human serum diluted 1:200 in 5% goat serum overnight at 4 °C. Next, the tissue was incubated with 200 µl biotinylated goat anti-human IgG Fcγ (1:1000, 109-066-008, Jackson laboratory) for 2 h at RT, followed by an incubation with the same amount of Vectastain Elite ABC kit (Vector lab., # PK 6100) mixture, for 1 h at RT and the reactivity developed using diaminobenzidine. Each staining included a negative control and a positive control serum for GAD65-Ab (serum from an autoimmune GAD-Ab encephalitis patient, which was tested GAD65 and GAD67-Ab positive by CBA and gave typical pattern on rat brain). Images were taken by the VENTANA iScan HT slide scanner (20 × objectives) and graded by 2 experienced observers separately (S. Zong, C. Hoffmann or M. Damas) by using Ventana Image Viewer for the hippocampal reactivity of sera as negative (0), borderline (1), weak positive (2), strong positive (3), based on the intensity and contrast of the staining. Staining was repeated and only if repeatedly was found positive with the same pattern by 2 observers, a final decision of positivity was made.

## Staining on live neurons

Rat hippocampal neuronal staining was performed as previously described [18]. Patients' sera (1:50 in Neurobasal with 1% BSA and 25 mM HEPES) were added to live neurons (cultured for 3 weeks before use) and incubated 1 h at RT followed by fixation with 3.5% formaldehyde. Bounded antibodies were labeled with goat-anti-human-IgG Fcγ-Alexa488 (1:1000, #109-546-170, Jackson) and visualized in a BX51 Olympus microscope. Samples with NMDAR and AMPAR autoantibodies from autoimmune encephalitis patients were used as positive controls and one sample from a healthy individual was used as negative control.

## Results

### CBA and IHC only detected sera with high level of GAD65-Ab (>396U/mL) by ELISA

Overall, 94% (47/50, 38 from the DM1/LADA group and 9 from the suspected autoimmune encephalitis/epilepsy group) were confirmed positive, and 100% (46/46, from the suspected autoimmune encephalitis/epilepsy group) were confirmed negative by ELISA for GAD65 (Figure 1). From the 9 samples confirmed positive by ELISA in the suspected autoimmune encephalitis/epilepsy group, only 7 patients had a clinical diagnosis of anti-GAD related encephalitis/epilepsy. As expected, the average GAD65-Ab levels in patients diagnosed as GAD-related encephalitis/epilepsy (n=7) was higher, than in patients with DM1/LADA (n=38; 103410.6 U/mL vs 7792.6U/mL; p=0.035, t-test; data were lognormal transformed before analysis). 11 patients had GAD65-Ab levels >396 U/mL in ELISA (8 were from the DM1/LADA group and 3 from the suspected autoimmune encephalitis/epilepsy group) and they were also positive for GAD65-Ab by CBA. From the 11 positive ELISA and CBA samples 8 samples (>3219 U/mL in ELISA) were positive by IHC for GAD65-Ab according to pattern (Figure 2 A and C); The strongest reactivity occurred on the cell bodies and there was a gradient staining on the outer layer of the dentate gyrus (DG). All samples (n=7) with antibody levels

above 10000 U/mL were scored positive by CBA and IHC. None of the negative samples was positive by CBA or showed a GAD-Ab staining pattern by IHC.

**Besides GAD65, other neuronal autoantibodies were found in both patient groups**

21% of the sera (10/47) which were confirmed positive by GAD65-Ab ELISA were also positive by GAD65-Ab and GAD-67-Ab CBAs. Moreover, All GAD-65 ELISA negative patients from the same cohort were also assessed negatively in both the GAD65-Ab and GAD67-Ab CBAs.

Three sera positive by ELISA gave staining patterns different from typical GAD65 by IHC (Fig 2, D, E, F). One belonged to a patient with anti-Hu antibodies diagnosed with paraneoplastic encephalitis (GAD65-Ab levels 19.3 U/mL) and two belonged to LADA patients (GAD65-Ab levels were 779.1 U/mL and 1136.5 U/mL respectively). The encephalitis

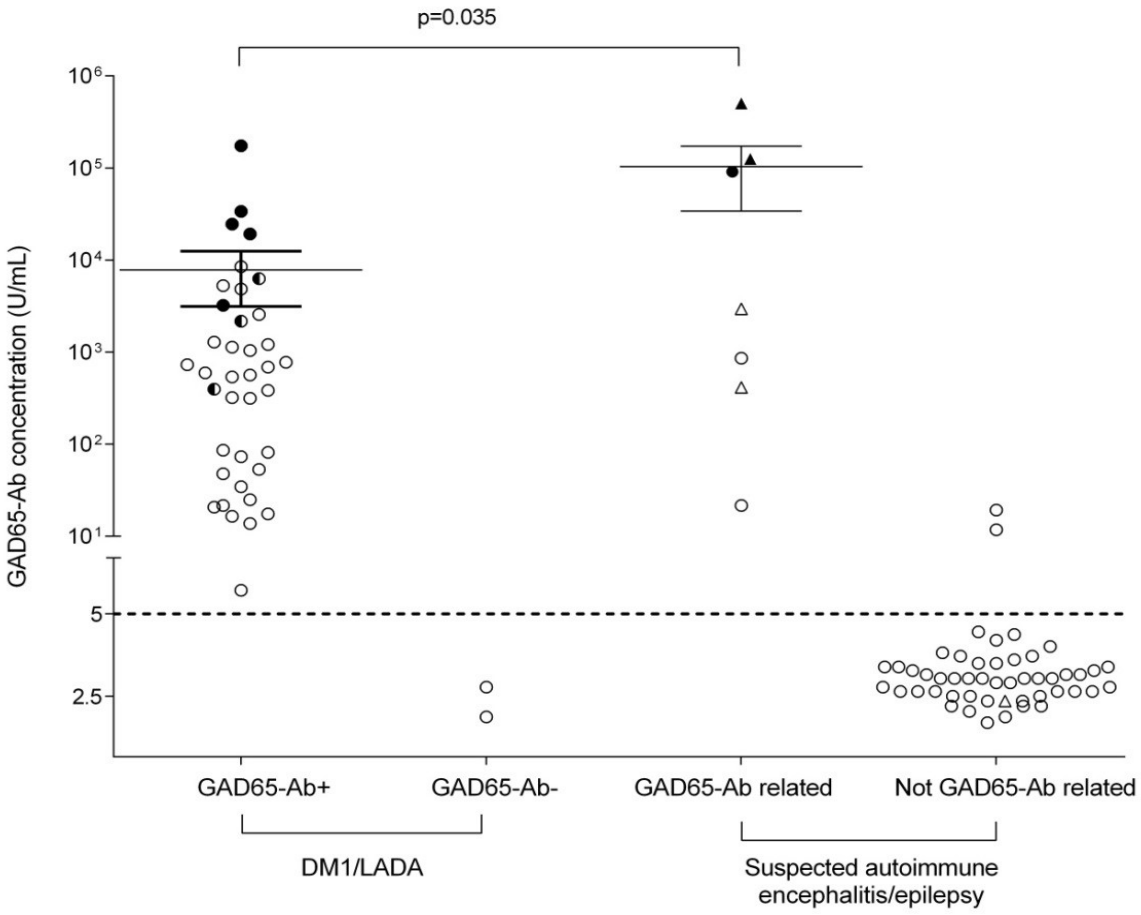


**Figure 1.** Analysis of GAD65-Ab by ELISA, CBA, and IHC in sera of patients with DM1/LADA or suspected autoimmune encephalitis/epilepsy. Circles indicate samples from both the DM1/LADA and the suspected autoimmune encephalitis/ epilepsy cohorts. Triangles represent patients with both DM1/LADA and suspected autoimmune encephalitis/ epilepsy. Samples with GAD65-Ab levels above 5 U/ml were considered positive by ELISA. Half-black symbols indicate samples which are also positive by CBA (CBA+), full black symbols indicate cases which were both positives by CBA and IHC (CBA and IHC+). All samples with GAD65-Ab levels ≥ 10000 U/mL were positive by CBA and IHC as well, which were found in both groups. The GAD65-Ab level in GAD-related encephalitis/epilepsy is higher than the antibody level in DM1/LADA, p=0.035, t-test.

patient tested anti-Hu positive had a lung tumor and the IHC staining pattern was typical for anti-Hu-antibodies [Fig 2, and table 2, (case 1)] [19, 20]. One of the patients with an unknown IHC pattern from the LADA cohort had clear reactivity in the area corresponding to the molecular layer of DG and CA3 regions, the second patient with unknown IHC pattern from LADA had a diffuse staining over the hippocampus (Fig 2, E and F; Table 2, Case 2 and 3). Clinically the last patient also suffered from panic attacks/anxiety disorders. These two samples were further tested negative on live neurons.

Additionally, to investigate whether the GAD65-Ab negative cases suspected with autoimmune encephalitis/epilepsy had autoantibodies to other neuronal antigens; we further analyzed the IHC staining for other neuronal autoantibody patterns, besides the standard patterns identified in autoimmune encephalitis. A sample was identified which gave a strong reactivity in the CA3 and DG area of the hippocampus and the molecular layer of the

**Table 2: 4 Clinical characteristics of 4 cases with other neuronal autoantibodies identified by IHC**

| | Sex | Age (year) | ELISA GAD65-Ab | CBA GAD65/67-Ab | IHC | Diagnosis | Comorbidity | Treatment | Response to treatment |
|---|---|---|---|---|---|---|---|---|---|
| Case 1 | F | 64 | 19.3 | Negative | Anti-Hu | Anti-Hu encephalitis | Lung cancer | Chemo-radiation therapy and antiepileptic drugs | Unknown |
| Case 2 | M | 50 | 779.1 | Negative | Weak positive to unknown antigens | LADA | Anxiety | NA | NA |
| Case 3 | M | 65 | 1136.5 | Negative | Weak positive to unknown antigens | LADA | no | NA | NA |
| Case 4 | F | 51 | 3.4 | Negative | Anti-mGluR1 | Refractory epilepsy (anti-mGluR1 encephalitis)* | no | Valproic acid (gamma globulins)* | Seizure reduction; (further reduction)* |

* After the finding of anti-mGluR1 by IHC in the serum of case 4, the patient was re-diagnosed as autoimmune encephalitis, subsequently treated by gamma globulins and with a short follow-up, she responded modestly [no seizures and decrease of general symptoms (fatigue)].

Abbreviations: GAD65/67-Ab: glutamate decarboxylase 65/67 autoantibody; ELISA: enzyme-linked immunosorbent assay; CBA: cell-based assay; IHC: immunohistochemistry; Anti-Hu: anti-Hu autoantibodies; Anti-mGluR1: autoantibodies against metabotropic glutamate receptor 1; LADA: Latent Autoimmune Diabetes in Adults (a form of diabetes mellitus type 1 that occurs in adulthood, often with a slower course of onset than type 1); NA: not applicable.

cerebellum (Fig 3). The sample corresponded to an epilepsy patient (Table 2, Case 4) with focal seizures and onset with auditory symptoms. The staining pattern was similar to anti-mGluR1 as previously reported [21] and it was confirmed to be mGluR1 by CBA. This serum sample was further analyzed on live neurons, which only gave a relatively weak neuronal surface staining and was considered negative.

## Discussion

This study confirms that CBA and IHC are only found positive when ELISA levels of GAD65-Ab are high. In our hands, all samples with GAD65-Ab > 10000 U/mL were positive both by CBA and IHC with a few exceptions. Our results confirmed that on average, DM1/LADA

patients have lower titers of GAD65-Ab than those with neurological symptoms, while samples with the highest levels could belong to both groups. Because of the strong comorbidity of DM1 in GAD65-Ab positive patients with neurological disorders, the presence of GAD65-Ab and their correlation to neurological manifestations have to be interpreted cautiously.

Whether other coexisting autoantibodies, as well as GAD67-Ab, might be more relevant to disease status than GAD65-Ab can only be assessed case by case. In our study, three cases

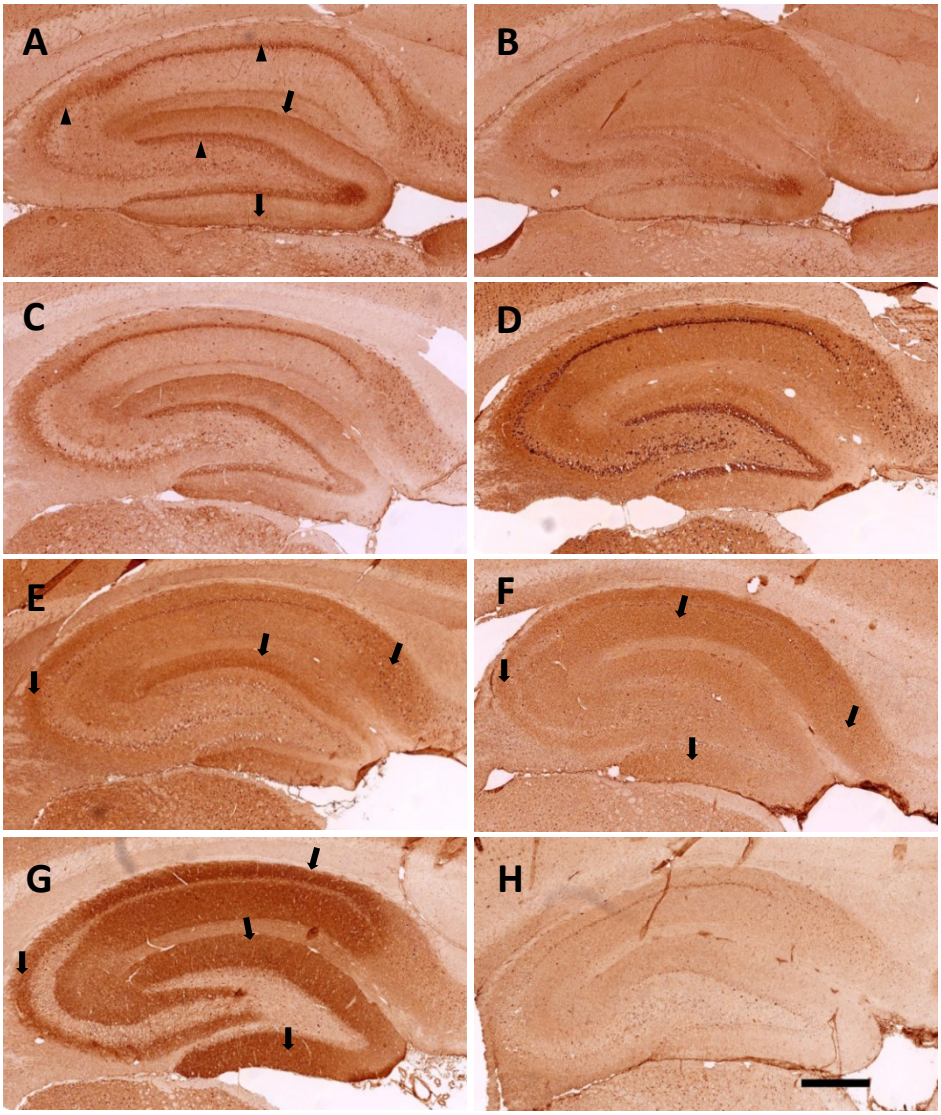


**Figure 2**. IHC staining patterns on rat brain hippocampus given by sera of patients with DM1/LADA or suspected autoimmune encephalitis/epilepsy. Commercial antibodies to GAD65 or GAD67, or human sera were incubated on rat brain slices, followed with biotinylated secondary antibodies, ABC kit and DAB to develop the color reaction. A. Commercial antibodies specific to GAD65 showed intracellular granular staining in the hippocampus (triangles) and neuropil staining in the outer layer of DG region (arrows). B. The commercial antibody directed against GAD67 did not give strong staining in the hippocampus region. C. Positive serum with high GAD65-Ab levels by ELISA (>200000 U/mL) gave the same pattern as the commercial GAD65-Ab. D. Positive serum from an encephalitis patient showed nuclei staining (Anti-Hu) and overall reactivity in the hippocampus. E, F. 2 samples from DM1/LADA patients showed neuropil staining (arrows) in the hippocampus (E, F). G. Positive control serum for NMDAR-Ab gave strong neuropil staining (arrows) through the hippocampus. H. Negative control serum from a healthy individual showed overall background staining. (Scale bar = 500 µm).

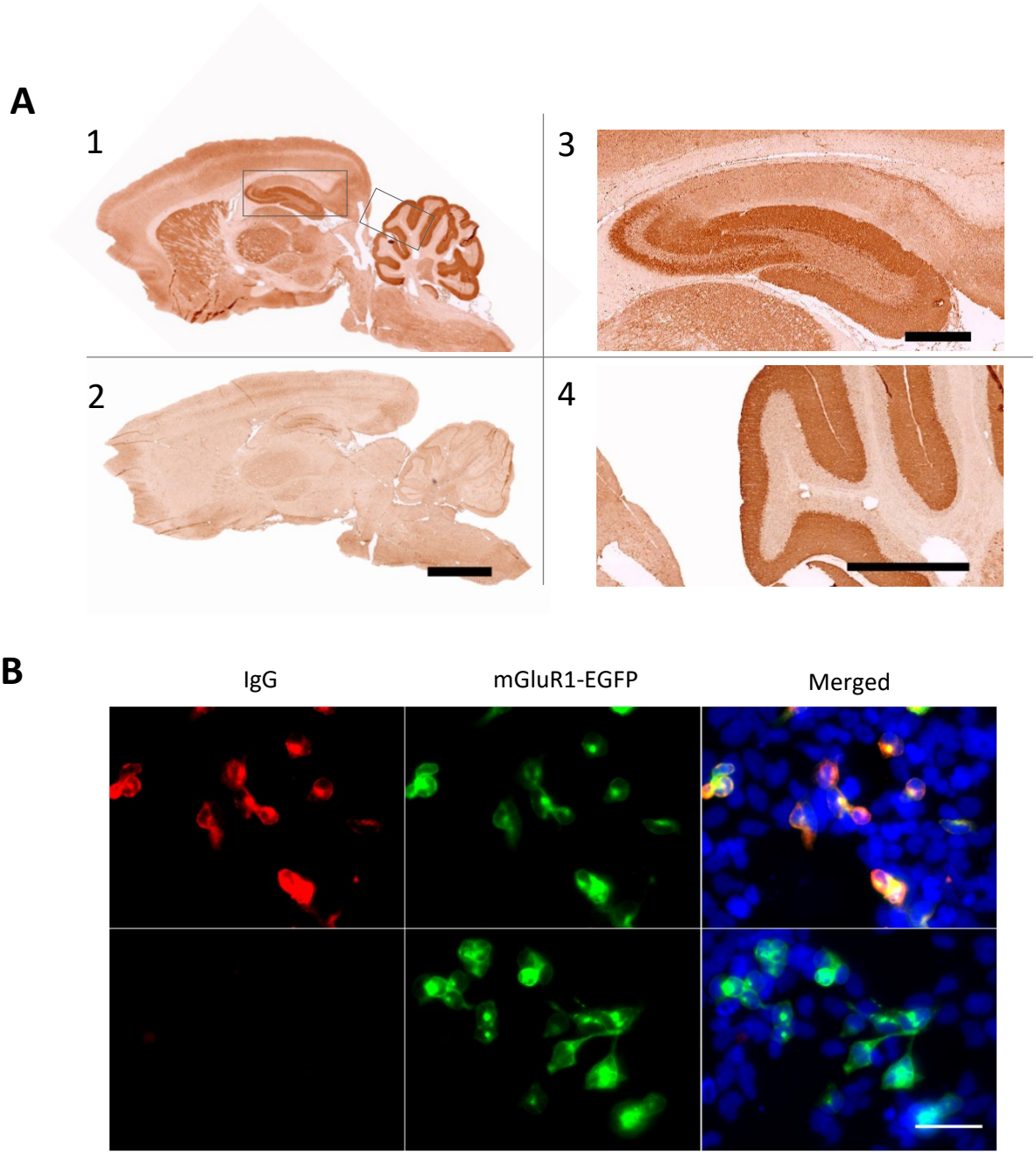


**Figure 3.** Identification of a serum positive for mGluR1 by IHC (A) and CBA (B) in the cohort of patients suspected for an autoimmune brain disorder which was tested GAD65-ab negative. A. IHC**:** Human sera were incubated on rat brain slices, followed with biotinylated secondary antibodies, ABC kit, and DAB to develop the color reaction. 1 and 2 show a rat brain sagittal slice (scale bar=2 mm) and in 3 and 4, zoom from the area marked with a gray square in 1 is shown (scale bar=500 µm). A serum sample from a refractory epilepsy patient (GAD65-Ab-) gave strong reactivity on rat brain by IHC (1) compared to the negative control (2). A zoomed area of the hippocampus showed reactivity mainly in the DG and CA3 areas but was nearly absent in the CA1 area (3). A zoom of the cerebellum showed staining in the molecular layer (4). B. CBA: The patient serum and a control serum were incubated on mGluR1-EGFP (green) transfected HEK cells and labeled with goat anti-human IgG-594 (red), nuclei were stained in DAPI (blue). The patient serum showed a strong reaction to transfected cells (merged) compared to control serum. Scale bar=50 µm.

giving IHC patterns, which were not-typical for GAD65, found among the GAD65-Ab positive cases indicated that they had other coexisting autoantibodies in the sera. One patient had anti-Hu antibodies as confirmed also in clinical tests while her GAD65-Ab titer was low and was finally diagnosed as anti-Hu associated encephalitis. While the other 2 cases had autoantibodies targeting two different unknown antigens according to the IHC staining patterns. Both cases had LADA and one patient also suffered from anxiety. Diabetes had high comorbidity with anxiety or depression disorders but the reason for this correlation is not clear [22, 23]. Reactivity against unknown antigens on IHC was not significantly different between the group with and without anxiety, though numbers were small (IHC positive for other brain autoantibodies in anxiety vs none anxiety DM/LADA patients: 1/3 vs 1/37, p=0.150). Thus, the clinical relevance of these autoantibodies remains unclear.

The anti-mGluR1 positive case (Case 4) found among the 46 ELISA GAD65-Ab negative patients who were suspected with autoimmune encephalitis/epilepsy emphasizes the value of IHC for detecting autoantibodies to antigens that were not routinely tested for clinical diagnoses in neuropsychiatric patients. The anti-mGluR1 positive patient had symptoms of focal epilepsy with focal seizures and auditory symptoms. Interictal EEG showed sporadic focal epileptiform discharges in the left temporal area, MRI revealed no abnormalities, and initial CSF assessment showed 10 leukocytes per microliter and oligoclonal bands. The patient had a partial response to anti-epileptic drugs and still had symptoms with features of chronic encephalitis 4 years later (persisting seizures and cognitive difficulties and fatigue). After the IHC results were known, autoimmune encephalitis was diagnosed. Her symptoms already improved modestly after the first round of immunoglobulins. A clear relationship between anti-mGluR1 and encephalitis/epilepsy was established in this case. It demonstrates that the use of IHC as a supplementary diagnostic tool, may give clinicians timely information and help to better diagnose and treat this type of patients [24-26].

Despite our findings, this study has some limitations. First, the retrospective design led to a dependency on the clinical notes in the electronic patient files, possibly leading to under-reporting of symptoms. Secondly, the false positive rate of the IHC results for detecting novel neuronal autoantibodies in not known. Thus the autoantibodies found positive only by IHC would need always further confirmation if possible with additional tools. Furthermore, we were dependent on the availability of sera in the archives of the participating centers, which led to a relatively small sample size of patients with neurological GAD65-Ab related diseases and also to a low number of patients with high antibody levels.

In conclusion, serum autoantibody levels in patients with GAD65-Ab related encephalitis or epilepsy were higher than in patients with DM1/LADA although high levels of GAD65-Ab (>10000 U/mL) that could be detected by CBA and IHC existed in both groups. Besides, a small portion of patients suspected with GAD related autoimmune encephalitis/epilepsy disorders had other neuronal autoantibodies and their clinical significance should be studied individually.

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# Chapter 7

## General discussion

Over the past decade, the spectrum of antibody-mediated brain disorders has been largely expanded; this is due to the discovery of novel pathogenic neuronal surface autoantibodies (NSAbs) in the last years, relevant to the fields of neurology and psychiatry. While increasing studies focus on autoantibodies against N-methyl-d-aspartate receptor (NMDAR) in a subgroup of psychotic disorder patients, few studies exist which have thoroughly analyzed the presence of other neuronal autoantibodies in mental disorders. To evaluate if neuronal autoantibodies (known and novel) are more common in neuropsychiatric disorders including psychotic, depression and anxiety disorders, we screened for neuronal autoantibodies in plasma of 1739 depression or anxiety patients and 492 controls. Further, we investigated these autoantibodies in sera of 621 patients with psychotic disorders, 70 individuals with affective disorders, 41 with other mental disorders and 257 controls. The approach used in this thesis is designed to investigate the presence of different autoantibodies in the peripheral circulation system of patients with a combination of immunohistochemistry (IHC), cell-based assay (CBA) and staining on live cultured neurons. Furthermore, in a cohort of GAD-Ab related disorders patients, we aim to compare GAD65-Ab detection methods and to search for other neuronal autoantibodies besides GAD. We tested 96 consecutive sera for GAD65-Ab from suspected diagnosis of autoimmune encephalitis, epilepsy, diabetes mellitus type 1 or latent autoimmune diabetes in adults (DM1/LADA) patients.

**Known neuronal surface autoantibodies are rare in neuropsychiatric disorders**

NSAbs are well characterized with a large number of cases reported and most of their pathogenic roles are demonstrated in autoimmune encephalitis (AE). When extending NSAbs detection to psychiatric disorders, prior knowledge about NSAbs in AE should be kept in mind. AE is a rare group of brain disorders with a prevalence of 13.7/100,000 and an incidence of 0.8/100,000 person-per year. NMDAR encephalitis is the most common type in this group, with a prevalence of 0.6/100,000 [1]. According to an observational study by Kayser et al, only 4% of cases of NMDAR encephalitis presented with isolated psychiatric symptoms [2].

In **chapter 4** and **chapter 5**, we report our findings on the detection of neuronal autoantibodies in 3316 blood (plasma or serum) samples from mental disorder patients and controls. The spectrum of diseases covered from psychosis, schizophrenia (SZ) to bipolar disorder, depression, and anxiety including also disorder and non-disorder controls. The initial step was using optimized immunohistochemistry (IHC) method on rat brain tissue, which could detect most of the known neuronal autoantibodies as well as autoantibodies to unidentified antigens that share the same epitope across species [3]. However, except for six samples which were clearly anti-GAD65 positive and one weak positive sample for anti-Caspr2, we did not find a typical pattern for other known neuronal autoantibodies by this method.

In accordance with our results, Snijders et al. using the same method did not find any known neuronal autoantibodies in a subpopulation of patients with bipolar disorder type I (BD-I) (n=104) [4]. Similarly, in our earlier study where we analyzed the presence of anti-NMDAR IgG autoantibodies in schizophrenia plasma samples, the only two putative positive samples detected by a commercial cell-based assay (CBA) were neither confirmed by an in house CBA nor by IHC [5].

We further screened all the IHC positive samples by fixed CBA for eight known neuronal autoantibodies. Only one anti-Caspr2 case and a few GAD65-Ab positive cases were confirmed.

Fixed CBA is the most widely used method for detecting neuronal autoantibodies in AE patients. It is a sensitive method which employs a low sample dilution (blood 1: 10 or CSF 1:1) which potentially increases the rate of false positives [6]. Studies that used fixed CBA found up to 10% neuronal autoantibodies in psychiatric disorders but also in controls [7, 8]. Thus, the presence of these autoantibodies in the circulation tested positive only by one method, obviously does not allow a firm conclusion as to whether those autoantibodies play a pathophysiological role in any of these psychiatric disorders. Besides, it would not justify by itself immunotherapy to the patients. One requirement to exert a brain effect is that the autoantibodies can cross the blood-brain barrier (BBB) and be detectable in CSF. The largest study using CSF from psychotic patients showed that 0.8% (1 out of 125) samples had anti-NMDAR autoantibodies [9] but another study from Oviedo-Salcedo et al failed to find any neuronal autoantibodies in the CSF in 124 psychotic patients [10], unfortunately, both studies did not include controls.

In contrast to the results of IHC and fixed CBA, we detected weak positives NSAbs including autoantibodies to LGI1, Caspr2, GABAAR, and GABABR in the plasma of our cohorts with no prevalence difference between the disorder group and controls by live CBA. Thus. we concluded that those autoantibodies detected by live CBA were not disease-specific. NSAbs detected by live CBA has been mostly reported in first-episode psychosis [11-13]. In the largest study using only live CBA methodology by Lennox BR et.al identified 3% (7 out of 228) of patients with first-episode psychosis with autoantibodies against NMDAR compared to none was found in controls (n=105) [13]. While we did not detect any anti-NMDAR positive cases in our studies by live or fixed CBA. The difference in results might be due to different strategies of cohort selection or varying method sensitivity/specificity between laboratories. From our experience, the NSAbs weakly positive samples found by live CBA from mental disorder patients only reacted to a few of the transfected cells (Figure 1), which is obviously different from the positive control we used (from autoimmune encephalitis patients) which normally showed autoantibody reactivity on all the transfected cells and could be confirmed by fixed CBA and IHC. Category these samples equally as NSAbs positive is somehow misleading. It is important to establish standard criteria in how to read out the live CBA results in the future so that we can separate samples only that are positive on live CBA from the ones that could react to all the transfected cells and be positive by different methods.

**2. Novel neuronal surface autoantibodies were detected in patients with anxiety**

Although no difference in the prevalence of known neuronal autoantibodies between psychiatric disorder groups and controls was found, we detected several autoantibodies against unknown antigens by IHC (Chapter **4** and **Chapter 5**). There are several explanations. First, the disorder and non-disorder control groups are not entirely free from other somatic diseases. Some (systemic) autoimmune conditions may contribute to the existence of the autoantibodies. Secondly, it is known that neuronal autoantibodies may occur in the general population and by no means are necessarily associated with disease [6, 14]. Lastly, as former studies revealed the importance of BBB integrity in antibody-related brain disorders [14, 15]; it is still an open question as to whether those circulating autoantibodies could cross the BBB and reach the brain in sufficient levels to cause disease. Previous studies have been mainly focused on the question as to whether neuronal autoantibodies are related to psychotic (especially anti-NMDA) or bipolar disorders [4, 16, 17]. The relation of novel NSAbs to depression or anxiety has barely

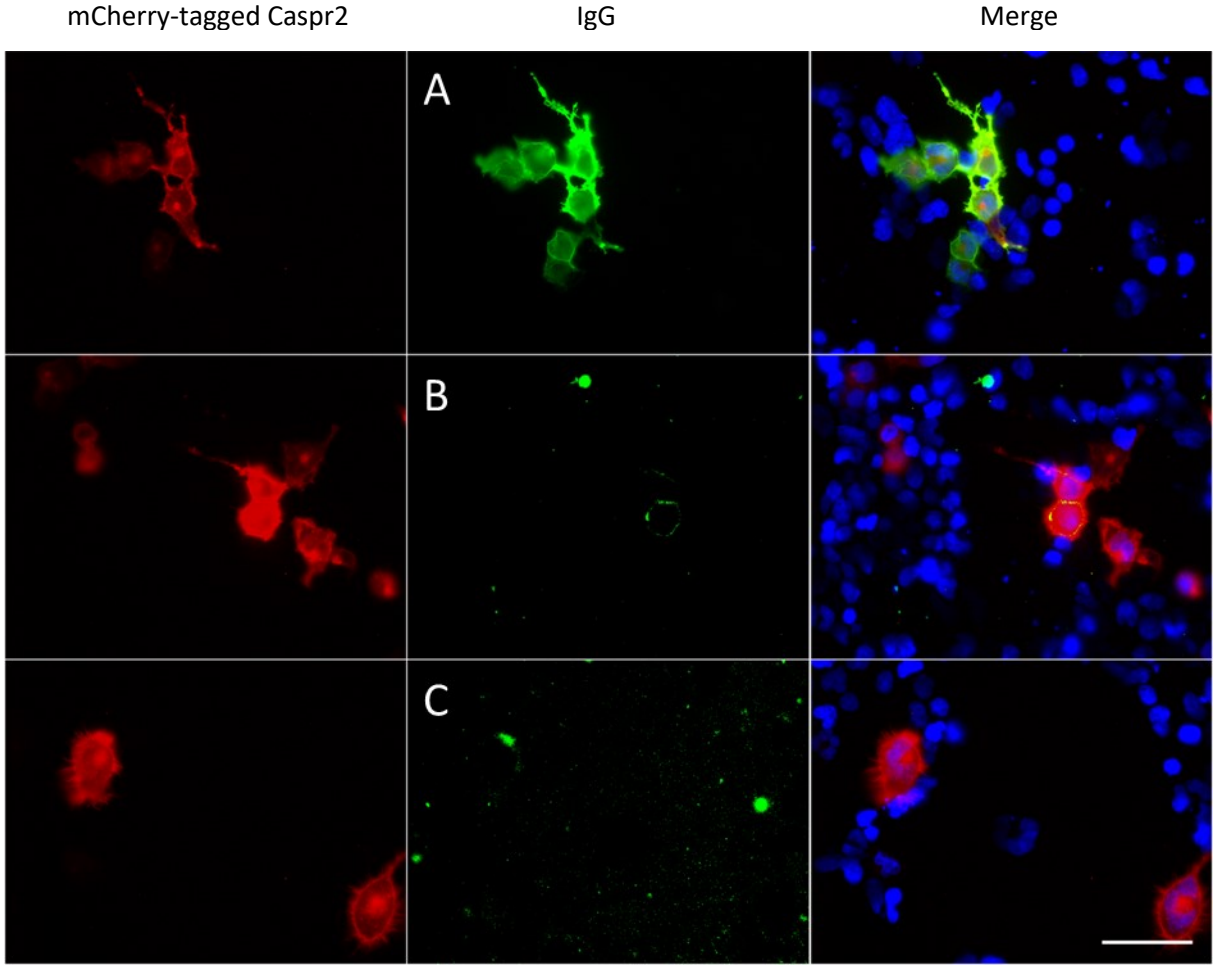


**Figure 1.** The live CBA staining images given by anti-Caspr2 autoantibodies positive serum/plasma from different patients and control. HEK cells were transfected with mCherry tagged Caspr2 (red), human serum/plasma was incubated on the cells and the IgGs binding to Caspr2 proteins were probed by goat-anti-human IgG-488 (Green). (A) serum from an autoimmune encephalitis patient showed strong reactivity on the transfected cells (positive control). (B) Plasma from anxiety and depression patient. (C) Plasma from a control (C). Nuclei were stained by DAPI (blue). Scale bar=50 µm.

been studied. Our further research applying these samples on live cultured neurons showed only that some of them had autoantibodies targeting unknown neuronal surface proteins (novel NSAbs), all of which were from current anxiety patients (**Chapter 4**). This suggests a possible pathophysiological involvement of autoantibodies in a subgroup of psychiatric disorders patients and warrants further research to identify what the autoantibodies are targeting.

### 3.Analysis of intracellular autoantibodies with a special focus on anti-GAD autoantibodies

**In chapter 5**, besides neuronal autoantibodies, we investigated the prevalence of systemic autoimmune rheumatic disease-related antibodies in sera of a sub-cohort consisting of 199 patients with psychotic disorders, 45 with affective disorders, 23 patients with other mental disorders and 152 healthy individuals. The anti-nuclear antibody (ANA) reactivity of sera to ANAs on HEp-2 cells was increased in schizoaffective individuals compared to healthy (p= 0.032). However, the number of sera tested positive (the possibility of false positives should

also be taken into consideration) for specific antigens here was too low for relevant statistical analysis

**In chapter 6**, we confirmed that serum levels of GAD65-Ab in patients with GAD65-Ab related encephalitis or epilepsy were higher than in patients with DM1/LADA although high levels of GAD65-Ab (>10000 U/mL) that could be detected by CBA and IHC existed in both groups [18]. Thus, the presence of GAD65-Ab and their correlation to neurological manifestations have to be interpreted with caution, since the direct involvement of the autoantibodies in encephalitis or epilepsy is debated. We need to analyze this in a more mechanistic fashion and analyze what is the putative disease mechanism caused by the anti-GAD autoantibodies. Besides, a small portion of patients suspected of GAD65-Ab related disorders had other neuronal autoantibodies and their clinical significance should be studied. We detected a case with anti-Hu and another with anti-mGluR1 autoantibodies in patients suspected to be autoimmune, which emphasizes the value of IHC for detecting autoantibodies that are not tested routinely in the clinic [16, 19, 20].

**Limitations of our study**

As described above, the main findings of this thesis are based on the results from **chapter 4, 5 and 6**. In **Chapter 4** and **5,** although we reported that autoantibodies to unknown neuronal antigens were found, not all the known antigens that have been reported were tested by CBA in this study (including D2R, mGluR5, mGluR1, neurexin-3α, IgLON5, DNER (Tr), Glycine receptor and amphiphysin) but were excluded according to the IHC patterns empirically; 2) our study did not include other potential pathogenic brain autoantibodies, including autoantibodies targeting other cell types (astrocytes, microglia); 3) we only analyzed peripheral blood samples, while no CSF material was available, so the question as to whether those antibodies pass the blood-brain-barrier in sufficient amounts still remains to be investigated. In **Chapter 6**, besides the IHC methods limitation mentioned above, the GAD positive neuropsychiatric cohort is relatively small.

**Further perspectives**

**Detection of novel neuronal surface autoantibodies in a pure anxiety or depression cohort**

In **Chapter 4**, we reported that all the novel NSAbs positive patients had anxiety but not depression which generally has not been reported earlier. Still the small positive numbers and high comorbidity of depression and anxiety make it difficult to claim they are more common in anxiety than in depression. Therefore, whether those autoantibodies specifically are associated with anxiety needs to be further investigated in a follow-up study including purely current anxiety patients without a depression diagnosis. The estimated size of the cohort according to our findings (1.7% positives) should be of approximately 300 patients to find at least 5-6 antibody-positive patients.

**To identify the antigens targeted by the autoantibodies and investigate if autoantibodies bind to other cell types besides neurons in neuropsychiatric cohorts**

**In chapter 4, a**ntigen identification and further analysis of autoantibody effector functions are needed for further demonstrate the initial hypothesis that NSAbs ca be causative of a subgroup of psychiatric disorders. Immunoprecipitation and mass spectrometry are commonly used to identify novel antigens. When we performed the immunoprecipitation method with all NSAbs

positive samples, none of them gave a specific band compared to healthy controls. One possible reason is that some reagents used during this procedure break the antibody-antigen interaction and thus render the autoantibody ineffective in pulling down the antigen. Additionally, the density/ concentration of the antigen and limitations in sample processing and analysis during mass spectrometry might have hindered the process.

Besides, there are autoantibodies which target other proteins that may have been neglected. Such antigens could be present on the surface of other cell types such as microglia and astrocytes. Test samples may potentially be positive on IHC but negative on live neurons simply because they target other cells in the brain. We have evidence for this in the case of a patient with depression whose serum showed strong staining on glial cells (Figure 2).

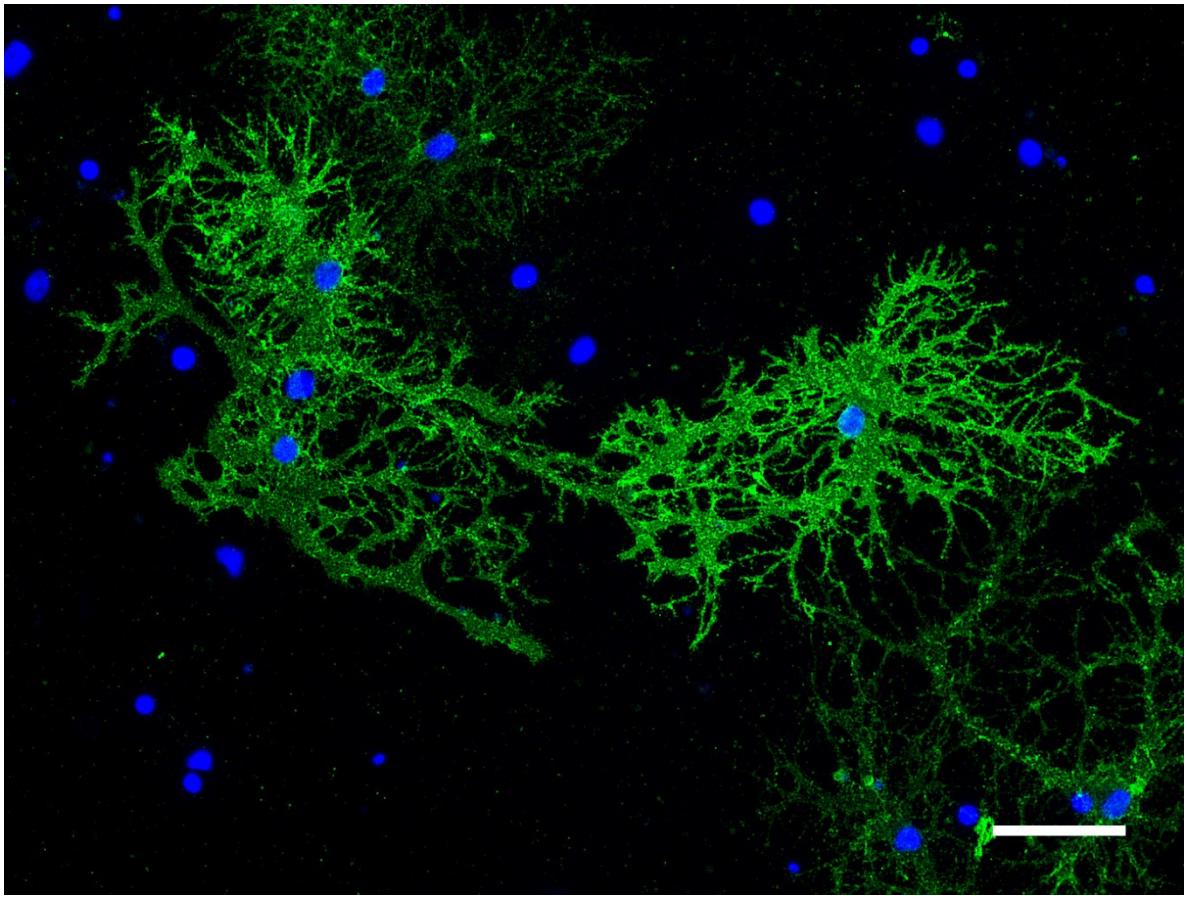

**Figure 2.** Plasma autoantibodies reacted strongly on cells other than neurons when performing live neuronal stainings. Primary cultured hippocampus cells were used. Plasma was incubated on the cells and the IgGs binding to cell surface proteins were probed by goat-anti-human IgG-488 (Green). Nuclei were stained by DAPI (blue). (Scale bar=50 µm).

Because we did not include cultures of these types of cells, we are uncertain about how many positive samples exist in our cohort targeting those cells.

**Autoimmune mechanisms beyond autoantibodies to GAD should be studied further in patients suspected of GAD-Ab related disorders**

GAD is a rate-limiting enzyme that catalyzes the decarboxylation of glutamate to GABA and GAD-Abs showed impairment in GABAergic neurons. In contrast, previous studies did not support that autoantibodies could pass through the cell membrane and target the cytoplasmic

antigens. Thus, it suggested that other associated neuronal surface autoantibodies co-exist with GAD-Abs [21, 22]. In **Chapter 6**, we detected only one case with anti-Hu autoantibodies in ten GAD-Abs positive patients with neurological symptoms. So far, it has been reported that GAD-Abs can be a useful biomarker for stiff person syndrome and related disorders; the causative factor, however, still needs to be identified.

## Overall conclusion:

The observation that a small subgroup of current anxiety but not psychotic disorders patients had novel NSAbs in the peripheral circulation opens the possibility that some patients are in fact autoimmune patients and thus would benefit from immunotherapy. This finding is important because the autoimmune subgroup of patients can be treated targeting the cause of the problem in contrast to traditional pharmacological approaches which treat only the symptomatology. It would, therefore, be important to replicate the current findings using robust methods and paired serum and CSF samples. The identification of novel antigens targeted by the autoantibodies will help to understand their cause-effect. Besides, a small portion of patients suspected of GAD65-Ab related disorders had other neuronal autoantibodies and their clinical significance should be studied individually.

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# Appendix I Valorization

Note: Different from a formal discussion on the academic way, I would like to write the valorization part into a story.

5 years ago in 2014, when the first time I had a skype with Prof. Pilar Martinez, who was an assistant professor then, and she said there was an ongoing project about detecting neuronal autoantibodies in psychiatric disorders in her group, I should have never imagined that one day, we would search those autoantibodies from neurological patients with psychiatric symptoms to psychosis, schizophrenia, bipolar, depression and anxiety! Nevertheless, now we found that a subgroup of patients with anxiety potentially had a relation to novel neuronal surface autoantibodies! At the beginning, I was fascinated immediately when Dr. Carolin Hoffmann, who was still a PhD student, and later became my colleague and one of my closest friend in Maastricht, explained me the hypothesis that there might be a subgroup of psychiatric patients who were actually caused by neuronal autoantibodies and thus could be treated by immunotherapy. As a resident in neurosurgery department, I personally faced the question that why some patients developed psychiatric symptoms after traumatic injury or head surgeries and never gave my patients a really satisfying answer. I knew the etiology of psychiatric disorders were so diverse and hard to clearly mapped. I thought, wow, this would be a chance to approve a direct causative of those sticky disorders, at least in some of them. Then I decided to come and gave a shot. In the second year in 2015, our first-round test of anti-NMDAR autoantibodies in psychosis turned out to be negative [1]. Still, I brought our idea to a famous hospital in Beijing to seek collaboration, I met Prof. Guan. He welcomed me well and gave me a lot of positive feedbacks including sharing their autoantibodies positive samples to us, which in the end did not work out because of the ethical rules. He also asked me a question which I remembered for a while: “Do you really think those autoantibodies could be found in purely psychiatric patients? You may go too far”. At that time, I said I was not sure.

If we had a chance to go back to 12 years ago, when 2007, Prof. Dalmau described the first cases-series of NMDAR autoantibodies encephalitis [2], I guess at that moment, no one could imagine that his research was actually lightening the whole field of autoantibodies mediated central nervous disorders and followed by the discovery of more than 13 novel neuronal surface autoantibodies [3]! Autoimmune encephalitis is a rare disease. When we apply the concept of neuronal autoantibodies mediated disorder to psychiatric disorders, it is still a rare condition as indicated in **Chapter 4** and **Chapter 5** as well as previous studies [4, 5]. Be as it may, it is already a solid fact of their existence. There is no second condition that ties psychology and neurology so close that specialists from both fields are working together to gain the knowledge about it and thus helping the patients. Some patients have already benefited from it and the trend of enclosing more input in research is going on. Another aspect is using those functional autoantibodies as a tool to study the basic biological changes in psychiatric disorders [6]. Those basic mechanism might be common in psychiatric patients without autoantibodies. In this way, not only the patients who have autoantibodies but all would benefit from it.

Over the years, there are contradictories exist in this field as we described in **Chapter 1**, **Chapter 2** and **Chapter 3**. One of the main possible reasons is the methodology problems. I worked years in developing and comparing the autoantibody detecting methods as showed in **Chapter 4**, **Chapter 5** and **Chapter 6** and deeply understand the method gaps exist between researchers, labs and countries. The development of new techniques is coming up but still needs

to be optimized, the communication between researchers is ongoing but still needs to boost and the barriers between countries have never been broken. Even though, I personally have seen lot of improvements already. Last November on a lancet summit, I asked Prof. Dalmau of the diagnostic value of Immunohistochemistry method he developed, which led the findings of all the novel neuronal autoantibodies in his group. He mentioned he always used this method in his lab for diagnosis and believed that it still was a very useful method in finding unknown autoantibodies [7]. I also met Prof. Guan there. Different from the opinion he gave years ago, his group brought 3 posters all of which gave a large space for psychiatric symptoms. He also wrote a letter to explain the pictographs of encephalitis in Chinese characters in Lancet Neurology this year [8]. He showed his social medium later that they actually worked very closely with psychiatrists from Beijing and tried to diagnose some anti-NMDAR encephalitis at the early stage. Another thing has to mention, their lab has already put anti-GAD65 tests in the routine neuronal autoantibody test panel which they did not perform before. What will be the future of other even rare neuronal autoantibodies that have not been covered by commercial kits? As we showed in current dissertation, before better methods developed, the tissue-based assay is still a good choice.

All in all, as a novel field, our current research takes out the first step. It emphasizes the importance of continuously studying autoimmunity in neuropsychiatric disorders, the comparing between methods and developing new techniques as well as the communicating between researchers, researchers to clinicians and societies. Still there are many confusions that need to be clarified in the future. It would be of great that we look back 10 years later and I am looking forward to it!

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# Appendix II Acknowledgements

Throughout the writing of this dissertation, I have received a great deal of support and assistance from my supervisors, colleagues, friends, and families. Firstly, I would like to thank my supervisor, Prof. Dr. **Pilar Martinez**, whose expertise was invaluable in the formulating of the research topic, the guidelines to conduct most of my experiments and methodology in particular. I still remember the first day when we met, your passionate smile and practical suggestions calmed me so much, especially at that moment I was still shaking about my decision of coming to the Netherlands to start my real research life. During the past five years, you advised me on multiple projects, you discussed with me on all the results step by step, you carefully corrected on each of my manuscripts. I can't imagine how could I complete these though work without your guidance.

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The same thanks go to my co-supervisor Dr. **Rob Rhoul**. As you may know, I was a clinical doctor and proud of what I was doing before. Also, my study topic here actually is so close to neurology although we always claim we are working on psychiatry disorders. For these reasons, I always want a supervisor who is working in the hospital, best from neurology department. Then when we have the GAD project and increasingly meetings between us make this possible. You may not notice I was so happy that you can be one of my co-supervisors. Definitely a lot of important and valuable advises from you. Thank you!

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year and experienced similar stages in the lab. Because both of us living with families and had a daughter, your suggestions were always very practical and tailored for my situation. Thanks a lot! Now, we almost will defend our dissertation on the same day (in the end, I will be 1-week earlier!), good luck with everything and hope to see you soon in Maastricht! **Caterina**, **Qian**, **Daan**, **Nikita** and **Tanya**, so glad that we worked together in the same group and had the chance to discuss our results, share our opinions and helped each other in the lab! Here I also would like to thank **Koen** as well when so many times we went to the same events and made jokes. Keep on your boxing and Cheers bro! **Artemis**, for many times, small talks with you really made me feel relax and deepen my understanding of Europe cultures. Also, I would like to thank other colleagues, **Roy**, **Fred**, **Maarten**, **Joao**, **Ehsan**, **Gusta**, **Marion**, **Shannen**, for many times, we went to the same events and shared stories, a lot of pleasant memories build my life in Maastricht! For many other colleagues, **Britt**, **Marion**, **Sandra**, **Sarah**, **Anne**, **Wouter**, **Christian**, **Roel**, **Glenn**, **Pim**, **Nick**, **Milaine**, **Dean**, **Elentina Sylvana**, **Alix**, **Bethany**, **Perla**, **Rose**, **Ellis**, **Kyonghwan**, **Gowoon**, **Majed**, **Mohammed**, **Faris**, **Faisal and many** may not mentioned here who belong to our big lunch group, I really enjoyed the talks, greetings and jokes you have made! There were also my students doing their intership here such as **Nils**, **Nienke**, and **Sofia** who did part of the work and I reaaly enjoyed the time that we worked together. Thanks!

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heart, I always feel lucky that we have the chance to study in the same university far away from home. The life in Maastricht is great but sometimes also could be hard, nothing could compare the help from you guys that we do share the same values and backgrounds.

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In the end, I would thank the most my wife Qing. Words will never express enough about what I feel but still, I will try gathering sentences here to describe it. People always say life is like a box of cholates. When I look back that many amazing things happened during my PhD study, there were 2 things always jumped out immediately, one was that you married me in the summer of 2015 and another was later you gave birth to Yumi in the spring of 2017! You just ignored the uncertainty of life and bravely moved forward to me which really swept away all my hesitation and worries! I would say my PhD study is largely part of your work because, the really truth is, your courage inspires my courage and your determination builds my determination……Looking forward to tasting the next box of chocolates with you!

# Appendix III Publications

**Refereed articles**

1. **Zong, S**. *, Hoffmann, C.*, Mane-Damas M., et al. **Absence of autoantibodies against neuronal surface antigens in sera of patients with psychotic disorders.** JAMA Psychiatry (accepted) (IF 15.9)
2. Mané-Damas M., Hoffmann C., **Zong S.**, et al. **Autoimmunity in psychotic disorders. Where we stand, challenges and opportunities.** *Autoimmunity Reviews* (2019) **18**(9):102348. doi: https://doi.org/10.1016/j.autrev.2019.102348. (IF 8.7)
3. Hoffmann, C., Stevens, J.*, **Zong, S***., et al. (2018). **Alpha7 acetylcholine receptor autoantibodies are rare in sera of patients diagnosed with schizophrenia or bipolar disorder.** PLOS ONE, 13(12), [e0208412]. dio: https://doi.org/10.1371/journal.pone.0208412 (IF 2.8)
4. **Zong, S**., Hoffmann, C., Mane-Damas, M., Molenaar, P., Losen, M., & Martinez-Martinez, P. (2017). **Neuronal Surface Autoantibodies in Neuropsychiatric Disorders: Are There Implications for Depression?** Frontiers in Immunology, 8, [752]. DOI: 10.3389/fimmu.2017.00752. (IF 6.4)
5. Hoffmann, C.*, **Zong, S**.*, Mane-Damas, M.*, Molenaar, P., Losen, M., & Martinez-Martinez, P. (2016). **Autoantibodies in Neuropsychiatric Disorders.** ANTIBODIES, 5(2), [9]. DOI: 10.3390/antib5020009. (IF N/A)

**Manuscripts in process:**

1. Shenghua Zong, Carolin Hoffmann, Marina Damas, Nils Kappelmann, Peter Molenaar, Gerard van Grootheest, Brenda W.J.H. Penninx, Rob P.W. Rouhl, Mario Losen1, Pilar Martinez-Martinez. **Novel neuronal surface autoantibodies in plasma of depression and Anxiety** (in process, submitted to JAMA Psychiatry, IF 15.9).
2. Shenghua Zong, Carolin Hoffmann, Marina Damas, Anita M.Vinke, Xingzhen Zhang, Jan G.M.C. Damoiseaux, Rob P.W.Rouhl, Mario Losen, Pilar Martinez Martinez. **Detection of Glutamic acid decarboxylase (GAD) and other neuronal autoantibodies in suspected GAD related disorders** (in process).
3. Shenghua Zong, Nijs, Laurence de., Marina Damas, B. Rutten, Pilar Martinez. **Higher prevalence of Low reactivity anti-neural (hippocampal) autoantibodies in the serum of PTSD.** (In process).
4. Shenghua Zong, Anita Vinke, Carolin Hoffmann, Marina Damas, Jan G.M.C. Damoiseaux, Rob P.W.Rouhl, Mario Losen, Pilar Martinez Martinez. **A case report of encephalitis patient with mGluR1 autoantibodies.** (In process)
5. 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** (in process, Neurology - Neuroimmunology & Neuroinflammation, impact factor 7.4)

**Oral presentations:**

- **Searching for novel neuronal surface autoantibodies in depression/anxiety.** By: Zong, S. Maastricht Immunology Seminar, January 17th, 2019, Maastricht.

**Posters:**

1. **Neuronal autoantibodies in psychotic disorder.** By: Hoffmann, C., Zong, S., Damas, M., et al. Conference: The Lancet Summit: Inflammation and Immunity in Disorders of the Brain and Mind, November 15-17th, 2018, Barcelona (Spain).
2. **Unidentified neuronal surface IgG autoantibodies in a case of Hashimoto encephalopathy.** By: Damas, M., Hoffmann, C., Zong, S., et al. Conference: The Lancet Summit: Inflammation and Immunity in Disorders of the Brain and Mind, November 15-17th, 2018, Barcelona (Spain).
3. **Tracking anti-neuronal surface autoantibodies at different time points (waves 1, 3 and 5) and their clinical significance.** By: Zong, S., Damas, M., Hoffmann, C., et al. Conference: 15th annual NESDA day, June 5th, 2018, Amsterdam.
4. **Screening for neuronal autoantibodies in plasma from the Netherlands study of depression and anxiety.** By: Zong, S., Hoffmann, C., Damas, M., et al. Conference: 14th annual NESDA day, May 31st, 2017, Groningen.
5. **Detection of neuronal autoantibodies in plasma from the Netherlands study of depression and anxiety.** By: Zong, S., Hoffmann, C., Damas, M., et al. Conference: MHeNS 9th annual research day, November, 30th 2016, Maastricht.
6. **Analysis of auto-antibodies in psychotic disorder.** By: Hoffmann, C., Zong, S., Damas, M., et al. Conference: The Lancet Neurology Autoimmune Disorders Conference, March 27th, 2015, Barcelona (Spain).

# Appendix IV Curriculum Vitae

Shenghua Zong was born on the 11th of October, 1987 in Ruzhou, Henan province, China. He grew up and received his pre-university education in his hometown. In 2006, he was admitted to the medical school of Zhengzhou University and majored in clinical medicine. After 7 years' study including 3 years clinical training in the first affiliated hospital of Zhengzhou University, in July 2013, he got his master's degree. In the last year (2012) of the medical education, he passed the Practitioners Exam in China and obtained the Medical practitioner's qualification certificate which licensed him as a medical doctor. Then he worked one year as a resident (on the training to become a neurosurgeon) in Nanjing Benq hospital, an affiliated hospital of the Nanjing Medical University. In October 2014, he came to the Netherlands and worked in Prof. Pilar Martinez's lab, Maastricht University, as a research assistant and become a PhD candidate later. In 2015, he was awarded a national scholarship from China Scholarship Council which supported 3 years of his PhD study at the Department of Psychiatry and Neuropsychology, School for Mental Health and Neuroscience, Division III (Neuroscience), Maastricht University. He worked on the project of detecting neuronal autoantibodies in neuropsychiatric disorders and also got involved in teaching and grand application under the supervision of Prof. Pilar Martinez, Dr. Mario Losen and Dr. Rob. P.W. Rouhl. The results of his research are presented in the current dissertation and parts of them have been published. During his PhD study, he also organized the department lab day-out as one of the committee members in 2016. He was also one of the department PhD student representatives during 2016-2017. He is applying for grants at the moment to continue his research.

# Appendix V Thesis defenses from MHeNs - School for Mental Health and Neuroscience

2013

Rob Havermans: Bipolar disorder in daily life; Mood and cortisol responses to naturally occurring events. Supervisor: Prof.dr. M. de Vries; Co-Supervisor: Dr. N. Nicolson.

Véronique Moers-Hornikx: Deep brain stimulation and the cerebellum. Supervisors: Prof.dr. J. Vles / Prof.dr. Y. Temel; Co-Supervisor: Dr. G. Hoogland.

Nicole Veldhorst-Janssen: Intranasal delivery of rapid acting drugs.Supervisors: Prof.dr. M. Marcus / Prof.dr. C. Neef; Co-Supervisor: Dr. P.H. van der Kuy.

Stéphanie Knippenberg: Vitamin D and Multiple Sclerosis: immunological and clinical outcome. Supervisor: Prof.dr. J. Cohen-Tervaert; Co-Supervisors: Dr. J. Damoiseaux / Dr. Y. Bols.

Erik D. Gommer: Dynamic Cerebral Autoregulation: from methodology towards clinical application. Supervisors: Prof.dr. W.H. Mess / Prof.dr. R.B. Panerai, UK; Co-Supervisor: Dr.ir. J.P.H. Reulen.

Olga A.H. Reneerkens: Can PDE inhibition improve cognition?Translational insights. Supervisor: Prof.dr. H.W.M. Steinbusch; Co-Supervisor: Dr. J. Prickaerts;.

Lyzel S. Elias-Sonnenschein: Clinical and biomarker correlates of genetic risk factors for Alzheimer's disease. Supervisor: Prof.dr. F.R.J. Verhey; Co-Supervisor: Dr. P.J. Visser.

Diego F. Mastroeni: Epigenetic Dysregulation and the Pathophysiology ofof Alzheimer's Disease. Supervisors: Prof.dr. H.W.M. Steinbusch / Prof.dr. P.D. Coleman, Sun City, Arizona; Co-Supervisors: Dr. B.P.F. Rutten / Dr. D.L.A. van den Hove.

Leonidas Chouliaras: Epigenetic Regulation in Aging and Alzheimer's disease: A translational perspective. Supervisor: Prof.dr. H.W.M. Steinbusch; Co-Supervisors: Dr. B.P.F. Rutten / Dr. D.L.A. van den Hove.

Liesbeth Knaepen: Perinatal events and altered pain sensitivity in later life. Supervisors: Prof.dr. E.A.J. Joosten / Prof.dr. D. Tibboel, EUR; Co-Supervisor: Dr. J. Patijn.

Marisela Martinez-Claros: Hippocampal plasticity and corticosterone: From dendrites to behaviour. Supervisor: Prof.dr. H.W.M. Steinbusch; Co-Supervisors: Dr. J.L. Pawluski / Dr. J. Prickaerts.

Marcus D. Lancé: A circle of improvement in bleeding management: from laboratory to clinic and back. Supervisors: Prof.dr. M.A.E. Marcu / Prof.dr. J.W.M. Heemskerk; Co-Supervisor: Dr. Y.M.C. Henskens.

Hilde Braakman: Imaging the brain; neuronal correlates of cognitive impairment in children with frontal lobe epilepsy. Supervisors: Prof.dr. A.P. Aldenkamp / Prof.dr. J.S.H. Vles; Co-Supervisors: Dr.ir. W.H. Backes / Dr. P.A.M. Hofman.

Willem H. van Zwam: Aneurysmal subarachnoid hemorrhage: imaging strategies and cost-effectiveness aspects in diagnostic work-up and post-therapeutic follow-up. Supervisors: Prof.dr. J.T. Wilmink / Prof.dr. J.E. Wildberger; Co-Supervisor: Dr. P.A.M. Hofman.

Klara De Cort: The Pathogenesis of Panic Disorder. Supervisors: Prof.dr. I. Myin-Germeys

/ Prof.dr. E.J.L. Griez; Co-Supervisors: Dr. K.R.J. Schruers / Dr. I. Van Diest, Leuven.

Kim van Wijck: Mind the Gap; experimental studies on splanchnic hyperfusion and gastrointestinal integrity loss in man. Supervisors: Prof.dr. W.A. Buurman / Prof.dr. C.H.C. Dejong; Co-Supervisor: Dr. K. Lenaerts.

Yvette Roke: Antipsychotic-induced hyperprolactinemia in children and adolescents with mainly autism spectrum disorders. Prevalence, symptoms, clinical consequences and genetic risk factors. Supervisors: Prof.dr. P.N. van Harten / Prof.dr. J.K. Buitelaar (RUN); Co-Supervisor: Dr. A. Boot (UMCG).

Fleur Goezinne: Retinal detachment surgery: pre and postoperative prognostic factors. Supervisors: Prof.dr. F. Hendrikse / Prof.dr. C.A.B. Webers; Co-Supervisor: Dr. E.C. La Heij (Amsterdam).

Ralph L.J.G. Maassen: The Merits of Videolaryngoscopy during Glottic Visualisation for Endotracheal Intubation. Supervisors: Prof.dr. M. Marcus / Prof.dr. A. van Zundert (University of Queensland).

Maria J. de Sousa Guerreiro: The role of sensory modality in age-related distraction. Supervisor: Prof.dr. C.M. van Heugten; Co-Supervisor: Dr. P.W.M. van Gerven.

Ine Rayen: Effects of developmental fluoxetine exposure on neurobehavioral outcomes.

Supervisor: Prof.dr. H.W.M. Steinbusch; Co-Supervisors: Dr. J.L. Pawluski / Dr. T.D. Charlier (Ohio University, USA).

Nynke M.G. Bodde: Psychogenic non-epileptic seizures; a separate disorder or part of a continuum? Supervisors: Prof.dr. R. van Oostenbrugge / Prof.dr. K. Vonck (UZ Gent);Co-Supervisors: Dr. R. Lazeron / Dr. A. de Louw (Epilepsiecentrum Kempenhaeghe, Heeze).

Alejandro M. Gomez: Novel strategies for making myasthenia less gravis: targeting plasma cells and the neuromuscular junction. Supervisor: Prof.dr. M.H. De Baets; Co-Supervisors: Dr. M. Losen / Dr. P. Martinez-Martinez.

Mohammad S. Rahnama'i: Prostaglandins and Phosphodiesterases in the Urinary Bladder Wall. Supervisors: Prof.dr. Ph. Van Kerrebroeck / Prof.dr. S. de Wachter (Universiteit Antwerpen); Co-Supervisor: Dr. G. van Koeveringe.

Mariken B. de Koning: Studying biomarkers in populations at genetic and clinical high risk for psychosis. Supervisors: Prof.dr. T. Amelsvoort / Prof.dr. J. Booij (AMC).

Fabien Boulle: Epigenetic regulation of BDNF/TrkB signaling in the pathophysiology and treatment of mood disorders. Supervisors: Prof.dr. H.W.M. Steinbusch /Prof.dr. L. Lanfumey (Universiteit Parijs); Co-Supervisors: Dr. D. van den Hove / Dr. G. Kenis.

2014

Iris Nowak-Maes: Tinnitus; assessment of quality of life & cost-effectiveness. Supervisors: Prof.dr. M. Peters / Prof.dr. B. Kremer; Co-Supervisors: Dr. M. Joore / Dr. L. Anteunis.

Marjolein Huijts: Cognitive function in patients with cerebral small vessel disease. Supervisor: Prof.dr. R.J. van Oostenbrugge; Co-Supervisors: Dr. A.A. Duits / Dr. J. Staals.

Markus Gantert: Fetal inflammatory injury as origin of long term disease: Lessons from animal models. Supervisors: Prof.dr. B. Kramer / Prof.dr. L. Zimmermann; Co-Supervisor: Dr. A. Gavilanes.

Elke Kuypers: Fetal development after antenatal exposures: Chorioamnionitis and maternal glucocorticoids. Supervisors: Prof.dr. B.W. Kramer / Prof.dr. H.W. Steinbusch / Prof.dr. Suhas G. Kallapur (University of Cincinnati, Ohio, USA).

Pieter Kubben: Ultra low-field strength intraoperative MRI for Glioblastoma Surgery. Supervisor: Prof.dr. J.J. van Overbeeke; Co-Supervisor: Dr. H. van Santbrink.

Laura Baijens: Surface electrical stimulation of the neck for oropharyngeal dysphagia in Parkinson's disease: therapeutic aspects and reliability of measurement. Supervisor: Prof.dr. B. Kremer; Co-Supervisor: Dr. R. Speyer, Townsville.

Janneke Hoeijmakers: Small fiber neuropathy and sodium channels; a paradigm shift. Supervisor: Prof.dr. R.J. van Oostenbrugge; Co-Supervisors: Dr. C.G. Faber / Dr. I.S.J. Merkies.

Stephanie Vos: The Role of biomarkers in preclinical and prodromal Alzheimer's disease. Supervisor: Prof.dr. F.R. Verhey; Co-Supervisor: Dr. P.J. Visser.

Muriël Doors: The Value of Optical Coherence Tomography in Anterior Segment Surgery. Supervisors: Prof.dr. R.M. Nuijts / Prof.dr. C.A. Webers; Co-Supervisor: Dr. T.T.J.M. Berendschot.

Anneke Maas: Sleep problems in individuals with genetic disorders associated with intellectual disability. Supervisors: Prof.dr. I. Curfs / Prof.dr. R. Didden.

Sebastiaan van Gorp: Translational research on spinal cord injury and cell-based therapies; a focus on pain and sensorimotor disturbances. Supervisors: Prof.dr. B. Joosten/ Prof.dr. M. van Kleef; Co-Supervisors: Dr. J. Patijn /Dr. R. Deumens, KU Leuven.

Andrea Sannia: High risk newborns and brain biochemical monitoring. Supervisor: Prof.dr. J.S.H. Vles; Co-Supervisors: Dr. D. Gazzolo, Alessandria, Italy / Dr. A.W.D. Gavilanes.

Julie A.D.A. Dela Cruz: Dopamine mechanisms in learning and memory: Evidence from rodent studies. Supervisors: Prof.dr. H.W.M. Steinbusch / Prof.dr. R.J. Bodnar, New York; Co-Supervisor: Dr. B.P.F. Rutten.

René Besseling: Brain wiring and neuronal dynamics; advances in MR imaging of focal epilepsy. Supervisors: Prof.dr. A.P. Aldenkamp / Prof.dr.ir. W.H. Backes; Co-Supervisor: dr. J.F.A. Jansen.

Maria Quint-Fens: Long-term care after stroke; development and evaluation of a long- term intervention in primary care. Supervisors: Prof.dr. J.F.M. Metsemakers / Prof.dr. C.M. van Heugten / Prof.dr. M. Limburg, Almere; Co-Supervisor: dr. G.H.M.I. Beusmans.

Veronique Moulaert: Life after survival of a cardiac arrest; the heart of the matter. Supervisors: Prof.dr. J.A. Verbunt / Prof.dr. C.M. van Heugten / Prof.dr. D.T. Wade, Oxford, UK.

Feikje Smeets: The hallucinatory-delusional state: a crucial connection in the psychosis symptom network. Supervisor: Prof.dr. J. van Os; Co-Supervisor: Dr. T. Lataster.

Lies Clerx: Alzheimer's disease through the MR-eye; novel diagnostic markers and the road to clinical implementation". Supervisor: Prof.dr. F. Verhey; Co-Supervisors: Dr. P.J. Visser / P. Aalten.

Sonny Tan: The subthalamic nucleus in Parkinson's disease. Supervisors: Prof.dr. Y. Temel / Prof.dr. H.W.M. Steinbusch / Prof.dr. T. Sharp, Oxford, UK / Prof.dr. V. Visser-Vandewalle, Koln.

Koen van Boxem: The use of pulsed radiofrequency in the management of chronic lumbosacral radicular pain. Supervisors: Prof.dr. M. van Kleef / Prof.dr. E.A.J. Joosten; Co-Supervisor: Assoc. Prof.dr. J. van Zundert.

Jérôme Waterval: Hyperostosis cranialis interna. Supervisors: Prof.dr. J.J. Manni / Prof.dr. R.J. Stokroos.

Sylvie Kolfschoten-van der Kruijs: Psychogenic non-epileptic seizures; the identification of neurophysiological correlates. Supervisors: Prof.dr. A.P. Aldenkamp / Prof.dr. K.E.J. Vonck, Universiteit Gent; Co-Supervisors: Dr. J.F.A. Jansen / Dr. R.H.C. Lazeron, Kempenhaeghe.

Wouter Pluijms: Spinal cord stimulation and pain relief in painful diabetic: polyneuropathy, a translational approach. Supervisors: Prof.dr. M. van Kleef / Prof.dr. E.A. Joosten; Co-supervisor: Dr. C.G. Faber.

Ron Handels: Health technology assessment of diagnostic strategies for Alzheimer's disease. Supervisors: Prof.dr. F.R.J. Verhey / Prof.dr. J.L. Severens (EUR); Co-Supervisor: Dr. M.A. Joore / Dr. C.A.G. Wolfs.

Evelyn Peelen: Regulatory T cells in the pathogenesis of Multiple Sclerosis: potential targets for vitamin D therapy. Supervisors: Prof.dr. R.M.M. Hupperts / Prof.dr. J.W. Cohen Tervaert; Co-Supervisor: Dr. J.G.M.C. Damoiseaux / Dr. M.M.G.L.Thewissen, Diepenbeek.

Reint Jellema: Cell-based therapy for hypoxic-ischemic injury in the preterm brain. Supervisors: Prof.dr. B.W.W. Kramer / Prof.dr. H.W.M. Steinbusch; Co-Supervisor: Dr. W.T.V. Germeraad / Dr. P. Andriessen, Veldhoven.

Maria Wertli: Prognosis of Chronic Clinical Pain Conditions: The Example of Complex Regional Pain Syndrome 1 and Low Back Pain. Supervisors: Prof.dr. M. van Kleef; Co-Supervisor: Dr. F. Brunner, Zürich / Dr. R. Perez, VUmc.

Dagmar Zeef: An experimental model of Huntington's disease: Validation & Stimulation. Supervisors: Prof.dr. Y. Temel / Prof.dr. H.W.M. Steinbusch; Co-supervisor: Dr. A. Jahanshahi.

Jeroen Decoster: Breaking Down Schizophrenia into phenes, genes and environment. Supervisors: Prof.dr. I. Myin-Germeys / Prof.dr. M. De Hert, KU Leuven; Co-Supervisor: Dr. R. van Winkel.

Eaja Anindya Sekhar Mukherjee: Fetal Alcohol Spectrum Disorders: exploring prevention and management. Supervisor: Prof.dr. L.M.G. Curfs; Co-Supervisor: Prof. S. Hollins, St. George's University of London, UK.

Catherine van Zelst: Inside out; On stereotype awareness, childhood trauma and stigma in psychosis. Supervisors: Prof.dr. Ph. Delespaul / Prof.dr. J. van Os.

Ibrahim Tolga Binbay: Extended Psychosis Phenotype in the Wider Social Environment. Supervisor: Prof.dr. J. van Os; Co-Supervisor: Dr. M. Drukker.

Frank Van Dael: OCD matters in psychosis. Supervisors: Prof.dr. J. van Os / Prof.dr. I. Myin-Germeys.

Pamela Kleikers: NOXious oxidative stress: from head toe too and back. Supervisors: Prof.dr. H.H.H.W. Schmidt / Prof.dr. H.W.M. Steinbusch; Co-Supervisor: Dr. B. Janssen.

José Luis Gerardo Nava: In vitro assay systems in the development of therapeutic interventions strategies for neuroprotection and repair. Supervisors: Prof.dr.med. J. Weis / Prof.dr. H.W.M. Steinbusch; Co-Supervisor: Dr. G.A. Brook, RWTH Aachen.

Eva Bollen: Cyclic nucleotide signaling and plasticity. Supervisors: Prof.dr. H.W.M. Steinbusch / Prof.dr. R. D'Hooge, KU Leuven; Co-Supervisor: Dr. J. Prickaerts.

2015

Jessica A. Hartmann: A good laugh and a long sleep; Insights from prospective and ambulatory assessments about the importance of positive affect and sleep in mental health. Supervisor: Prof.dr. J. van Os; Co-Supervisors: C.J.P. Simons / Dr. M. Wichers.

Bart Ament: Frailty in old age; conceptualization and care innovations. Supervisors: Prof.dr. G.I.J.M. Kempen / Prof.dr. F.R.J. Verhey; Co-Supervisor: Dr. M.E. de Vugt.

Mayke Janssens: Exploring course and outcome across the psychosis-continuum. Supervisor: Prof.dr. I. Myin-Germeys; Co-Supervisor: Dr. T. Lataster.

Dennis M.J. Hernau: Dopayours is not dopamine: genetic, environmental and pathological variations in dopaminergic stress processing. Supervisor: Prof.dr. I. Myin- Germeys; Co-Supervisors: Prof.dr. F.M. Mottaghy / Dr. D. Collip.

Ingrid M.H. Brands: The adaptation process after acquired brain injury Pieces of the puzzle. Supervisors: Prof.dr. C.M. van Heugten / Prof.dr. D.T. Wade, Oxford UK; Co-Supervisors: Dr. S.Z. Stapert / Dr. S. Köhler.

Francesco Risso: Urinary and salivary S100B monitoring in high risk infants. Supervisor: Prof.dr. J.S.H. Vles; Co-Supervisors: Dr. D. Gazzolo, Genoa,Italy / Dr. A.W.D. Gavilanes.

Alessandro Borghesi: Stem and Progenitor Cells in Preterm Infants: Role in the Pathogenesis and Potential for Therapy. Supervisor: Prof.dr. L. Zimmermann; Prof.dr. B. Kramer; Co-Supervisors: Dr. D. Gazzolo, Genoa,Italy / Dr. A.W.D. Gavilanes.

Claudia Menne-Lothmann: Affect dynamics; A focus on genes, stress, and an opportunity for change. Supervisor: Prof.dr. J. van Os; Co-Supervisors: Dr. M. Wichers / Dr. N. Jacobs.

Martine van Nierop: Surviving childhood new perspectives on the link between childhood trauma and psychosis. Supervisors: Prof.dr. I. Myin-Germeys / Prof.dr. J. van Os; Co-Supervisor: Dr. R. van Winkel.

Sylvia Klinkenberg: VNS in children; more than just seizure reduction. Supervisors: Prof.dr. J. Vles / Prof.dr. A. Aldenkamp; Co-Supervisor: Dr. H. Majoie.

Anouk Linssen: Considerations in designing an adult hearing screening programme. Supervisor: Prof.dr. B. Kremer; Co-Supervisors: Dr. L. Anteunis / Dr. M. Joore.

Janny Hof: Hearing loss in young children; challenges in assessment and intervention. Supervisors: Prof.dr. B. Kremer / Prof.dr. R. Stokroos / Prof.dr. P. van Dijk, RUG; Co-Supervisor: Dr. L. Antheunis.

Kimberly Cox-Limpens: Mechanisms of endogenous brain protection; Clues from the transcriptome. Supervisors: Prof.dr. J. Vles / Prof.dr. L. Zimmermann; Co-Supervisor: Dr. A. Gavilanes.

Els Vanhoutte: Peripheral Neuropathy outcome measures; Standardisation (PeriNomS) study part 2: Getting consensus. Supervisors: Prof.dr. C. Faber / Prof.dr. P. van Doorn; Co-Supervisor: Dr. I. Merkies, Spaarne ziekenhuis Hoofddorp.

Mayienne Bakkers: Small fibers, big troubles; diagnosis and implications of small fiber neuropathy. Supervisors: Prof.dr. C. Faber / Prof.dr. M. de Baets; Co-Supervisor: Dr. I. Merkies, Spaarne ziekenhuis Hoofddorp.

Ingrid Kramer: Zooming into the micro-level of experience: An approach for understanding and treating psychopathology. Supervisor: Prof.dr. J. van Os; Co-Supervisors: Dr. M. Wichers, UMC Groningen / Dr. C. Simons.

Esther Bouman: Risks and Benefits of Regional Anesthesia in the Perioperative Setting. Supervisors: Prof.dr. M. van Kleef / Prof.dr. M. Marcus, HMC, Qatar / Prof.dr. E. Joosten; Co-Supervisor: Dr. H. Gramke.

Mark Janssen: Selective stimulation of the subthalamic nucleus in Parkinson's disease; dream or near future. Supervisors: Prof.dr. Y. Temel / Prof.dr. V. Visser-Vandewalle, Keulen / Prof.dr. A. Benazzouz, Bordeax, France.

Reina de Kinderen: Health Technology Assessment in Epilepsy; economic evaluations and preference studies. Supervisors: Prof.dr. S. Evers / Prof.dr. A. Aldenkamp; Co-Supervisor: Dr. H. Majoie / Dr. D. Postulart, GGZ O-Brabant.

Saskia Ebus: Interictal epileptiform activity as a marker for clinical outcome. Supervisors: Prof.dr. A. Aldenkamp / Prof.dr. J. Arends, TUE / Prof.dr. P. Boon, Universiteit Gent, België.

Inge Knuts: Experimental and clinical studies into determinants of panic severity. Supervisor: Prof.dr. I. Myin-Germeys; Co-Supervisor: Dr. K. Schruers; Influencing panic.

Nienke Tielemans: Proactive coping post stroke: The Restored4Stroke Self-Management study. Supervisors: Prof.dr. C. van Heugten / Prof.dr. J. Visser-Meily, UMC Utrecht; Co-Supervisor: Dr. V. Schepers, UMC Utrecht.

Tom van Zundert: Improvements Towards Safer Extraglottic Airway Devices. Supervisors: Prof.dr. A.E.M. Marcus / Prof.dr. W. Buhre / Prof.dr. J.R. Brimacombe, Queensland, Australia / Prof.dr. C.A. Hagberg.

Tijmen van Assen: Anterior Cutaneous Nerve Entrapment Syndrome Epidemiology and surgical management. Supervisors: Prof.dr. G.L. Beets / Prof.dr. M. van Kleef / Dr. R.M.H. Roumen / Dr. M.R.M. Scheltinga, MMC Veldhoven.

Rohit Shetty: Understanding the Clinical, Immunological and Genetic Molecular Mechanisms of Keratoconus. Supervisors: Prof.dr. R.M.M.A. Nuijts / Prof.dr. C.A.B. Webers.

Christine van der Leeuw: Blood, bones and brains; peripheral biological endophenotypes and their structural cerebral correlates in psychotic disorder. Supervisor: Prof.dr. J. van Os; Co-supervisor: Dr. M. Marcelis.

Sanne Peeters: The Idle Mind Never Rests; functional brain connectivity across the psychosis continuum. Supervisor: Prof.dr. J. van Os; Co-supervisor: dr. M. Marcelis.

Nick van Goethem: α7 nicotinic acetylcholine receptors and memory processes: mechanistic and behavioral studies. Supervisor: Prof.dr. H.W.M. Steinbusch; Co- supervisor: Dr. J. Prickaerts.

Nicole Leibold: A Breath of fear; a translational approach into the mechanisms of panic. Supervisor: Prof.dr. H.W.M. Steinbusch; Co-supervisors: Dr. K.R.J. Schruers / Dr. D.L.A. van den Hove.

Renske Hamel: The course of mild cognitive impairment and the role of comorbidity. Supervisor: Prof.dr. F.R.J. Verhey; Co-supervisors: Dr. I.H.G.B. Ramakers / Dr. P.J. Visser.

Lucia Speth: Effects of botulinum toxin A injections and bimanual task-oriented therapy on hand functions and bimanual activities in unilateral Cerebral Palsy. Supervisors: Prof.dr. J. Vles; Prof.dr. R. Smeets; Co-supervisor: Dr. Y. Janssen-Potten, Adelante Hoensbroek.

Yuan Tian: The effects of Lutein on the inflammatory pathways in age-related macular degeneration (AMD). Supervisors: Prof.dr. C. Webers; Prof.dr. A. Kijlstra, WUR; Co- supervisor: Dr. M. Spreeuwenberg; Dr. H. Tange.

Peggy Spauwen: Cognition and Type 2 diabetes; the interplay of risk factors. Supervisors: Prof.dr. F. Verhey; Prof.dr. C. Stehouwer; Co-supervisor: Dr. M. van Boxtel

Marc Hilhorst: Crescentic glomerulonephritis in ANCA associated vasculitis. Supervisors: Prof.dr. J. Cohen-Tervaert; Co-supervisor: Dr. P. van Paassen

Martin Gevonden: The odd one out: exploring the nature of the association between minority status and psychosis. Supervisors: Prof.dr. J-P. Selten; Prof.dr. J. Booij, Uva; Prof.dr. I. Myin-Germeys

Bart Biallosterski: Structural and functional aspects of sensory-motor Interaction in the urinary bladder. Supervisors: Prof.dr. Ph. Van Kerrebroeck; Prof.dr. S. De Wachter, UvAntwerpen; Co-supervisors: Dr. G. van Koeveringe; Dr. M. Rahnama'i.

Alexandra König: The use of information and communication technologies (ICT) for the assessment of patients with Alzheimer's Disease and related disorders. Supervisors: prof.dr. F. Verhey; prof.dr. Ph. Robert, Nice, Fr; Co-supervisors: dr. P. Aalten; dr. R. David, Nice. Fr.

Michelene Chenault: Assessing Readiness for Hearing Rehabilitation. Supervisors: prof.dr. M.P.F. Berger; prof.dr. B. Kremer; Co-supervisor: dr. L.J.C. Anteunis.

Anand Vinekar: Retinopathy of Prematurity. Recent advances in tele-medicine screening, risk factors and spectral domain optical coherence tomography imaging. Supervisor: prof.dr. C.A.B. Webers; Co-supervisor: dr. N.J. Bauer

Fleur van Dooren: Diabetes and Depression: exploring the Interface between Pathophysiological and Psychological factors. Supervisors: prof.dr. F.R.J. Verhey; prof.dr. J.K.L. Denollet, UvT; prof.dr. F. Pouwer, UvT; Co-supervisor: dr. M.T. Schram.

Gabriëlla Pons van Dijk: Taekwondo and physical fitness components in middle-aged healthy volunteers; the Sekwondo study. Supervisors: prof.dr. J. Lodder; prof.dr. H. Kingma; Co-supervisor: dr. A.F. Lenssen.

Yara Pujol López: Development and psychoneuroimmunological mechanisms in depression. Supervisor: prof.dr. H.W.M. Steinbusch; Co-supervisors: Dr. G. Kenis; Dr. D. van den Hove; Dr. Aye Mu Myint, München.

Romina Gentier: UBB+1; an important switch in the onset of Alzheimer's disease. Supervisors: Prof. H. Steinbusch; Prof. D. Hopkins; Co-supervisor: Dr. F. van Leeuwen.

Sanne Smeets: Insights into insight: studies on awareness of deficits after acquired brain injury. Supervisor: Prof. C. van Heugten; Prof. R. Ponds; Co-supervisor: Dr. I. Winkens

Kim Beerhorst: Bone disease in chronic epilepsy: fit for a fracture. Supervisor: Prof. A. Aldenkamp; Prof. R. van Oostenbrugge; Co-supervisor: Dr. P. Verschuure.

Alex Zwanenburg: Cerebral and cardiac signal monitoring in fetal sheep with hypoxic- ischemic encephalopathy. Supervisor: Prof. T. Delhaas; Prof. B. Kramer; Co-supervisors: Dr. T. Wolfs; Dr. P. Andriessen, MMC.

Ismail Sinan Guloksuz: Biological mechanisms of environmental stressors in psychiatry. Supervisor: Prof. J. van Os; Co-supervisors: Dr. B. Rutten; Dr. M. Drukker.

Seyed Ehsan Pishva MD: Environmental Epigenetics in mental health and illness. Supervisor: Prof.dr. J. van Os; Co-supervisors: Dr. B.P.F. Rutten; Dr. G. Kenis.

Ankie Hamaekers: Rescue ventilation using expiratory ventilation assistance; innovating while clutching at straws. Supervisors: Prof.dr. W.F. Buhre; Prof.dr. M. van Kleef.

Rens Evers. 22q11.2 deletion syndrome: intelligence, psychopathology and neurochemistry at adult age. Supervisors: Prof.dr. L.M.G. Curfs; Prof.dr. T. v. Amelsvoort.

Sarah-Anna Hescham. Novel insights towards memory restoration. Supervisor: Prof.dr. Y. Temel; Co-supervisor: Dr. A. Blokland; Dr. A. Jahanshahi.

João P. da Costa Alvares Viegas Nunes. Insulin receptor sensitization improves affective pathology in various mouse models. Supervisor: Prof.dr. H.W.M. Steinbusch; Co- supervisors: Dr. K-P. Lesch; Dr. T. Strekalova; Dr.B.H. Cline, Oxford.

Yanny Ying-Yee Cheng. Clinical Outcomes After Innovative Lamellar Corneal Transplantation Surgery. Supervisor: Prof.dr. R.M.M.A. Nuijts; Co-supervisor: Dr. J.S.A.G. Schouten.

2016

Oliver Gerlach. Parkinson's disease, deterioration during hospitalization. Supervisor: Prof.dr. R. van Oostenbrugge; Co-supervisor: Dr. W. Weber.

Remo Arts. Intracochlear electrical stimulation to suppress tinnitus. Supervisor: Prof.dr. R.J. Stokroos; Co-supervisor: Dr. E.L.J. Georg.

Mitchel van Eeden. The €- Restore4stroke study: Economic evaluation of stroke care in the Netherlands. Supervisors: Prof.dr.mr. S.M.A.A. Evers; Prof.dr. C.M. v. Heugten; Co-supervisor: dr. G.A.P. van Mastrigt.

Pim Klarenbeek. Blood pressure and cerebral small vessel disease. Supervisor: Prof.dr. R.J. van Oostenbrugge; Co-supervisor: Dr. J. Staals.

Ramona Hohnen. Peripheral pharmacological targets to modify bladder contractility. Supervisor: Prof.dr. Ph.E.V. van Kerrebroeck; Co-supervisors: Dr. G.A. van Koeveringe; Dr. M.A. Sahnama'i; Dr. C. Meriaux.

Ersoy Kocabicak. Deep brain stimulation of the subthalamic nucleus: Clinical and scientific aspects. Supervisors: Prof.dr. Y. Temel; Prof.dr. K. van Overbeeke; Co-supervisor: Dr. A. Jahanshahi.

Sven Akkerman. Temporal aspects of cyclic messenger signaling in object recognition memory; a pharmalogical approach. Supervisor: Prof.dr. H.W.M. Steinbusch;Co-supervisors: dr. J. Prickaerts; dr. A. Blokland.

Anja Moonen. Emotion and Cognition in Parkinson's disease; etiology and neurobiological mechanisms. Supervisor: Prof.dr. F.R.J. Verhey; Co-supervisor: dr. A.F.G. Leentjens.

Anna Schüth. Three-dimensional bladder tissue morphology. Supervisors: Prof.dr. G.A. van Koeveringe; Prof.dr. M. v. Zandvoort, Aachen; Prof.dr. Ph. V. Kerrebroeck.

Elisabeth van der Ven. Ethnic minority position as risk indicator for autism- Spectrum and psychotic disorders. Supervisors: Prof.dr. J.P. Selten; Prof.dr. J. van Os.

Zuzana Kasanova. Environmental reactivity for better or worse; The impact of stress and reward on neurochemistry, affect and behavior across the psychosis continuum. Supervisor: Prof.dr. I. Myin-Germeys, KU Leuven/UM; Co-supervisor: dr. D. Collip.

Danielle Lambrechts. Ketogenic diet therapies; treatment for children and adults with refractory epilepsy. Supervisors: Prof.dr. H.J.M. Majoie; Prof.dr. J.S.H. Vles; Prof.dr. A.P. Aldenkamp; Co-supervisor: dr. A.J.A. de Louw, Kempenhaghe, Heeze.

Frank van Bussel. Advanced MRI in diabetes; cerebral biomarkers of cognitive decrements. Supervisors: Prof.dr.ir. W.H. Backes; Prof.dr. P.A.M. Hofman; Co-supervisor: dr. J.F.A. Jansen.

Lisa Schönfeldt. Neurostimulation to treat brain injury? Supervisors: Prof.dr. Y. Temel; Prof.dr. S. Hendrikx, Hasselt; Co-supervisor: dr. A. Jahanshahi.

Rianne Geerlings. Transition in patients with childhood-onset epilepsy; a long way to adulthood. Supervisor: Prof.dr. A.P. Aldenkamp; Co-supervisors:dr. A.J.A. de Louw, dr. L.M.C. Gottmer, Kempenhaeghe.

Nele Claes. B cells as multifactorial players in multiple sclerosis pathogenesis: insights from therapeutics. Supervisors: Prof.dr. V. Somers, Hasselt; Prof.dr. R. Hupperts Co-supervisors: Prof.dr. P. Stinissen, dr. J. Fraussen, Hasselt.

Olaf Schijns. Epilepsy surgery and biomarkers from history to molecular imaging. Supervisors: Prof.dr. J.J. van Overbeeke; Prof.dr. H. Clustermann, Aachen; Co-supervisors: dr. G. Hoogland; dr. M.J.P. v. Kroonenburgh.

Lizzy Boots. Balanced and Prepared; development and evaluation of a supportive e- health intervention for caregivers of people with early-stage dementia. Supervisors: Prof.dr. F.R.J. Verhey; Prof.dr. G.I.J.M. Kempen; Co-supervisor: dr. M.E. de Vugt.

Wouter Donders. Towards patient-specific (cerebro-) vascular model applications. Supervisors: Prof.dr. T. Delhaas; Prof.dr.ir. F.N. van de Vosse, TUE; Co-supervisor: dr.ir. W. Huberts.

Sizzle Vanterpool. The implications of intrauterine invasion by microbes for placental Pathology and the occurrence of adverse pregnancy outcomes. Supervisor: Prof.dr. B.W. Kramer. Co-supervisors: dr. J.V. Been, Erasmus MC Rotterdam, dr. U von Rango.

Manuela Heins. The Relationship between Social Adversity, Psychosis, and Depression across an Individual's Life Span. Supervisor: Prof.dr. I. Myin-Germeys.

Christianus van Ganzewinkel. NEONATAL PAIN; Out of Sight, Out of Mind? Supervisor: Prof.dr. B.W.W. Kramer; Co-supervisor: dr. P. Andriessen, MMC Veldhoven.

Anne-Hilde Muris. Hype or hope? Vitamin D in multiple sclerosis; A clinical and immunological perspective. Supervisor: Prof.dr. R.M.M. Hupperts; Co-supervisor: dr. J.G.M.C. Damoiseaux.

Gerard Bode. The link between ceramide transporters, innate Immunity and Alzheimer's disease. Supervisor: Prof.dr. M.H.V. de Baets; Co-supervisors: dr. P. Martinez, dr. M. Losen.

Jo Stevens. Advanced diagnostics and therapeutics for Alzheimer's disease. Supervisor: Prof.dr. M. de Baets; Co-supervisors: dr. M. Losen, dr. P. Martinez-Martinez.

Rosan Luijcks. Stress and pain in muscles and brain; developing psychophysiological paradigms to examine stress and pain interactions. Supervisors: Prof.dr. J.J. van Os; Prof.dr.ir. H.J. Hermens, UT; Co-supervisor: dr. R. Lousberg.

M.C. Haanschoten. Towards efficient cardiac surgery – the integrating role of anesthesiology and intensive care. Supervisors: Prof. dr. W. Buhre; Prof. dr. A. van Zundert (Queensland); Co-supervisors: Dr. M.A. Soliman Hamad; Dr. A. van Straten (Catharina zkhs.)

Harmen Jan van de Haar. Microvascular and blood-brain barrier dysfunction in Alzheimer's disease. Supervisor: Prof.dr.ir. W. Backes; Prof.dr. F. Verhey; Co-supervisor: Dr. J. Jansen; Dr.ir. M. v. Osch, LUMC.

Coenraad Itz. Chronic low back pain, considerations about: Natural Course, Diagnosis, Interventional Treatment and Costs. Supervisor: Prof.dr. M. van Kleef; Prof.dr. F. Huygen, EUR; Co- supervisor: Dr. B. Ramaekers.

Willemijn Jansen. The Path of Alzheimer's disease: from neuropathology to clinic. Supervisor: Prof.dr. F. Verhey; Co-supervisors: Dr. P.J. Visser; Dr. I. Ramakers.

Ligia dos Santos Mendes Lemes Soares. Phosphodiesterase inhibitors: a potential therapeutic approach for ischemic cerebral injury. Supervisor: Prof.dr. H.W.M. Steinbusch; Co-supervisors: Dr. R.M. Weffort de Oliveira, Brazil; Dr. J. Prickaerts

Martijn Broen. Anxiety and depression in Parkinson's disease. Supervisor: Prof.dr. R.J. van Oostenbrugge; Co-supervisors: Dr. A.F.G. Leentjens; Dr. M.L. Kuijf.

Sandra Schipper. Extrasynaptic receptors as a treatment target in epilepsy. Supervisor: Prof.dr. J.H.S. Vles; Co-supervisors: Dr. G. Hoogland; Dr. S. Klinkenberg; Dr. M.W. Aalbers, RUG.

João Casaca Carreira. Making sense of Antisense Oligonucleotides Therapy in Experimental Huntington's disease. Supervisor: Prof.dr. Y. Temel; Co-supervisors: Dr. A. Jahanshahi; Dr. W. van Roon-Mom, LUMC.

Dominique IJff. Trick or Treat? Cognitive side-effects of antiepileptic treatment. Supervisors: Prof.dr. A.P. Aldenkamp; Prof.dr. M. Majoie; Co-supervisors: Dr. J. Jansen; Dr. R. Lazeron, Kempenhaeghe.

Alfredo Ramirez. Neurogenetic approach in neurodegenerative disorders. Supervisors: Prof.dr. B.P.F. Rutten; Prof.dr. H.W.M. Steinbusch; Prof.dr. M.M. Nöthen, University of Bonn.

Nienke Visser. Toric Intraocular lenses in cataract surgery. Supervisor: Prof.dr. R.M.M.A. Nuijts; Co-supervisor: Dr. N.J.C. Bauer.

Jakob Burgstaller. Prognostic indicators for patients with degenerative lumbar spinal stenosis. Supervisor: Prof.dr. M. van Kleef; Co-supervisors: Dr. M.M. Wertli, University of Zurich; Dr. H.F. Gramke.

Mark van den Hurk. Neuronal Identity and Maturation: Insights from the Single-Cell Transcriptome. Supervisors: Prof.dr. H.W.M. Steinbusch; Prof.dr. B.P.F. Rutten; Co- supervisors: Dr. G. Kenis; Dr. C. Bardy, Adelaide.

Maria Nikiforou. Prenatal stress and the fetal gut. Potential interventions to prevent adverse outcomes. Supervisors: Prof.dr. B.W. Kramer; Prof.dr. H.W. Steinbusch; Co- supervisor: Dr. T.G. Wolfs.

Janneke Peijnenborgh. Assessment of cognition, time perception, and motivation in children. Supervisors: Prof.dr. J.S.H. Vles; Prof.dr. A.P. Aldenkamp; Co-supervisors: Dr. J. Hendriksen; Dr. P. Hurks.

Joany Millenaar. Young onset dementia; towards a better understanding of care needs and experiences. Supervisors: Prof.dr. F. Verhey; Prof.dr. R. Koopmans, RUN; Co- supervisors: Dr. M. de Vugt; Dr. C. Bakker, RUN.

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Adriana Smits. Perinatal factors and hearing outcome. Supervisors: Prof.dr. R.J. Stokroos; Prof.dr. B.W. Kramer; Prof.dr. B. Kremer.

Angela Bouwmans. Transcranial sonography in parkinsonian disorders: clear window or blurred vision. Supervisor: Prof.dr. W.H. Mess; Co-promotores: Dr. W.E.J. Weber; Dr. A.F.G. Leentjens.

Björn K. Stessel. Patient centred care after day surgery: scope for improvement. Supervisors: Prof.dr. W. Buhre; Prof.dr. B. Joosten. Co-supervisor: Dr. A.H. Gramke.

Jan Guy Bogaarts. Quantitative EEG and machine learning methods for the detection of epileptic seizures and cerebral asymmetry. Supervisor: Prof.dr. W.M. Mess; Co-supervisor: Dr.ir. J.P.H. Reulen; Dr.ir. E.D. Gommer.

Martin M. Müller. Pregnancy derived products for treatment of perinatal brain injuries. Supervisors: Prof.dr. B.W.W. Kramer; Prof.dr. D. Surbek, Bern; Co-supervisors: Dr. T. Wolfs; Dr. G. Gavilanes.

Daan Ophelders. Novel treatment strategies for the protection of the preterm brain; Re- balancing inflammation and regeneration. Supervisor: Prof.dr. B. Kramer; Co-supervisor: Dr. T. Wolfs; Dr. R. Jellema.

Rosalie van Knippenberg. Experience sampling in dementia care; an innovative intervention to support caregivers in daily life. Supervisors: Prof.dr. F. Verhey; Prof.dr. R. Ponds; Prof.dr. I. Myin-Germeys, KU Leuven; Co-supervisor: Dr. M. de Vugt.

Claudia Vingerhoets. Investigating neurobiological mechanisms underlying comorbid cognitive symptoms in psychosis and substance use. Supervisors: Prof.dr. T. van Amelsvoort; Prof.dr. J. Booij, UvA; Co-supervisor: Dr. O. Bloemen

Dennis Oerlemans. Evolution of Neuromodulation for Lower Urinary Tract Dysfunction; Past, Present and Future. Supervisors: Prof.dr. Ph. van Kerrebroeck; Prof.dr. G. van Koeveringe. Co-supervisors: Dr. E. Weil; Dr. T. Marcelissen.

Marion Levy. Evaluation of BDNF/TrkB signaling as a common target in the treatment of major depression and Alzheimer's disease. Supervisors: Prof.dr. H. Steinbusch; Prof. L. Lanfumey, Université Paris Descartes, France. Co-supervisors: Dr. G. Kenis; Dr. D. van den Hove.

Patrick Domen. Stay connected: a family-based diffusion imaging study in psychotic disorder. Supervisor: Prof.dr. J. van Os. Co-supervisor: Dr. M. Marcelis

Geor Bakker. Innovative Approaches to Understanding the Neurobiology of Psychosis. Supervisors: Prof.dr. T. van Amelsfoort; Prof.dr. J. Booij, UvA. Co-supervisor: dr. M. Caan, UvA; dr. O. Bloemen.

Wilma Boevink. HEE! Over Herstel, Empowerment en Ervaringsdeskundigheid in de psychiatrie. Supervisors: Prof.dr. J. van Os; Prof.dr. Ph. Delespaul. Co-supervisor: dr. H. Kroon.

Nataliia Markova . Modified swim test as a mouse depression paradigm of enhanced Cognitive processing: the role of GSK3β. Supervisor: Prof.dr. H. Steinbusch; Prof.dr. K-P. Lesch, University of Wuerzburg. Co-supervisor: Dr. T. Strekalova.

Merijn van de Laar. Individual differences in insomnia; implications of Psychological factors for diagnosis and treatment. Supervisor: Prof.dr. A. Aldenkamp; Prof.dr. D. Pevernagie, Universiteit Gent. Co-supervisor: Dr. S. Overeem, TUE.

Willem Buskermolen. If only I could tell …; Measuring predictors for challenging behaviour in people with both intellectual disability and hearing impairment. Supervisor: Prof.dr. A. Aldenkamp. Co-supervisor: Dr. J. Hoekman, UL.

Kay Deckers. The role of lifestyle factors in primary prevention of dementia; an epidemiological perspective. Supervisor: Prof.dr. F. Verhey. Co-supervisor: Dr. M. van Boxtel; Dr. S. Köhler.

Brechje Dandachi-FitzGerald. Symptom validity in clinical assessments. Supervisors: Prof.dr. R. Ponds; Prof.dr. F. Verhey.

Maurice Theunissen. Understanding factors affecting postoperative Quality of Life. Supervisors: Prof.dr. M. Peters, Prof.dr. M. Marcus. Co-supervisor: Dr. H. Gramke.

Anna Cleutjens. COgnitive-Pulmonary Disease? Neuropsychological functioning in patients with COPD. Supervisors: Prof.dr. E. Wouters, Prof.dr. R. Ponds. Co-supervisors: Dr. D. Janssen, Horn, Dr. J. Dijkstra.

Laura Serpero. Next Generaton Biomarkers in Perinatal Medicine: S100B Protein. Supervisors: Prof.dr. D. Gazzalo, Alessandria, Italy; Prof.dr. B..W.W. Kramer. Co-supervisor: Dr. A.W.D. Gavilanes.

Alessandro Varrica. S100B Protein and Congential Heart Diseases: Brain Aspects. Supervisors: Prof.dr. D. Gazzalo, Alessandria, Italy; Prof.dr. J.S.H. Vles; Prof.dr. L.J.I. Zimmermann. Co-supervisor: Dr. A.W.D. Gavilanes.

Pim R.A. Heckman. Targeting phosphodiesterase type 4 for improving cognitive fronto- striatal function: a translational approach. Supervisor: Prof.dr. J.G. Ramaekers. Co- supervisors: Dr. J.H.H.J.. Prickaerts; Dr. A. Blokland.

Sven van Poucke. Platelets, form sample to big data; exploring granularity in platelet research. Supervisors: Prof.dr. M.A.E. Marcus; Prof.dr. W. Buhre. Co-supervisor: Dr. M. Lancé.

Désirée M.J. Vrijens. Dysfunctions of the Lower Urinary Tract and Affective Symptoms. Supervisors: Prof.dr. Ph.E.V. van Kerrebroeck; Prof.dr. G.A. van Koeveringe. Co- supervisors: Dr. C. Leue.

Tamar van Veenendaal. Neurotransmitters & Networks. An MR view on epilepsy and antiepileptic drugs. Supervisors: Prof.dr.ir. W.H. Backes; Prof.dr. A.P. Aldenkamp. Co- supervisor: Dr. J.F.A. Jansen.

Evelien M. Barendse. Autism Spectrum Disorders in High functioning Adolescents; Diagnostic considerations (AHA). Supervisors: Prof.dr. A.P. Aldenkamp; Prof.dr. R.P.C. Kessels, Radboud University.

Roy Lardenoije. A venture into the epigenetics of aging and Alzheimer's Disease. Supervisors: Prof.dr. B.P.F. Rutten; Prof.dr. H.W.M. Steinbusch. Co-supervisors: Dr. D. van den Hove; Dr. C.A. Lemere, USA.

Charlotte L. Mentzel. The course recognition and treatment of movement disorders in severe mental illness. Supervisors: Prof.dr. P.N. van Harten; Prof.dr. M.A.J. de Koning- Tijssen, UMCG. Co-supervisor: Dr. P.R. Bakker.

Tim Batink. Third Wave Behaviour Therapy: Process Measures and Contextual Interventions. Supervisors: Prof.dr. F.P.M.L. Peeters; Prof.dr. J.J. van Os; Prof.dr. M.C. Wichers, UMC Groningen.

Kevin L.J. Rademakers. Detrusor Underactivity: From Theory To Clinical Assessment. Supervisors: Prof.dr. G.A. van Koeveringe; Prof.dr. Ph.E.V. van Kerrebroeck. Co-supervisor: Dr. M. Oelke.

Iris M.J. Lange. Should I stay or should I go ? Brain mechanisms underlying fear and safety learning, and explosure therapy outcome. Supervisors: Prof.dr. K.R.J. Schruers; Prof.dr. T.A.M.J. van Amelsfoort. Co-supervisor: Dr. L. Goossens.

Ruben G.F. Hendriksen. Evidence for a dystrophin-associated encephalopathy in Duchenne Muscular Dystrophy. Supervisor: Prof.dr. J.S.H. Vles. Co-supervisors: Dr. G. Hoogland; Dr. M.W. Aalbers, UMC Groningen.

Michael Gofeld. Strengths and limitations of the lumbar spine ultrasound-guided interventions. Supervisor: Prof.dr. M. van Kleef. Co-supervisor: Dr. M. Sommer.

Willem A.R. Zwaans. Strategies for chronic inguinal pain. Supervisor: Prof.dr. M. van Kleef. Co-supervisors: Dr. R.H.M. Roumen; Dr. M.R.M. Scheltinga, MMC Veldhoven.

Linda M. Rolf. Mapping the effects of vitamin D in multiple sclerosis A 3D Perspective. Supervisor: Prof.dr. R.M.M. Hupperts. Co-supervisors: Dr. J.G.M.C. Damoiseaux; Dr. J.J.F.M. Smolders, CWZ Nijmegen.

Maarten van Beek. Spinal Cord Stimulation in Clinical and Experimental Painful Diabetic Polyneuropathy. Supervisors: Prof.dr. E.A. Joosten; Prof.dr. M. van Kleef. Co- supervisor: Dr. S.M.J. van Kuijk.

Melina Barkhuizen. Genetic and perinatal risk factors for movement disorders. Supervisors: prof.dr. B.W.W. Kramer, prof.dr. H.W.M. Steinbusch, Prof.dr. A.F. Grobler. Co- supervisor: dr. A.W.D.Gavilanes-Jimenez.

Renske Uiterwijk. Cognitive function and cerebral small vessel disease in hypertension. Supervisor: prof.dr. R.J. van Oostenbrugge. Co-supervisor: Dr. J.E.A. Staals.

Elles Douven. Depression and apathy after stroke. Supervisor: prof.dr. F.R.J. Verhey. Co- supervisors: Dr. P. Aalten, dr. J. Staals.

Mauro Pessia. Brain K+ Channels: from molecular and physiological features to autism spectrum disorder and intellectual disability. Supervisors: prof.dr. H.W.M. Steinbusch, prof.dr. M.B. Donati, It.

Carsten Leue. Hyperarousal in the Hospital and what to do about it: the MED-PSYCH- NET - a transitional network approach fostering personalized care in psychosomatic medicine. Supervisors: Prof.dr. J. van Os, Prof.dr. A. Masclee. Co-supervisors: Dr. J. Strik, Dr. J. Kruimel

Andrea S. Herrera Soto. Aminochrome, an endotoxin for inducing a new rat model of Parkinson's Disease. Supervisor: prof.dr. H.W.M. Steinbusch. Co-supervisors: Prof.dr. Juan Segura-Aquilar; prof. G. Diaz-Veliz, Santiago of Chile

Eline E.B. de Clerck. Ocular neurodegenerative changes and macular cysts in prediabetes and type 2 diabetes. Supervisors: Prof.dr. C.A.B. Webers, Prof.dr. C.D.A. Stehouwer. Co-supervisor: Dr. J.S.A.G. Schouten

Steven T.H. Honings. Exploring psychosis and multidirectional violence: a prospective study in the general population. Supervisor: Prof.dr. J. van Os. Co-supervisor: Dr. M. Drukker

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Sau May Wong. Advances in Microvasculair MRI Techniques: Breaking the Pathophysiological Barriers in Cerebral Small Vessel Disease. Supervisor: Prof.drir. W.H. Backes, Porf.dr. R.J. van Oostenbrugge. Co-supervisor: Dr. J.F.A. Jansen

Mark B.N. van Winkel. Lonely at heart and stressed in company of Others; the influence of daily life social experiences and emotions on depression. Supervisors: prof.dr. F. Peeters; prof.dr. I. Myin-Germeys, KU Leuven/UM; prof.dr. M. Wichers, UMC Groningen

Harsha Birur Laxmana Rao. Revisiting the vascular theory of glaucoma using optical coherence tomography angiography. Supervisors: prof.dr. C.A.B. Webers; prof.dr. R.N. Weinreb, University of California, San Diego

Babette L.R. Reijs. Cognitive correlates of cerebrospinal fluid biomarkers for Alzheimer's disease. Supervisor: prof.dr. F.R.J. Verhey. Co-supervisors: Dr. P.J. Visser; dr. I.H.G.B. Ramakers

Rachel Slangen. Spinal cord stimulation in painful diabetic peripheral Neuropathy. Clinical- and cost-effectiveness. Supervisors: prof.dr. M. van Kleef; Prof.dr. C. Dirksen; prof.dr. C. Faber

Ganne Chaitanya. Epilepsy: A network disorder. Supervisors: prof.dr. A.P. Aldenkamp; prof. P. Satishchandra, NIMHANS, Bangalore, India. Co-supervisors: Dr. J.F.A. Jansen; Dr. S. Zinger, TUE

Sumitha Rajendrarao. New Insight into the Multifaceted Pathogenic Mechanisms of Sporadic Amyotrophic Lateral Sclerosis. Supervisors: prof.dr. B.W. Kamer; prof.dr. H.W. Steinbusch. Co-supervisor: prof. T.R. Raju, NIMHANS, Bangalore, India

Suzanne Roggeveen. Interference of mobile phone with electrophysiology and emotions; results from short-term experimental studies. Supervisor: Prof.dr. J. van Os. Co-supervisor: Dr. R. Lousberg.

Matthias Walter. Multi-methodological approaches to investigate lower urinary tract function in health and disease. Supervisors: Prof.dr. Ph.E.V.A. van Kerrebroek; Prof.dr. G.A. van Koeveringe; Prof.dr. A. Curt, Zürich, CH.

Lalit Gupta. Inhomogeneities in spontaneous brain fluctuations. Supervisors: Prof.dr.ir. WH. Backes; Prof.dr. P.A.M. Hofman. Co-supervisor: Dr. J.F.A. Jansen.

Chaitra Jayadev. Impact of imaging the pediatric retina. Supervisor: Prof.dr. C.A.B. Webers. Co-supervisor: Dr. N.J.C. Bauer; Dr. A. Vinekar.

Annelie Klippel. Navigating through complexity; processes and mechanisms underlying the development of psychosis. Supervisors: Prof.dr. I. Myin-Germeys, KU-Leuven; Prof.dr. M.C. Wichers, UMC Groningen. Co-supervisor: Dr. U. Reininghaus.

Kürşat Altinbaş. Reconstructing The Diagnostic Framework of Bipolarity. Supervisor: Prof.dr. J. van Os. Co-supervisor: Dr. I.S. Gülöksüz.

Andrea J.R. Balthasar. Eyes of the needle; Spectral tissue sensing, an innovative technology for detecting various tissue types during percutaneous needle-based procedures in locoregional anesthesia and pain medicine. Supervisor: Prof.dr. M. van Kleef. Co-supervisor: Dr. G-J. van Geffen, Radboud UMC Nijmegen.

Walmari Pilz. Shedding light on oropharyngeal dysphagia in myotonic dystrophy type 1. Supervisor: Prof.dr. B. Kremer. Co-supervisors: Dr. L.W.J. Baijens; Dr. V. Lima Passos.

Nynke J. van den Hoogen. Repetitive painful procedures in the neonate: Treatment and adult pain sensitivity. Supervisors: Prof.dr. E.A.J. Joosten, Prof.dr. D. Tibboel, Erasmus MC-Sophia, Rotterdam. Co-supervisor: Dr. J. Patijn.

Carlota Mestres Gonzalvo. Medication optimisation; Methodological aspects and new strategies. Supervisors: Prof.dr. F.R.J. Verhey, Prof.dr. P.H.M. van der Kuy, Erasmus MC Rotterdam. Co-supervisors: Dr. R. Janknegt, Zuyderland MC.

Carolin Hoffmann. The Brain under Attack: Autoantibodies in Psychotic Disorders. Supervisors: Prof.dr. P. Martinez, Prof.dr. B. Rutten, Prof.dr. J. van Os, UU/UM.

Jindra M. Bakker. On the bumpy road of happiness: Mechanisms of daily life reward processing and how it can be changed. Supervisors: Prof.dr. M. Wichers, UMC Groningen, Prof.dr. I. Myin-Germeys, KU Leuven/UM. Co-supervisor: Dr. L. Goossens.

Marasha-Fiona de Jong. Between mood and matter; studies on the interface between mood disorders and physical conditions. Supervisor: Prof.dr. F.P.M.L. Peeters. Co- supervisors: Prof.dr. Mischoulon.

Anouk Smeets. New insights in deep brain stimulation for Tourette syndrome. Supervisor: Prof.dr. Y. Temel. Co-supervisors: Dr. L. Ackermans, Dr. A.A. Duits, de. A.F.G. Leentjens.

Margaretha Skowron. Cisplatin resistance in urothelial carcinoma; Understanding and targeting inherent and acquired mechanisms. Supervisors: Prof.dr. G.A. van Koeveringe, Prof.dr. P. Albers, Heinrich-Heine Univ. Düsseldorf. Co-supervisors: Dr. J.G.H. van Roermund, Dr. A. Romano.

Thierry Mentzel. Capturing the cacophony of movement. Supervisors: Prof.dr. P.N. van Harten, Prof.dr. H.A.M. Daanen, VUA. Co-supervisor: Dr.mr. O.J.N. Bloemen, GGZ Hilversum/UM.

Petronella de Meij. Quality indicators for the assessment of pain clinic care: A step forward? Quality from professionals and pain patients' perspective (QiPPP). Supervisors: Prof.dr. G.D.E.M. van der Weijden, Prof.dr. M. v. Kleef. Co-supervisor: Dr. A.J.A. Köke.

Thomas Vaessen. Stress sensitivity in psychosis: assessment, mechanism & intervention. Supervisor: Prof.dr. I. Myin-Germeys, KU Leuven/UM.

Yori van der Steen. Dissecting the psychosis continuum; risk factors along the pathway from experiences to disorder. Supervisor: Prof.dr. I. Myin-Germeys, KU Leuven/UM, Prof.dr. R. van Winkel, KU Leuven.

Aryo Zare. Unveiling the sensory connections between the bladder and the brain that involve the periaqueductal gray matter. Supervisor: Prof.dr. G.A. van Koeveringe; Co- supervisor: Dr. A. Jahanshahi.

Magdalena Weidner. Brain serotonin throughout development – for better and for worse. Supervisors: Prof.dr. H.W.M. Steinbusch, Prof.dr. K.P. Lesch, JM.Univ. Würzburg. Co- supervisor: Dr. D.L.A. van den Hove.

Catherine Vossen. Cortical processing of pain; the role of habituation. Supervisors: Prof.dr. E.A. Joosten, Prof.dr. J. van Os, UU/UM. Co-supervisor: Dr. R. Lousberg.

Whitney Freeze. Microvascular contributions to dementia; Exploring the role of blood- brain barrier leakage in cerebral small vessel disease and Alzheimer disease. Supervisors: Prof.dr. F.R.J. Verhey, Prof.dr.ir. W.H. Backes. Co-supervisor: Dr. H.I.L. Jacobs.

Simone Schüller. Characterization of Stem and Immune Cell Ontogeny to Inform Prevention and Treatment of Infections in Preterm Newborns. Supervisors: Prof.dr. B.W.W. Kramer, Prof.dr.med. A. Berger, Wien. Co-supervisor: Dr. E. Villamor.

Michael J. Kemna. Predicting relapses in ANCA associated vasculitis. Supervisor: Prof.dr. J.W. Cohen Tervaert. Co-supervisors: Dr. J. Damoiseaux, Dr. P. van Paassen.

Artemis Iatrou. Epigenetics in mental and neurodegenerative disorders. Supervisor: Prof.dr. B.P.F. Rutten. Co-supervisors: Dr. D.L.A. van den Hove, Dr. G. Kenis.

Laura Wielders. Prevention & Treatment of Cystoid Macular Edema after Cataract Surgery. Supervisor: Prof.dr. R.M.M. Nuijts. Co-supervisors: Dr. J.S.A.G. Schouten, CWZ Nijmegen, Dr. B. Winkens.

Daisy Hoofwijk. The way to understanding Chronic Postsurgical Pain; From clinical and psychological predictors to incorporating genetics. Supervisor: Prof.dr. W.F.F.A. Buhre; Prof.dr. E.A.J. Joosten; Co-Supervisor: dr. H.-F. Gramke; dr. A.A.A. Fiddelers.

Loes Leenen. Self-management in Epilepsy; The Goal is: "Live with a Z(s)mile. Supervisors: Prof.dr. H.J.M. Majoie; Prof.dr.mr. S.M.A.A. Evers; Prof.dr. C.M. van Heugten.

Chiara Peila. 'Effects of Pasteurization and Refrigerated Storage on Human Milk Neurobiomarkers Concentrations. Supervisors: Prof.dr. D. Gazzallo, Alessandria, It./MUMC+; Prof.dr. G. Visser, UU; Prof.dr. E. Bertino, Alessandria, It.

Raymond van de Berg. The Vestibular Implant: Feasibility in humans. Supervisor: Prof.dr. H. Kingma; Co-supervisor: dr. J.-P. Guyot, Université de Genève, CH.

Nils Guinand. The Vestibular Implant: a more stable horizon for patients with a bilateral vestibular deficit? Supervisors: prof.dr. H. Kingma; Prof.dr. J.-P. Guyot, Université de Genève, CH.

Jasper Smit. Exploring deep brain stimulation as a treatment for tinnitus. Supervisors: Prof.dr. R.J. Stokroos; Prof.dr. Y. Temel; Co-supervisor: dr. Jahanshahianvar.

Bindu Paravil Sankaran. Brain MRI in Mitochondrial Disorders: Correlating the Phenotype with Genotype. Supervisor: Prof.dr. H. Smeets; Prof.dr. A. Taly, NIMHANS, Bangalore, India.

Syenna Schievink. Vascular cognitive impairment; at the heart of the matter. Supervisor: Prof.dr. F.R.J. Verhey; Prof.dr. R.J. van Oostenbrugge; Co-supervisor: dr. S. Köhler.

Isabelle Bos. Biomarkers of Alzheimer's disease; relations with vascular factors and cognition in the pre-dementia stages. Supervisor: Dr. P.J. Visser; Prof.dr. F.R.J. Verhey; Co-supervisor: dr. S.J.B. Vos.

Stijn Michielse. Road work ahead; cerebral pathways mediating Psychological mechanisms underlying the psychosis spectrum. Supervisor: Prof.dr. J.J. van Os; Co- supervisor: dr. M.C. Marcelis.

Georgios Schoretsanitis. Risperidone-based therapeutic regimens; Drug interactions and adverse drug reactions. Supervisor: prof.dr. K.R.J. Schruers; Co-supervisor: dr. M. Bak .

Alieske Dam. INLIFE; An innovative online social support intervention for caregivers of persons with dementia. Supervisor: Prof.dr. M.E. de Vugt; Prof.dr. F.R.J. Verhey; Co- supervisor: Dr. M.P.J. van Boxtel.

Roel Haeren. Vascular ventures; Analysis of vascular structures and function in epilepsy. Supervisor: Prof.dr. Y Temel; Co-supervisor: dr. K. Rijkers; Dr. G. Hoogland.

Chiara Fabbri. Pharmacogenomics of antidepressant drugs: perspectives for the personalization of treatment in depression. Supervisors: Prof.dr. K. Schruers; Prof.dr. A. Serretti, Bologna.

Esther van Duin. Dancing in the (B)rain'; neurobiology of reward, stress & Information processing in 22q11.2 deletion syndrome. Supervisors: Prof.dr. T. van Amelsvoort; Prof.dr. J. Booij, UvA. Co-supervisor: dr. D. Hernaus.

Rob Verdonschot. Oropharyngeal dysphagia and its psychiatric Comorbidities; The prevalence of affective symptoms and the unmet clinical need for integrated care in medically unexplained symptoms. Supervisor: Prof.dr. B. Kremer; Co-supervisors; Dr. L. Baijens; dr. S. Vanbelle.

Lisanne Breuer. Accelerated Cognitive Ageing in Epilepsy' Does it Exists? Supervisors: Prof.dr. A. Aldenkamp; Prof.dr. P. Boon, UZ Gent; Co-supervisors: dr. A. de Louw, Kempenhaeghe, Heeze; dr.ir. S. Zinger, TUE.

Liselot Kerpershoek. Access to formal dementia care; A European perspective. Supervisors: Prof.dr. F. Verhey; Prof.dr. M. de Vugt; Prof. B. Woods, Bangor University, UK Co-supervisor: Dr. C. Wolfs.

Henrietta Steinhart. Same Same but Different; Psychological Interventions and how to Mind the Knowledge Practice Gap. Supervisor: Prof.dr. I. Myin-Germeys. Co-supervisor: Dr. U. Reininghaus.

Ulrich Mehnert. The management of urine storage dysfunction in the neurological patient. Supervisors: Prof.dr. G. van Koeveringe; Prof.dr. Ph.van Kerrebroeck; Prof.dr. S. Wachter, Antwerpen; Prof.dr E. Chartier-Kastler, Sorbonne, Paris.

Giovanne B. Diniz. Weaning-induced alterations on neuropeptidergic populations of the rat hypothalamus. Supervisors: Prof.dr. H. Steinbusch; Prof.dr. J. Bittencourt, ICB/USP, Brasil.

Rajani Ravindra Battu. Inherited Retinal Diseases: New Imaging and Molecular Genetics. Supervisor: Prof.dr. C.A.B. Webers. Co-supervisors:Dr. J.S.A.G. Schouten, CWZ; dr. T.T.J.M. Berendschot.

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Jans van Ool. Diagnostic and neuropsychiatric considerations in epilepsy and intellectual disability; Psychological perspectives. Supervisor: Prof.dr. A. Aldenkamp. Co-supervisors: Dr. J. Hendriksen; Dr. H. Schelhaas, Kempenhaeghe.

Eveline Janssen. Depression in the elderly: focus on high risk groups. Supervisors: Prof.dr. F. Verhey; Prof.dr. M. de Vugt. Co-supervisor: Dr. M. Schram.

Cécile Kicken. Extreme blood coagulation; investigating the influence of physiological extremes on thrombin generation and platelet activation. Supervisor: Prof.dr. W. Buhre Co-supervisors; Dr. B. de Laat; Dr. M. Lancé, Qatar.

Martinus van Eerd. Diagnosis and Interventional Pain Treatment of Cervical Facet Joint Pain. Supervisor: Prof.dr. M. van Kleef. Co-supervisor; Dr. J. Patijn, Eindhoven; Dr. M. Sommer.

Chenxing E. Zhang. Novel insights in the pathophysiology of cerebralsmall vessel disease – a study using advanced imaging techniques. Supervisors: Prof.dr. R.J. van Oostenbrugge; Prof.dr.ir. W.H. Backes; Co-supervisor: dr. J. Staals.

Ivo Eijkenboom. A zebrafish model of small-fiber neuropathy. Supervisors: Prof.dr. H.J.M. Smeets; Prof.dr. C.G. Faber; Co-supervisor: dr. J. Vanoevelen.

Bianca de Greef. Small fiber neuropathy: from underlying conditions to treatment. Supervisor: Prof.dr. C.A. Faber; Co-supervisor: Dr. I.S.J. Merkies; Dr. J.G.J. Hoeijmakers.

Lotte Berk. MINDFULNESS AND AGING: Exploring Mechanisms and Interventions. Supervisors: Prof.dr. J. van Os; Prof.dr. M.W. de Vugt; Co-supervisor: dr. M.P.J. van Boxtel.

Mor Dickman. Practice patterns and outcomes of corneal transplantation. Supervisor: Prof.dr. R.M.M.A. Nuijts; Co-supervisors: Dr. T.J.M. Berendschot; dr. F.J.H.M. van den Biggelaar.

Thyagi Ponnamperuma. Mental Health Problems in Sri Lankan Adolescents Exposed to the Tsunami and Other Traumatic Events. Supervisor: Prof.dr. M.W. De Vries; Co-supervisor: Dr. N.A. Nicolson.

Robbert C. Maatman. Anterior cutaneous nerve entrapment syndrome (acnes): an analysis of various subtypes and alternative treatment modalities. Supervisor: Prof.dr. M. van Kleef; Co-supervisors: Dr. R.M.H. Roumen, dr. M.R.M. Scheltinga.

Mari Elshout. Neovascular Age-Related Macular Degeneration in the Era of Value-Based Health Care. Supervisor: Prof.dr. C.A.B. Webers; Co-supervisor: Dr. J.S.A.G. Schouten.

Jeroen Deenik. Thinking inside the box; Changing lifestyle to improve the health status of inpatients with severe mental illness. Supervisor: Prof.dr. P.N. Harten; Co-supervisors: Dr. D.E. Tenback; dr. I.J.M. Hendriksen.

Thomas Draak. Peripheral Neuropathy outcome measures Standardisation (PeriNomS) study part 3: Capturing the Patient's Voice. Supervisor: Prof.dr. C.G. Faber; Co-supervisor: Dr. I.S.J. Merkies.

Ana Luisa Gil Martínez. Neuroprotection in neurodegenerative processes associated with Parkinsonism and aging. Correlation between dopaminergic neuronal death and glial activation. Supervisor: Prof.dr. H.W.M. Steinbusch, Prof.dr. Maria-Trinidad Herrero Ezquerro, University of Murcia.

Bernice J.A. Gulpers. Anxiety in older adults; Correlates, comorbidities and prognosis with lifespan perspectives. Supervisor: Prof.dr. F.R.J. Verhey, Prof.dr. R.C. Oude Voshaar; Co-supervisor: Dr. S. Köhler.

Elke Devocht. Combining a cochlear implant and a hearing aid in opposite ears: The best of both worlds. Supervisor: prof.dr. H. Kingma; co- supervisor: dr. E.I.J. George.

Gillian Townend. Rett Syndrome: Recognising the Communication Challenges, Needs and Potential of Individuals Living with a Rare Disease. Supervisor: Prof.dr. L.M.G. Curfs; co- supervisor: Dr. P.B. Marschik, Med. University of Graz, Austria.

Takashi Koizumi. Genetic and neuroinflammatory components of familial and sporadic cerebral Small Vessel Disease. Supervisor: Prof.dr. H. Steinbusch,Prof.dr. T. Mizuno,Japan; co-supervisor: Dr. S. Foulquier

Muhammad Ali. Integrative network-based approaches for modelling Human disease. Supervisor: Prof.dr. J. Kleinjans; co-supervisor: Dr. D. van den Hove; Dr. E. Pishva.

Guillaume Durand. The adaptive side of psychopathy. Investigating adaptive characteristics associated with the psychopathic personality. Supervisor: Prof.dr. B. Rutten; co-supervisor: Dr J. Lobbestael.

Darius C. Henatsch. Honey: A Novel Treatment in Chronic Ear Infections. Supervisor: Prof.dr. R.J. Stokroos; UMC Utrecht/UM; co-supervisor: Dr. J.J. Briedé.

Reinhilde J. Melles. Vaginal penetration: pain or pleasure? The role of fear and sexual arousal. Supervisor: Prof.dr. M.L. Peters; co-supervisor: Dr. M. ter Kuile, LUMC, Dr. M. Dewitte.

Raul Felipe Abella Antón. Cardiac Surgery Biochemical Monitoring in Congenital Heart Diseases Infants. Supervisors: Prof. dr. D. Gazzolo, Prof. dr. L.J.I. Zimmermann, Prof. dr. J.S.H. Vles, co-supervisor; Dr. A.W.D. Gavilanes.

Francesca M. Snoeijen-Schouwenaars. Diagnostic, neuropsychiatric and therapeutic considerations in epilepsy and intellectual disability – medical perspectives –. Supervisor: prof.dr. A.P. Aldenkamp, co-supervisors: Dr. H.J. Schelhaas, SEIN Zwolle; dr. J.G.M. Hendriksen, Kempenhaeghe, Heeze

Mariëlle H.J. Pruppers. Peripheral Neuropathies: Standardizing Functional Assessment. Supervisors: prof.dr. C.G. Faber; prof.dr. N.C. Notermans, UU;Dr. I.S.J. Merkies, ius promovendi.