Generation and Characterization of Human Monoclonal Autoantibodies from Patients with *N*-Methyl-D-Aspartate Receptor Autoimmune Encephalitis

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Content

Summary

Approximately 7 % to 9 % of people in western populations suffer from one of 80 inflammatory disorders that are collectively defined as autoimmune diseases. This translates into 24 million people in the United States of America alone. During the development of an autoimmune disease, the immune system is not able to distinguish self from foreign antigens and attacks its own healthy tissue. About a decade ago, a new autoimmune disease, the *N*-methyl-D-aspartate receptor (NMDAR) encephalitis, has been discovered. NMDARs are frequently expressed in nerve cells in the brain and autoantibodies targeting this receptor are thought to influence neuronal brain functions. The disease is associated with a prodromal phase which includes fever and headache and can lead to psychotic syndromes, seizures, decreased levels of consciousness, dyskinesia and often to intensive care treatment with autonomic instability and hypoventilation. A trigger for some cases of the disease might be an ovarian teratoma.

Previously, in NMDAR encephalitis, the involvement of anti-NMDAR antibodies has been demonstrated in the disease process but an association with other neuronal autoantibodies could not be excluded. Therefore, we collected the cerebrospinal fluid (CSF) of eight women between 18 and 34 years, five with ovarian teratoma, either in the acute phase of the disease or in remission in order to study their antibody repertoire. With fluorescence-activated cell sorting (FACS) we separated CSF single memory B cells and antibody secreting cells and amplified their immunoglobulin heavy and light chains. We generated 170 human monoclonal antibodies of NMDAR encephalitis patients representing their antibody repertoire.

We tested all antibodies on mouse brain sections and human embryonic kidney cells (HEK293T) transfected with the NR1 subunit of the NMDAR, which was identified as the major autoantigen in NMDAR encephalitis. However, only nine antibodies displayed a specific anti-NMDAR reactivity. Previously, it was suggested that the target region of the autoantibodies lies in the amino-terminal domain (ATD) and a mutation at the amino acid N368Q abolishes binding. We could confirm a loss of binding with the N368Q mutation in all nine monoclonal NMDAR autoantibodies. *In vivo* binding of NMDAR epitopes by the monoclonal autoantibodies was further demonstrated by intravenous injections of the purified antibodies into mice and

subsequent immunohistochemistry (IHC) of brain sections. In addition, *functional in vitro* assays on primary hippocampal neurons revealed surface NMDAR downregulation and impairment of NMDAR-mediated glutamate-evoked currents and therefore proved the antibody's inhibitory function on NMDARs.

To investigate the origin and pathogenesis of NMDAR autoantibodies we analyzed whether somatic hypermutations and class switching had occurred. Here, we could show that in comparison to all non-NMDAR antibodies in the patients CSF, NMDAR autoantibodies presented less somatic hypermutations and some of them even had no mutations as compared to the germline. The latter are in general referred to as natural occurring antibodies, which indicate incomplete formation of tolerance against the NMDAR during B cell development. Besides antibody secreting cells, such as plasma cells and plasma blasts, we could also detect memory B cells, which are thought to be a risk factor for clinical relapses after therapy.

Interestingly, only a small amount of autoantibodies is NMDAR specific, over 95 % of the non-NMDAR antibodies demonstrated binding to mouse brain epitopes including neuronal surface antigens. In mass spectrometry analysis, we could detect autoantibodies against a variety of brain epitopes, such as alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subtype 2 (AMPAR2), inositol 1,4,5-trisphosphate receptor type 1 (ITPR1), and glial fibrillary acidic protein (GFAP). Further investigations of these and additional autoantibodies could reveal novel targets, implying a contribution to the diverse clinical picture of NMDAR encephalitis.

In the future, the generated recombinant human monoclonal autoantibodies, against NMDAR and non-NMDAR targets, could be used as tools for additional research, e.g. together with high resolution synaptic imaging to study molecular mechanisms of disease pathogenesis. As titer correlations between patients are ambiguous, using these monoclonal NMDAR autoantibodies for standardization of autoantibody titers could improve its relevance in clinical settings and ultimately improve therapeutic intervention.

Zusammenfassung

Ungefähr 7 % bis 9 % der Menschen in westlichen Bevölkerungen leiden an einer von 80 entzündlichen Erkrankungen, die kollektiv als Autoimmunkrankheit definiert sind. Dies entspricht einer Anzahl von 24 Millionen Menschen allein in den Vereinigten Staaten von Amerika. Während der Entwicklung einer Autoimmunerkrankung ist das Immunsystem nicht in der Lage, sich selbst von fremden Antigenen zu unterscheiden und greift seine eigenen gesunden Zellen und Organe an. Vor etwa einem Jahrzehnt wurde eine neue Autoimmunkrankheit entdeckt, die *N*-Methyl-D-Aspartat-Rezeptor-(NMDAR)-Enzephalitis. Besonders häufig sind NMDA Rezeptoren in Nervenzellen im Gehirn exprimiert und es wird angenommen, dass Autoantikörper, die diesen Rezeptor erkennen, die neuronale Signalweiterleitung im Gehirn beeinflussen. Die Krankheit ist mit einer Prodromalphase assoziiert, die mit Fieber und Kopfschmerzen beginnt und zu psychotischen Syndromen, Anfällen, Bewusstseinsstörungen, Dyskinesien und oft zu einer Intensivbehandlung mit Symptomen von autonomer Instabilität und Hypoventilation führen kann. Bei einem Teil der NMDAR-Enzephalitis Patienten wird das Auftreten eines Ovarialteratoms als Auslöser angenommen.

Vorherige Studien haben eine mögliche Beteiligung von Anti-NMDAR-Autoantikörpern aufgezeigt, eine Assoziation mit anderen neuronalen Autoantikörpern konnte jedoch nicht ausgeschlossen werden. Daher haben wir den Liquor von acht Frauen zwischen 18 und 34 Jahren, fünf mit Ovarialteratom, entweder in der akuten Phase der Erkrankung oder in Remission gesammelt um das Antikörper-Repertoire zu untersuchen. Mit der Fluoreszenz aktivierten Zellsortierung (FACS) trennten wir einzelne B-Gedächtniszellen und Antikörper-sezernierende Zellen des Liquors von den Patienten und vervielfältigten ihre schweren und leichten Immunglobulinketten. Folglich erzeugten wir 170 humane monoklonale Antikörper von NMDAR-Enzephalitis Patienten, die deren Antikörper-Repertoire abbilden.

Alle monoklonalen Antikörper wurden an Gehirnschnitten von Mäusen und einer Zelllinie von menschlichen embryonalen Nierenzellen (HEK293T) getestet, die mit der NR1-Untereinheit des NMDA-Rezeptors transfiziert waren. Jedoch zeigten nur neun Antikörper eine spezifische Reaktivität gegen den NMDAR. Bisher wurde

vermutet, dass die potentielle Zielregion der Autoantikörper in der aminoterminalen Domäne (ATD) liegt und insbesondere eine Mutation an der Aminosäure N368Q eine Bindung der Antikörper verhindert. In unseren Experimenten mit den humanen monoklonalen NMDAR-Autoantikörpern konnten wir das bestätigen. Weiterhin konnte die *in vivo* Bindung an NMDA-Rezeptoren via intravenöser Injektionen der monoklonalen Autoantikörper in Mäusen gezeigt werden. Darüber hinaus wiesen funktionelle Experimente mit monoklonalen NMDAR Autoantikörpern an primären hippocampalen Neuronen darauf hin, dass zum einen NMDA-Rezeptoren herunterreguliert und zum anderen die durch NMDA-Rezeptoren vermittelten elektrischen Ströme reduziert werden. Diese Experimente belegen abschließend, dass NMDAR Autoantikörper alleine dazu in der Lage sind die inhibitorischen Funktionen auszuüben, die die primären Symptome der NMDAR Enzephalitis auslösen.

Die Entwicklung von Antikörper-sezernierenden Zellen umfasst Immunglobulin-Klassenwechsel und somatische Hypermutationen. Im Vergleich zu allen Nicht-NMDAR-Antikörpern im Patientenliquor zeigten NMDAR Autoantikörper eine geringere somatische Hypermutation und einige von ihnen wiesen sogar gar keine Mutationen im Vergleich zur Keimbahn auf. Diese werden im Allgemeinen als natürlich vorkommende Antikörper bezeichnet, die eine unvollständige Toleranzbildung gegenüber dem NMDAR während der B-Zell-Entwicklung anzeigen könnten. Neben Antikörper-sezernierenden Zellen, wie Plasmazellen und Plasmablasten, konnten wir auch B-Gedächtniszellen erkennen, die die Ursache für klinische Rückfälle sein könnten.

Interessanterweise war nur ein kleiner Teil aller Autoantikörper NMDAR spezifisch, über 95 % der Nicht-NMDAR-Antikörper zeigten eine Bindung an Mausgehirnepitope einschließlich neuronaler Oberflächenantigene. In massenspektrometrischen Analysen konnten wir Autoantikörper gegen eine Vielzahl von Gehirnepitopen nachweisen, wie den α-Amino-3-hydroxy-5-methyl-4-isoxazol-Propionsäure Rezeptor Subtyp 2 (AMPAR2), den Inositol-1,4,5-trisphosphat-Rezeptor Typ 1 (ITPR1) und saures Gliafaserprotein (GFAP). Weitere funktionelle Untersuchungen dieser und weiterer Autoantikörper könnten neue Zielantigene aufzeigen, die einen Beitrag zum vielfältigen Krankheitsbild der NMDAR-Enzephalitis liefern.

In zukünftigen Studien könnten die hergestellten rekombinanten humanen monoklonalen Autoantikörper gegen NMDA-Rezeptoren und Nicht-NMDA-Rezeptoren als Werkzeuge für zusätzliche Untersuchungen dienen, beispielsweise zusammen mit hochauflösender synaptischer Bildgebung zur Erforschung molekularer Mechanismen. Da die Titerkorrelationen zwischen den Patienten nicht eindeutig sind, könnte die Verwendung dieser monoklonalen NMDAR-Autoantikörper als Standardvergleich zwischen Patienten und Kliniken die Relevanz von Antikörpertitern in der therapeutischen Intervention verbessern.

1. Introduction

1.1. Autoimmune Encephalitis

The immune system is a highly effective host defense machinery and protects against various pathogens. Under certain conditions in which the immune system is not able to distinguish between self and foreign, a so-called autoimmune disease develops, where the body's defense mechanism attacks its own structures. Autoimmune diseases are defined as a collection of over 80 inflammatory disorders with a prevalence of 7-9 % in the population, affecting more women and often developing in middle-aged patients causing significant morbidity and mortality (Fairweather et al., 2008; Theofilopoulos et al., 2017; Whitacre, 2001).

Autoimmune encephalitis (AIE) can be described as an umbrella term of heterogeneous neuro-psychiatric disorders caused by the immune system. Symptoms associated with autoimmune encephalitis are present in a variable clinical picture, starting from mild memory or cognitive impairment, to personality changes, psychosis, seizures or movement disorders, leading - in some cases - to intensive care treatment (Leypoldt et al., 2015; Tüzün and Dalmau, 2007). In general, psychiatric symptoms occur quite often in AIE patients. According to a recently reported study, 60 % of the patients display psychiatric symptoms at the onset of the disease and 31 % were initially treated in a psychiatric hospital (Herken and Prüss, 2017). Several studies point out the importance of an early diagnosis in regards to a successful treatment outcome (Finke et al., 2012, 2016; Graus et al., 2016; Irani et al., 2011; Titulaer et al., 2013). As a consequence, several clinical symptoms have been established over the years to facilitate diagnosis of AIE in general and subsequently initiate screening for markers.

The three main diagnostic criteria for AIE are, at first, a subacute onset of less than 3 months with psychiatric symptoms, like working memory deficits or altered mental status such as personality change. Secondly, one or more of the following four criteria should apply: cerebrospinal fluid (CSF) pleocytosis, indicated by a count of more than five white blood cells per mm^3, new focal central nerves system (CNS) findings such as impairments of nerves, spinal cord or brain function, not previously known seizures and/or hyperintense signals in MRI features in specific areas of the

brain. Finally, the reasonable exclusion of alternative causes, for an example virus testing to exclude herpes simplex virus encephalitis, or metabolic, and toxic triggers (Graus et al., 2016; Ramanathan et al., 2014). Quite recently, it has been shown that neuroleptic intolerance should also raise suspicion to test for autoantibodies as a sign of AIE especially in psychiatric disorders (Herken and Prüss, 2017; Lejuste et al., 2016).

AIE was initially revealed through syndromes caused by cancer associated immune responses and termed as paraneoplastic syndromes. Because these diseases were associated with brain infiltrates of cytotoxic T cells attacking the neuronal tissue, the response to treatment and recovery was limited (Tüzün and Dalmau, 2007). In this case, the detection of autoantibodies functions as a diagnostic marker for the underlying tumor. Here, the autoantibodies are not thought to be pathogenic themselves (Guan et al., 2016). With the finding of a new category of autoantibodies against *N*-methyl-D-aspartate receptor (NMDAR), these neuronal receptors autoantibodies demonstrate to be themselves pathogenic via eliciting functional responses (Dalmau, et al., 2007). In addition, patients with NMDAR autoantibodies have been more responsive to immune therapy (Dalmau et al., 2008). Soon other synaptic or cell-surface autoantibodies were detected such as anti-AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor), anti-LGI1 (leucine-rich glioma inactivated 1), anti-Caspr2 (contactin-associated protein-like 2), and anti-GABAB receptor (γ-aminobutyric acid class B receptor) (Lancaster et al., 2011; Martinez-Hernandez et al., 2011). Compared to previous described AIE patients, NMDAR encephalitis patients indicated differences as they displayed infiltrates of B cells and plasma cells, microgliosis, but only a small amount of T cells (Dalmau et al., 2007; Tüzün et al., 2009). Therefore, AIE can be divided in two main groups. One with classic paraneoplastic neurological syndromes associated with neuronal autoantibodies that target intracellular antigens in combination with the occurrence of cytotoxic T cell responses and the other with limited T cell response but autoantibodies to neuronal surface or synaptic antigens that are able to change the structure and function of the target antigen themselves (Leypoldt et al., 2015).

Besides the important identification of autoantibodies, the ongoing research in the field could further reveal that the origin of AIE has several triggers, of which one is tumor association. Here, the exact mechanisms how tumors can trigger AIEs are

still unknown and need further investigations. The current hypothesis is, that the expression of neuronal proteins in tumors can contribute to the break of immune tolerance by initiating a B cell immune response towards production of autoantibodies (Höftberger, 2015; Lai et al., 2009; Lancaster et al., 2010).

Another trigger of AIE is presumed to be virus associated. For instance, in herpes simplex virus encephalitis (HSE), 20 % of patients who suffer a relapse display no viral detection and are not responsive to anti-viral medications but display behavioral changes, which represent a main symptom for AIE (Höftberger, 2015). In addition, several studies could detect NMDAR autoantibodies in relapsed patients with former HSE diagnosis (Armangue et al., 2014; Prüss et al., 2012; Schein et al., 2017). These observations suggest that neurologic autoimmunity might be triggered by viral infections through molecular mimicry of neuronal antigens by the virus or through virus induced lysis of neuronal cells which then activates the immune system to produce autoantibodies (Höftberger, 2015).

As an additional trigger vaccination has been discussed for its involvement in AIE and other autoimmune diseases. It has been reported for a few cases that after vaccination of H1N1 influenza, tetanus, diphtheria, pertussis, poliomyelitis and Japanese encephalitis, NMDAR encephalitis had occurred (Dalmau et al., 2011; Hofmann et al., 2011; Wang, 2017). However, this might be a coincidence and which other factors are also involved in the origin of autoimmune diseases, such as genetic predisposition has to be further investigated (Perdan-Pirkmajer et al., 2012; Shoenfeld et al., 2000; Vadalà et al., 2017; Wang, 2017).

Besides the possible triggers, such as tumor or viral association, the main cause for AIE for a large amount of patients remains unknown (Höftberger, 2015). Therefore, research of the immune system of patients and analysis of their autoantibody repertoire are necessary to improve the understanding of immunological deregulation that leads to AIE.

1.2. *N*-Methyl-D-Aspartate Receptor Encephalitis

About a decade ago, in 2007, a new form of autoimmune encephalitis was identified by Dalmau and colleges, known as NMDAR encephalitis. Since then, it has been

shown that in 70 % of patients, disease manifestation starts with a prodromal phase including headache, fever, nausea, vomiting, diarrhea or upper respiratory-tract symptoms (Dalmau et al., 2011). As the disease progresses many patients develop psychiatric symptoms such as anxiety, agitation, psychosis, memory deficits and speech reduction (Höftberger, 2015). In more severe cases, a multistage processing NMDAR encephalitis occurs. Here, patients develop abnormal movement, seizures, decreased consciousness, coma, breathing instability (hypoventilation) and display autonomic instability (Dalmau et al., 2007; 2009; Höftberger, 2015). Nonetheless, also milder forms of NMDAR encephalitis have been reported, whereas isolated psychiatric symptoms, seizures or repetitive movements (dystonia) appear (Niehusmann et al., 2009; Rubio-Agustí et al., 2011).

The exact frequency of NMDAR encephalitis is unknown, but epidemiological studies suggest that it is the most common cause of non-infectious encephalitis, apart from acute demyelinating encephalomyelitis (ADEM) in children (Granerod et al., 2010). A Californian encephalitis project, investigating encephalitis patients younger than 30 years, identified more cases with NMDAR encephalitis than viral triggers such as herpes simplex virus (Gable et al., 2012). Another study in Germany identified, that 1 % of all patients diagnosed with encephalitis of unknown origin between 18 and 35 years treated in the intensive care unit (ICU) were eventually diagnosed to have NMDAR encephalitis (Pruss et al., 2010).

About 80 % of patients with NMDAR encephalitis are women and the mean age is about 23 years of age (Dalmau et al., 2008; Titulaer et al., 2013). A study by Titulaer and colleges in 2013 link 38 % of patients with a tumor, mostly ovarian teratoma (94 %). The distribution among age, sex and the detection of a tumor reveals that the disease occurs in children of both sexes and in younger females. Interestingly, the discovery of the tumor and its subsequent removal perceptibly improves the disease outcome (Dalmau et al., 2008; Iizuka et al., 2008; Irani et al., 2010; Rypulak et al., 2016).

The disease can often be so severe that 77 % of NMDAR encephalitis patients have to be admitted to an ICU. However about half of the patients benefit of first-line therapy which is composed of immunotherapy with the administration of steroids and intravenous immunoglobulins and with plasma exchange (Titulaer et al., 2013).

Nonetheless, several studies outline that cognitive impairments in executive functions and memory persist after treatment, implicating that earlier and more aggressive treatment might be necessary (Finke et al., 2012; Houtrow et al., 2012). Second-line therapy consists of rituximab in combination with cyclophosphamide (Dalmau et al., 2011). Rituximab is a chimeric monoclonal antibody against CD20-positive B cells inducing B cell depletion that may involve antibody-dependent cytotoxicity, complement-mediated lysis, or apoptosis (Dalakas, 2008; Edwards et al., 2004; Gopal and Press, 1999; McLaughlin, 2001). Cyclophosphamide is an alkylating agent, functioning as a general cytostatic and is frequently used as a broad approach in cancer therapy as well as in treatment of autoimmune diseases (Teles et al., 2017). Second-line therapy led to a significant decrease of relapses observed in a study by Titulaer and colleges in 2013. However, as the disease discovery was quite recently in 2007, long-term outcome studies are still pending. Generally, NMDAR autoantibodies titers changes in CSF can be observed during relapses and might be useful to complement clinical assessment, (Gresa-Arribas et al., 2014). Yet, the serum titer correlation between patients is poor, and the correlation between CSF and serum titers is not fully understood.

The preliminary survival rate of NMDAR encephalitis is estimated at 93 – 96 %, whereas earlier treatment through sooner disease recognition might improve this number and probably the long-term outcome (Dalmau et al., 2008, 2011; Titulaer et al., 2013). A favorable outcome was accompanied by low severity of symptoms within four weeks of disease onset, early treatment, no ICU hospitalization and longer follow-up after treatment (Titulaer et al., 2013). Nonetheless, although about 80 % of NMDAR encephalitis patients recover completely or at least partially, it can take up to 18 months for signs of recovery to appear (Titulaer et al., 2013).

1.3. The *N*-Methyl-D-Aspartate Receptor

The NMDAR was named after one of its ligand, NMDA, which is an amino acid derivative originally used in neuroscience research to mimic the action of glutamate, which in turn is the natural ligand that leads to opening of the NMDAR ion channel (Curtis and Watkins, 1960; Curtis and Watkins, 1963; Stone and Burton, 1988). Generally, the NMDAR belongs to the family of glutamate receptors, which are

primarily located on neurons and are synaptic receptors. The family is represented by metabotropic glutamate receptors that indirectly activate ion channels through G coupled receptors and ionotropic glutamate receptors that form an ion channel and are activated by glutamate binding. The tetrameric NMDAR, an ionotropic glutamate receptor, is a heterogeneous structure composed of two NR1 subunits and two of various combinations of NR2 or NR3 subunits (Chazot et al., 1994; Monyer et al., 1992; Schorge and Colquhoun, 2003; Ulbrich and Isacoff, 2007, 2008) (Fig. 1B). The NR1 subunit is expressed by a single gene (GluN1) but splicing leads to the expression of eight different isoforms throughout the brain (Sugihara et al., 1992; Zukin and Bennett, 1995). The NR2 subunit is expressed by four (GluN2A-D) and the NR3 subunit by two genes (GluN3A-B) (Chatterton et al., 2002; Sucher et al., 1996). It is known that during development NR2B and NR2D are predominantly expressed earlier, their expression decreases while the expression of NR2A and NR2C increases (Monyer et al., 1994). All NMDARs harbor an extracellular amino-terminal domain (ATD), a agonist binding domain (ABD) with two chains (S1 and S2), a membrane domain with three transmembrane domains (M1, M3, M4), one membrane domain with a reentrant loop (M2) and a carboxyl-terminal domain (CTD) reachable for signaling proteins (Vyklicky et al., 2014) (Fig. 1 A).

There are several known antagonists targeting different domains of the NMDAR. Of note, magnesium ions are located in the membrane domain, phencyclidine (PCP) and ketamine are channel blockers and zinc ions and ifenprodil localize to the ATD of the NMDAR (Hansen et al., 2018) (Fig. 1 B). A previously reported study using CSF of NMDAR encephalitis patients has detected that the ATD region of the NMDAR and especially the amino acid N368 as crucial for autoantibody binding, as antibodies of patients do no longer bind to a N368Q mutated form of the NMDAR, where asparagine was replaced by glutamine (Gleichman et al., 2012).

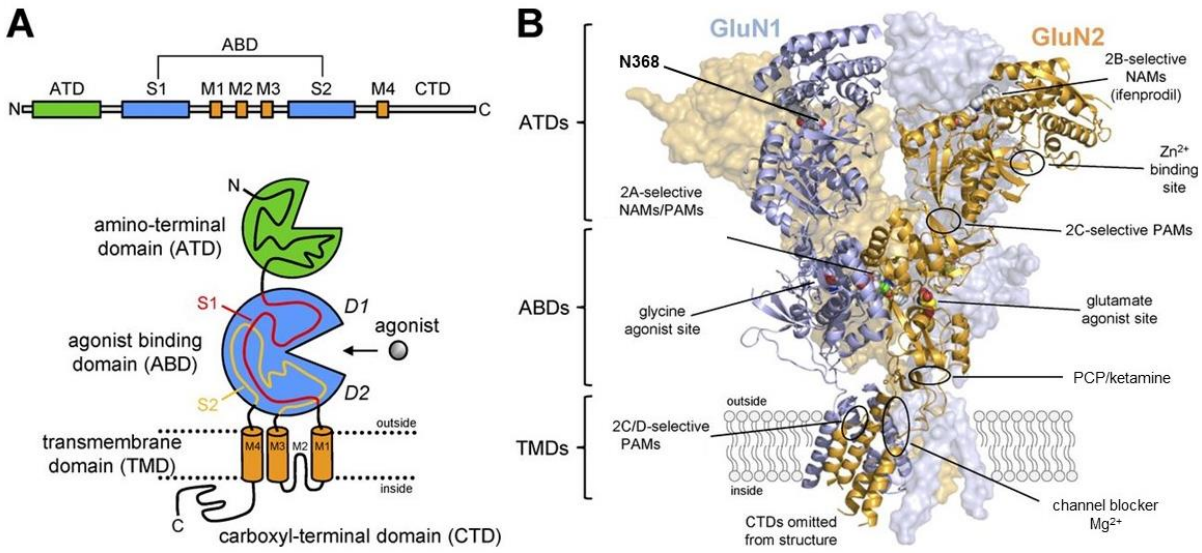

Figure 1 Structure of N-Methyl-D-aspartate receptors.

A: Linear and structural representation of the different domains of one NMDAR subunit. Each NMDAR subunit is comprised of an amino-terminal domain (ATD), a agonist binding domain (ABD) with the S1 and S2 segments forming a bilobed structure with an upper lobe (D1) and lower lobe (D2) and with linkers to the transmembrane domain (TMD). The cytoplasmatic carboxyl-terminal domain (CTD) contains signaling molecules that influence the NMDAR. **B:** The model of a NMDAR tetramer containing two NR1 (GluN1) and two NR2 (GluN2) subunits and a schematic view of its binding partners. Opening of the NMDAR pore needs the binding of both glycine (or D-serine) and glutamate at the ABD. Additionally, the depolarization via AMPA or kainate receptors is necessary to remove the magnesium (Mg^{2+}) that blocks the NMDAR channel in the TMD. The opening allows the sodium (Na^{+}) and calcium (Ca^{2+}) ions into the cell and enables efflux of potassium (K^{+}) ions. Further binding sites have been identified such as the noncompetitive receptor antagonist site for phencyclidine (PCP) and ketamine. Further binding partners in the ATD of the NMDAR, such as zinc and ifenprodil also modulate the receptor activity. For NMDAR encephalitis, several studies could reveal that mutation of the N368 amino acid in the ATD region of the NR1 subunit leads to a rejection of patients' antibodies bindings. Positive (PAMs) and negative allosteric modulators (NAMs) are highlighted indicated to which subunit they belong to. (adapted from Hansen *et al.,* 2018).

Functionally, the NMDAR, located at the postsynaptic membrane, is important for the communication between neurons through synaptic transmission. The NMDAR operates as a coincidence detector and therefore as an integrator of synaptic activity (Madden, 2002). In addition to the co-agonists glutamate and glycine, or D-serine, the NMDAR requires the depolarization of the membrane via AMPAR and kainate receptors. This initiate the release of the blocked magnesium ion in the NMDAR channel of its transmembrane domain and calcium and sodium ions can enter and potassium ions can leave the cell which leads to depolarization and thus resulting in communication between cells.

1.4. The *N*-Methyl-D-Aspartate Receptor in Health and Disease

Previously, the NMDAR has been associated, besides NMDAR encephalitis, with various diseases including certain neurodegenerative diseases such as Alzheimer's disease or Huntington's disease, schizophrenia, addiction, stroke and epilepsy (Lau and Zukin, 2007; Parsons and Raymond, 2014). An important function of the NMDAR is supposed to be its involvement in learning and memory due to a process called long-term potentiation (LTP) or long-term depression (LTD). LTP can be induced through simultaneous activation of pre- and postsynaptic neurons. The resulting maximal calcium influx stimulates intracellular signaling cascades, which subsequently lead to a strengthening of connection between neurons. LTD is caused by repeated activation of only the presynaptic neuron, consequently resulting in low stimulation of the postsynaptic neuron, which is presumably the cause of weakening connection between neurons. However, these processes are only possible due to the NMDAR functioning as a coincidence detector which is essential for several forms of synaptic plasticity (Lüscher and Malenka, 2012). For example, in Alzheimer's disease, the block of LTP and eliciting of LTD via amyloid beta (Aβ) oligomers is believed to be the reason for early cognitive decline (Kamenetz et al., 2003).

Besides its contribution to development of memory and learning, the NMDAR is also involved in a process named excitotoxicity leading to neurodegeneration (Beal, 1992). Excitotoxicity is caused via excessive activation of the NMDAR. These stimulations result in an huge influx of calcium that induces cell death throughout the brain (Choi, 1987; Choi et al., 1988). In Huntington's disease the degeneration of neurons are thought to be caused by excessive NMDAR signaling that induced excitotoxicity (Heng et al., 2009; Sepers and Raymond, 2014). Prevention of excitotoxicity plays also a major role in treatment of ischemic stroke in order to prevent neuronal damage. Research on NMDAR antagonists revealed their neuroprotective properties (Muir and Lees, 1995; Simon et al., 1984). However, a big challenge to treat patients is that the healthy tissue is also effected by NMDAR antagonists and drug specificity has to be further investigated (Dingledine et al., 1999).

The first identified antagonists of the NMDAR, such as PCP and ketamine, used as anesthetic drugs display various adverse side effects, which led to the removal of

PCP from clinical use (Javitt and Zukin, 1991). Mild intoxication of these anesthetic drugs could mimic the symptoms of schizophrenia suggesting that the disturbance of NMDAR function plays a major role in the disease (Javitt and Zukin, 1991; Krystal et al., 1994). Higher concentrations of PCP lead to severe rigidity and catatonia and severe PCP intoxication was accompanied by coma, respiratory failure and seizures (Burns et al., 1975; Liden et al., 1975; Pearce, 1976; Walberg et al., 1983). These symptoms are similar to different known disease stages of NMDAR encephalitis, which have been described as hallucination, agitation and psychosis. Later, the multistage clinical course of NMDAR encephalitis leads to unresponsiveness with catatonic features, coma and respiratory failure (Dalmau et al., 2011) (Fig. 2 A,B). A comparison of NMDAR encephalitis with research in PCP abuse as a schizophrenia model could also reveal new understandings of the disease pathomechanisms in NMDAR encephalitis (Masdeu et al., 2016) (Fig. 2C).

In previous studies, it was suggested that autoantibodies against the NMDAR are the main cause that induce the symptoms of the disease. *In vitro* experiments with patients' CSF revealed a reduction of synaptic NMDAR-mediated currents in whole-cell patch-clamp recordings of hippocampal neurons (Hughes et al., 2010). Similar to pharmacological NMDAR antagonists, patients' CSF samples reduced surface NMDAR levels through crosslinking and internalization of the receptor (Hughes et al., 2010; Moscato et al., 2014). *In vivo* injection of patients' CSF into mice and subsequent behavioral studies could partly mimic the patients' symptoms such as progressive memory deficits (Planagumà et al., 2015). Overall, NMDAR encephalitis has been explained as a multistage disease due to a progressive NMDAR reduction through binding, crosslinking and internalization of the patients' NMDAR via autoantibody binding. Here, varying titers are thought to be involved in its gradual internalization and worsening or progression of the disease (Dalmau et al., 2011) (Fig. 2C).

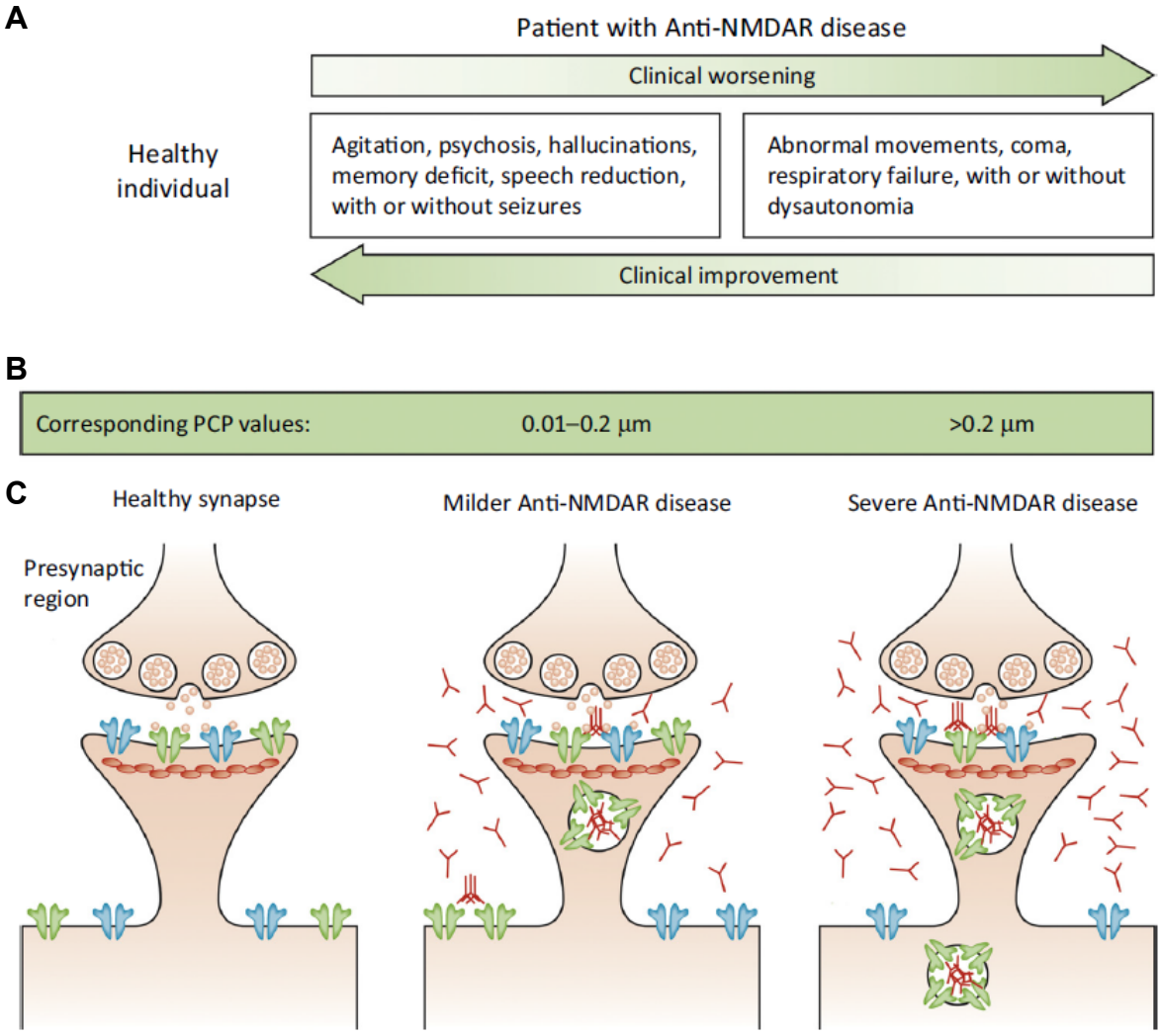

Figure 2 A psychoactive drug mimics symptoms of schizophrenia and NMDAR encephalitis: a common explanation for the pathogenesis of both diseases

A: In NMDAR encephalitis patients initially undergo a prodromal phase with fever. As the clinical course gets worse they display agitation, psychosis and hallucinations etc., which also in a later state of the disease can lead to abnormal movement, coma and respiratory failure etc. Clinical improvement of the patient displays symptoms in a reverse manner. **B:** Studies have demonstrated that phencyclidine (PCP) in correlation to the concentration presents similar symptoms in comparison to the different states of the NMDAR encephalitis. **C:** It has been suggested that the NMDAR autoantibodies disturb the signal transduction of neuronal cells, which is demonstrated via the release of vesicles of the presynapse with the neurotransmitter glutamate and an activation through receptors of the postsynapse, such as the NMDAR (*N*-methyl-D-aspartate receptor) (green) and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) (blue). It is believed that worsening depends on the autoantibody titer that induces internalization of the NMDAR, leading to a decrease of NMDAR in the long-term of the disease course. (Masdeu et al. 2016)

All these studies support the hypothesis that autoantibodies against the NMDAR are involved and causal in the pathogenicity of NMDAR encephalitis, but ultimate proof is missing as CSF samples contain a variety of different antibodies. In addition,

some symptoms such as epileptic seizures have been associated with increase or unchanged NMDAR expression at the synapse (Kalev-Zylinska et al., 2009; Wasterlain et al., 2013) and therefore cannot be explained by the current model. In addition, the variable clinical picture of NMDAR encephalitis raises the question, whether non-NMDAR binding antibodies do exist in the CSF and are involved in the disease picture. Furthermore, little is known about the origin of the disease, as antibodies are produced by B cells. The question arise which part of the B cell development might be deregulated to break central tolerance and is involved in the emergence of NMDAR autoantibodies.

1.5. Checkpoints of Central Tolerance that Might Induce the Generation of *N*-Methyl-D-Aspartate Receptor Autoantibodies

The human immune system is constituted of two subsystems. The fast innate immune system, which includes the anatomic barriers, the complement system and the cellular barriers as well as the adaptive immune system which allows for a stronger and highly specific reaction through recognition of individual pathogenic antigens. To initiate adaptive immunity, antigen presenting cells of the innate immune system, such as dendritic cells (DC), carry captured and degraded pathogen antigens to the peripheral lymphoid organs. Antigens of intracellular pathogens are presented on the major histocompatibility complex (MHC) class I, whereas extracellular pathogenic motives are phagocytosed and presented on MHC class II that activated $CD4^+$ T helper cells and are able to prime and activate B cells (Trombetta and Mellman, 2005). Activated B cells comprise the humoral immunity by secreting antibodies that can bind and neutralize pathogens and toxins (Owens and Zeine, 1989). Deregulated B cells are thought to be a major contributor of autoimmune encephalitis by secreting autoantibodies that target brain structures. Generally, highly effective immune responses are tightly controlled to differentiate between self and foreign antigens. In order to prevent autoimmune diseases several checkpoints in the development of B cells help to distinguish self and non-self-antigens (Meffre and Wardemann, 2008; Melchers, 2015).

During B cell development and maturation, a highly diverse repertoire of B cells and antibodies are generated through somatic recombination. At the immunoglobulin

heavy (IgH) chain of the variable (V), diversity (D) and joining (J) segments a process called VDJ recombination takes place, leading to the formation of a pre-B cell receptor (BCR). A first checkpoint to prevent autoimmunity is the recognition of nuclear antigens by the pre-BCR in the microenvironment which leads to positive or negative selection of the pre-B cells (Almqvist and Mårtensson, 2012; Keenan et al., 2008). In order to convert into immature B cells, similar to the VDJ recombination in the heavy chain, one of the light chain (IgL), kappa or lambda, rearranges its V and J segments and the second checkpoint follows. Presumably by a selection process depending on the strength of the interaction of BCRs with the autoantigens in the bone marrow, the autoreactivity decreases from early immature cells of 76 % to about 40 % in emigrated immature cells (Melchers and Rolink, 2006; Wardemann et al., 2003). At the third checkpoint, these immature B cells migrate to the spleen where autoantigens induce anergy or apoptosis leading to a further reduction of 20 % autoreactivity (Wardemann et al., 2003). As the now mature naïve B cells circulate the body, anergic B cells need to maintain their unresponsiveness to prevent autoimmunity (Merrell et al., 2006).

In order to activate naïve B cells to convert into antibody secreting cells, such as plasma blasts and plasma cells, or function as memory B cells, the T cell dependent activation takes place in the secondary lymphoid organs (Fig. 3). The naïve B cell takes up an antigen and presents it via the MHC class II to a matching T follicular helper cell (T_{FH}) at the border of the T cell zone and B cell follicle, leading to B cell proliferation (Lane et al., 1992) (Fig.3 a). There are two main pathways how B cells differentiate into plasma cells and memory B cells, depending on high affinity B cell-T cell interaction (Schwickert et al., 2011). The first pathway resulting in low affinity antibodies is located outside of the germinal center (GC) and these B cells differentiate into extrafollicular short-lived plasma cells and some convert to GC-independent memory B cells, which are both able to undergo class switch-recombination (CSR) from IgM to IgG, IgA, or IgE (Kaji et al., 2012; Kurosaki et al., 2015) (Fig. 3 b, c).

The second pathway inside the GC initiates the process of affinity maturation that generates even stronger affinity and non-autoreactive antibodies. Affinity and reactivity to self serve as a fourth checkpoint to prevent autoimmunity. Affinity maturation in the dark zone is mediated by the activation-induced cytidine

deaminase (AID), the main regulator of CSR and somatic hypermutation (SHM) in B cells (Muramatsu et al., 2000; Revy et al., 2000) (Fig. 3 d). In the light zone, follicular dendritic cells (FDC) present antigens to B cells. Depending on the interaction, low affinity leads to apoptosis of B cells and high affinity results in positive selection. B cells that recognize self-antigens are mostly eliminated in this process, an exception are rare antigens or antigens expressed in distal tissue such as brain epitopes, which might be a cause for autoimmunity (Chan et al., 2012). After each circle, the surviving B cells either differentiate into long-lived plasma cells, memory B cells or reenter the GC circle to continue affinity maturation (Heesters et al., 2014) (Fig. e, f). In NMDAR encephalitis, autoantibody producing cells are thought to be initially stimulated in the periphery, whereas priming of autoantibody producing B cells are suspected to begin in GC nearby NMDAR expressing tumors. Subsequently, B cells that were primed against the NMDAR in the germinal centers near to ovarian teratomas could reach through the deep cervical lymph node into the CSF (Titulaer, et al., 2013; Dalmau, 2016). Nonetheless, the exact underlying mechanism and at which checkpoint of B cell activation and maturation central tolerance might be disturbed to induce NMDAR encephalitis, is still incompletely understood.

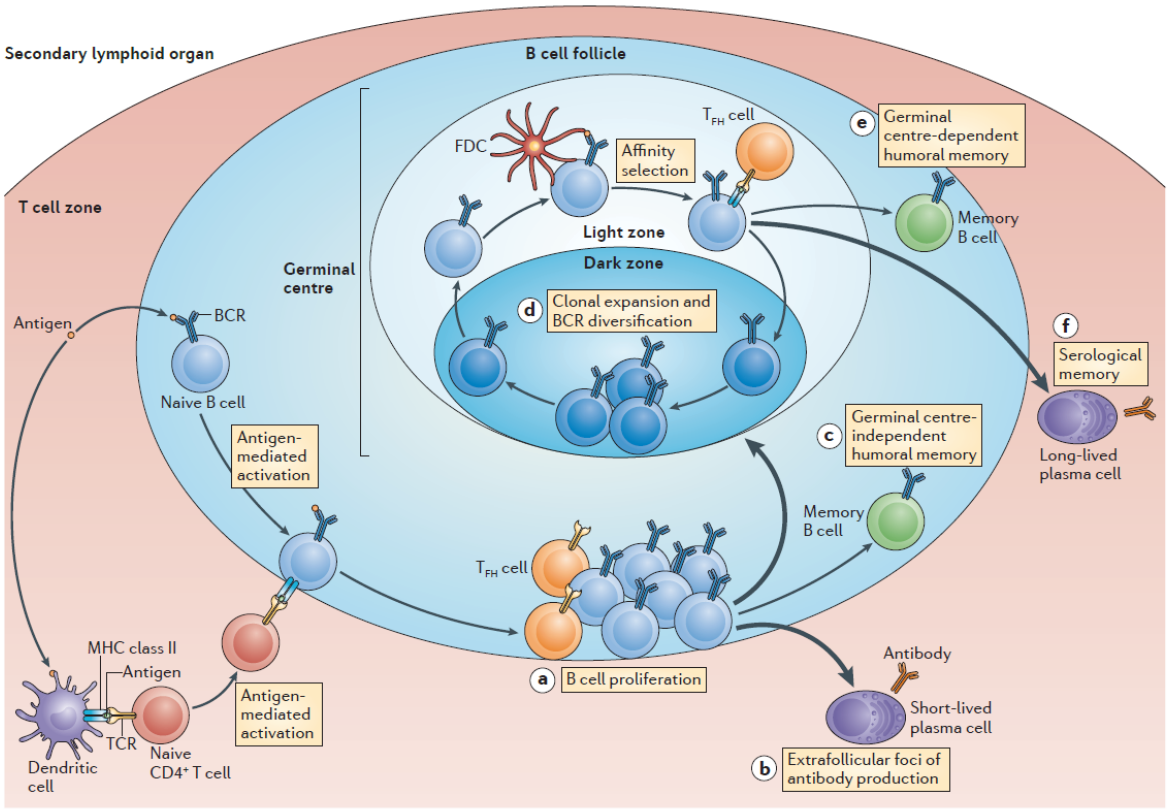

Figure 3 T cell dependent and independent B cell activation.

In the secondary lymphoid organs antigens are presented by dendritic cells to native T cell leading to migration to the border of the T cell zone and the B cell follicle. Antigen-activated B cells interact with the activated T cells and both move to the outer follicles, where proliferation of B cells take place (**a**). Afterwards, one of the three following differentiation occurs: (**b**) some B cells become short-lived plasma cells, (**c**) some convert into germinal center (GC) independent memory B cells and (**d**) other B cells undergo rapid proliferation and develop a GC. In the dark zone, clonal expansion together with somatic hypermutation improves the diversification of the B cell receptor (BCR). In the light zone, follicular dendritic cells (FDC) antigen complex and antigen specific T follicular helper (T_{FH}) cells interact with the B cells and affinity selection is accomplished. The B cell can either reenter the circle or leaves the GC as a memory B cell (**e**) or a long-lived plasma cell (**f**). The arrows symbolize the fate of the cells, whereas strong signals are represented by bold arrows, weak signals are depicted by narrow arrows. TCR (T cell receptor), MHC (major histocompatibility complex). (Kurosaki et al., 2015).

Another disruption in central tolerance might be comprised by natural occurring autoantibodies (NABs). These are generated by a presumed B cell subtype predominantly in the fetal omentum to protect newborns but also in adults in the liver and are thought to be generated spontaneously (Kantor and Herzenberg, 1993; Panda and Ding, 2015). NABs are often polyreactive and display germline immunoglobulin (Ig) genes with no or little somatic hypermutation and low affinity

(Baccala et al., 1989; Chen et al., 1991). Functionally, they are thought to clear the biological waste by monitoring the tissue homeostasis via clearance of cellular debris in order to neutralize the pro-inflammatory properties and therefore are thought to be beneficial (Binder, 2012). In conclusion, despite the benefit of these NAB producing B cells, the partial lack of central tolerance to ignore these autoantibody-producing B cells in the immune system might be a further explanation for the origin of autoimmune diseases, such as NMDAR encephalitis. However, if NABs targeting NMDAR do exist in NMDAR encephalitis patients and whether they are involved in the emergence of the disease is a highly discussed question.

1.6. Aim of this Thesis

NMDAR encephalitis has been identified as one of the most common autoimmune encephalitides targeting extracellular neuronal antigens. To date, the pathological mechanism of the emergence of this autoimmune encephalitis has not been defined, leaving therapeutic interventions to be quite broad. Despite treatment, recovery is often slow and patients may retain some disturbances in their cognition and impairments in memory can persist. So far, known disease associated triggers are quite diverse reaching from cancer to viral infections to unknown causes. As high levels of NMDAR autoantibodies titers in the CSF are a hallmark of the disease, it is thought that these autoantibodies against the NMDAR might be the main cause of the disease symptoms, but direct proof is missing, as further autoantibodies could also play a role in the disease process.

One way to a better understanding of the disease pathogenesis is to explore the human monoclonal autoantibody repertoire of NMDAR encephalitis patients.

In this study, we specifically aimed to:

1. Generate human monoclonal autoantibodies of NMDAR encephalitis patients to address the question if NR1 autoantibodies alone are sufficient to induce neurological changes.
    I. Characterize different NR1 autoantibodies by defining whether their target epitope is conserved to discuss possible therapeutic interventions.
    II. Analyze the autoantibody repertoire towards somatic hypermutations as indications for germinal center reactions to gain new insights in the origin and pathogenic process behind the development of NMDAR encephalitis.
    III. Further to elucidate the contribution of natural occurring autoantibodies (NABs) in the autoantibody repertoire to the emergence of NMDAR encephalitis and discuss whether NABs are pathogenic in the disease.
2. Define the contribution of antibody secreting cells and memory B cells in the CSF to the severity of NMDAR encephalitis. In detail, to determine the percentage of cells in the CSF that are NR1 autoantibody specific and characterize non-NR1 specific immune cells.

2. Research Articles

Human cerebrospinal fluid monoclonal *N*-methyl-D-aspartate receptor autoantibodies are sufficient for encephalitis pathogenesis.

Kreye J*, **Wenke NK*,** Chayka M, Leubner J, Murugan R, Maier N, Jurek B, Ly LT, Brandl D, Rost BR, Stumpf A, Schulz P, Radbruch H, Hauser AE, Pache F, Meisel A, Harms L, Paul F, Dirnagl U, Garner C, Schmitz D, Wardemann H, Prüss H.***Shared first authorship** Brain. 2016 Oct;139(Pt 10):2641-2652 PMID: 27543972

Reply: In vitro effects of a human monoclonal antibody against the N-methyl-d-aspartate receptor.

Kreye J, **Wenke NK**, Garner CC, Wardemann H, Prüss H.
Brain. 2016 Nov 17. pii: aww29. PMID: 27864269

2. Research Articles

https://doi.org/10.1093/brain/aww208

2. Research Articles

The original article is not published in the electronic version due to copyright to the publisher, but is available at:

https://doi.org/10.1093/brain/aww290

3. Discussion

3.1. Highlights of this Thesis

In this study, we examined the pathogenic mechanisms of NMDAR encephalitis by investigating the antibody repertoire of NMDAR encephalitis patients via the generation and characterization of patient derived monoclonal antibodies.

1. The human monoclonal NMDAR autoantibodies were subsequently used to perform functional assays. Here, incubation of hippocampal neurons with the NMDAR autoantibodies lead to downregulation of synaptic NMDAR clusters and a reduction in NMDA-induced calcium influx. Additionally, electrophysiological changes were observed. These findings constitute the final proof that NMDAR autoantibodies alone are able to induce pathological changes at critical neuronal structures.

    I. All identified NMDAR autoantibodies could no longer bind the NMDAR after a point mutation in a main immunogenic epitope, suggesting a common binding motive.
    II. Our study revealed high affinity NMDAR autoantibodies with minor somatic hypermutation highlighting the pathogenic potential of deregulated germinal center reactions.
    III. But we also detected low affinity NMDAR autoantibodies without somatic hypermutation, so called NABs, that showed pathogenic properties, indicating incomplete central tolerance.

2. The variable clinical disease picture of NMDAR encephalitis questioned whether additional autoreactivity besides NR1 specific autoimmunity might play a role in pathogenesis. Indeed, only 6 % of all detected antibody secreting cells and memory B cells were identified to be NR1 specific. Of note, the majority of the non-NR1 antibodies targeted particular epitopes on brain sections, pointing towards a possible role of these other autoantibodies in NMDAR encephalitis.

3.2. NR1 Autoantibodies Alone Are Sufficient to Decrease the Number of *N*-Methyl-D-Aspartate Receptors in Hippocampal Neurons

Previous work with NMDAR encephalitis patients' CSF or serum could demonstrate that synaptic NMDAR clusters were downregulated in hippocampal neurons and electrophysiological experiments revealed reductions of NMDAR-mediated currents upon autoantibody treatment (Hughes et al., 2010; Mikasova et al., 2012; Moscato et al., 2014; Planagumà et al., 2015; Pruss et al., 2010). However, in these studies human CSF, serum or whole Ig fractions of patients' CSF including possible non-NMDAR antibodies were used, making it questionable whether additional autoantibodies contribute to observed changes. Here, our study with purified human monoclonal NMDAR autoantibodies, generated from NMDAR encephalitis patients' CSF, substantiated the final proof that NMDAR autoantibodies are sufficient to induce electrophysiological and morphological changes, such as NMDAR cluster downregulation and decreased calcium influx when stimulated with NMDA in hippocampal neurons (Kreye and Wenke et al., 2016). In line, subsequent and independent studies using CSF samples as well as peripheral blood mononuclear cells (PBMCs) confirmed our results, verifying that human monoclonal NMDAR autoantibodies of NMDAR encephalitis patients specifically reduce NMDAR-mediated synaptic currents and initiate NMDAR cluster downregulation (Malviya et al., 2017; Sharma et al., 2018). In addition, *in vivo* experiments showed that autoantibodies can induce memory deficits and similar to the NMDAR inhibitor MK801, cause increased locomotor activity (Sharma *et al*., 2018). As a conclusion, data of independent experiments demonstrates that NMDAR autoantibodies alone can induce functional changes in neurons to disturb signaling and are sufficient to provoke the main symptoms of NMDAR encephalitis.

Discussing pathological mechanisms of NMDAR encephalitis in more detail, one has to point out the occurrence of memory B cells in the patients' CSF. Here, it is of interest, that relapses occur in about one quarter of NMDAR encephalitis patients (Gabilondo et al., 2011). It is not fully understood, why patients relapse after treatment. We could show that a third of all identified NR1 autoantibody producing cells are indeed memory B cells. These findings strengthen our hypothesis that relapses might occur due to reactivation and differentiation of NR1 specific memory B cells. This hypothesis is supported by the fact that therapeutic intervention with

rituximab, a CD20 autoantibody targeting B cells and memory B cells can prevent relapses in NMDAR encephalitis patients in clinical use (Titulaer et al., 2013).

In future, the human monoclonal autoantibodies obtained in this study could be interesting for clinical use. As efficient recovery from NMDAR encephalitis can be directly correlated with decreasing CSF autoantibody titers, the same is true for disease relapses which go in line with increased autoantibody titers (Gresa-Arribas *et al*., 2014). Yet, comparisons between patients' CSF titers are intricate (Gresa-Arribas *et al*., 2014). Using the identified monoclonal NR1 autoantibodies, molecular features such as the influence of avidity and affinity in NMDAR encephalitis could be investigated in detail in further studies. In long-term, this information might be leading to the use of monoclonal NMDAR autoantibodies as a comparable standard within clinics. Which in turn might help to compare NMDAR encephalitis patients among each other and might serve as a prediction standard of NMDAR autoantibody titers to determine treatment options.

3.2.1. The Target Region of *N*-Methyl-D-Aspartate Receptor Autoantibodies Might Be Broader than Expected

NMDAR autoantibodies have been described to target a specific epitope in the amino-terminal domain (ATD) of the NR1 subunit, thus we address the question if different monoclonal NMDAR autoantibodies display similar binding properties. In general, antibodies target their native antigen epitope through the variable chains of the Ig, the complementarity-determining regions (CDRs), whereby CDR3 is considered to be the most important structure for high affinity antigen binding (Xu and Davis, 2000). Contact between the antibodies paratop and the antigen epitope is established through 15-22 amino acid residues of each of them, whereas the binding energy is contributed mostly by only 5 to 6 of these residues (Laver et al., 1990). Except for the clonally expanded autoantibodies, the identified NMDAR autoantibodies in this study do not display similarities in their amino acid sequence of the CDR3. Nonetheless, they all seem to bind one small epitope in the ATD region of the NR1 subunit of the NMDAR. In 2012, a study by Gleichman and colleges demonstrated that binding of patients' CSF antibodies is abolished when a point mutation at the ATD region of the NR1 subunit was introduced, exchanging

asparagine to glutamine at position 368 (N368Q). Similarly, the binding of our purified human monoclonal NMDAR autoantibodies was eliminated in HEK293T cells expressing the N368Q mutated NMDAR form. Therefore, it has been hypothesized that NMDAR autoantibodies target a small, common, immunogenic region, a phenomenon that has also been described for nicotinic acetylcholine receptors in myasthenia gravis, another autoimmune disease. This hypothesis is meanwhile contrasted by newer findings in which competitive binding assay experiments revealed that there was no interference among the human monoclonal NMDAR autoantibodies, 003-102, 003-109, 007-168 (unpublished data). Similar results were reported by Sharma and colleges in 2018.

Interestingly, analyzing the protein structure of the ATD region, the identified amino acid N368 lies in the area that links two lopes resulting in a clamshell-like structure. The hinge between the clamshell-like structure forms binding sites for endogenous allosteric modulators of the NMDAR such as protons (H^+) and zinc ions (Zn^{2+}), which induce after binding structural rearrangements in the NMDAR agonist binding domain (Gielen et al., 2008). Thus, mutations in this area (N368) are potentially leading to conformational changes. In addition, a recent *in vitro* study with positive allosteric modulators could restore the NMDAR function altered by NMDAR encephalitis patients' CSF, suggesting that a special conformation is required for NMDAR autoantibody binding (Warikoo et al., 2018). Taking these findings together, it seems more likely that the NMDAR autoantibodies do not compete for the exact same epitope, but further studies are pending.

In conclusion, the area around the amino acid N368 is important for NMDAR autoantibody binding, but it is probably not the only binding domain for NMDAR autoantibodies. Additionally, conformational changes might prevent autoantibody binding and should be considered for potential therapeutic interventions.

3.2.2. *N*-Methyl-D-Aspartate Receptor Autoantibodies Showed Minor Somatic Hypermutation after Germinal Center Reactions

In general, somatic hypermutation, high affinity and class-switched IgG autoantibodies are indicative of germinal center reactions with the involvement of T cells, resembling a pathological process in which homeostatic pathway functions

are disturbed (Elkon and Casali, 2008). We could show, that compared to all antibodies in the repertoire, NR1 autoantibodies showed only minor hypermutation in their sequence, but still demonstrated high affinity binding that resulted in NMDAR internalization and functional inhibition of the receptor (Kreye and Wenke *et al*., 2016). A subsequent study also reported minor somatic hypermutations and validated autoantibody functionality *in vivo* (Malviya *et al*., 2017). Taken together, it has been independently shown that high affinity NMDAR autoantibodies can be generated with minor somatic hypermutations. The existing somatic hypermutations in NMDAR autoantibodies indicate an important role for affinity maturation via germinal center reactions as a pathogenic factor in the development of NMDAR encephalitis. At least in lymphoid aggregates, that were dissected of teratomas of NMDAR encephalitis patients, germinal center reactions have been reported previously (Dabner et al., 2012). In addition, a recent study could also emphasize the importance of germinal center reactions in NMDAR encephalitis. They identified continuous high levels of IgM and IgG NR1 autoantibodies in the sera of NMDAR encephalitis patients suggesting a consistent production of NR1-IgG from continuously circulating activated B cells in germinal centers (Makuch et al., 2018). As germinal center reactions are dependent on T_{FH} to sufficiently stimulate B cells during affinity maturation, it is of interest to investigate T cell dependency during the development of NMDAR encephalitis. Verification of the hypothesis that a continuous production of autoantibodies is necessary and subsequent research might even lead to new therapeutic interventions through targeting germinal center reactions.

3.2.3. Natural Occurring Autoantibodies of the *N*-Methyl-D-Aspartate-Receptor Are Present in the Repertoire of *N*-Methyl-D-Aspartate-Receptor Encephalitis Patients

During analysis we detected natural occurring autoantibodies (NABs) in the CSF of NMDAR encephalitis patients. In general, NABs are autoantibodies with no changes in the germline configuration, are present in the absence of known immunization and are thought to be a first line of defense against bacteria, viruses and parasites (Coutinho et al., 1995). Several studies could further show that NABs fulfill a

beneficial role in healthy individuals explaining their evolutionary existence and making them a possible therapeutic against diseases such as multiple sclerosis and Alzheimer's disease (for a detailed review, see Wootla et al., 2015). In contrast, we could demonstrate that the NAB (003-109), which displays lower affinity to the NR1 subunit than the mutated NR1 autoantibody (003-102) of the same patient, is still able to functionally disturb synaptic NMDAR-mediated currents (unpublished data). In line, a study by Malviya and colleges, in 2017, described a human monoclonal NR1 autoantibody with one hypermutation in each chain. Due to its low hypermutation, this antibody can be defined as a NAB (Coutinho et al., 1995). Intraventricular infusion of this NAB in mice demonstrated that synaptic NMDAR cluster density was decreased and memory impairment was observed (Malviya *et al*., 2017). Taken together, these results suggest that NR1 specific NABs can also be pathogenic, which is supported by the fact that NABs have been shown to play an important role in pathogenesis of other autoimmune diseases. For instance, in insulin-dependent diabetes mellitus, it has been shown that anti-insulin IgG autoantibodies arise from a natural antibody with germline configuration targeting insulin without affinity maturation (Ichiyoshi et al., 1995). In addition, NAB producing B cells can enter the germinal center pathway and mature, undergoing class switch and hypermutation, as a result converting to higher affinity autoantibodies (Casali and Schettino, 1996). Thus, regulation of NABs' maturation in a direct or indirect way might be contributing to the emergence of autoimmune diseases (Avrameas, 1991). In conclusion, NABs specifically targeting NR1 in the CSF repertoire of NMDAR encephalitis patients might contribute to disease manifestation in NMDAR encephalitis.

3.2. The Majority of Autoantibodies in the Cerebrospinal Fluid Target other Neuronal Structures

The detection of high NR1 autoantibody titers in the CSF of NMDAR encephalitis patients initially suggested a major contribution of these autoantibodies (Dalmau *et al*., 2008) and thus we presumed that a large proportion of antibody secreting cells in the CSF would be NR1 specific. Interestingly, we could only detect NR1 autoantibodies in three out of eight patients and found that among all patients only

6 % of CSF derived antibodies reacted against the NR1 subunit. In line, additional studies generating human monoclonal autoantibodies demonstrated that only a few of the antibody producing cells and memory B cells in the CSF are positive for NR1 autoantibodies. Earlier studies suggested that plasma cells of autoantibodies are also present in inflammatory brain infiltrates, indicating an additional location of antibody producing cells (Camdessanché et al., 2011; Martinez-Hernandez et al., 2011; Tüzün et al., 2009). As plasma cells are able to produce over several thousands of antibodies per second (Alberts et al., 2002), NMDAR autoantibodies could reach high concentrations in the CSF, whereas the plasma cells themselves might be located not only in the CSF but also in the brain parenchyma. Using laser capture microdissection in the parenchyma of tissue samples of NMDAR encephalitis patients and subsequent messenger RNA purification and Ig sequencing could unveil whether cells secreting NR1 autoantibodies might be also and largely located in the brain tissue.

Focusing on all CSF derived non-NR1 specific antibodies, it is of note that 95 % bound specifically to diverse brain-expressed epitopes, providing first indications for the strong variability in the clinical picture of NMDAR encephalitis patients. Unpublished data of immunoprecipitation and mass spectrometry experiments of the non-NR1 specific antibodies shows binding to already known antigens involved in other autoimmune encephalitis diseases, such as AMPAR 2, glial fibrillary acidic protein (GFAP) and inositol 1,4,5-trisphosphate receptor type 1 (ITPR1) (Fang et al., 2016; Jarius et al., 2014; Lai et al., 2009). Additionally, these three targets have been verified via cell based assays. However, if these human monoclonal autoantibodies disturb neuronal function is still under investigation.

Interestingly, a young NMDAR encephalitis patient additionally suffered from asystole, repeated failure in heart contraction, and needed a transient pacemaker during the stay in the ICU. The patient fully recovered from NDMAR encephalitis after treatment and also no longer needed the pacemaker. One autoantibody of this patient targeted both, ITPR1 and ryanodine receptor 2, known to be expressed in myocardial cells. Indeed, the autoantibody could alter the contraction rates of myocardial rat cells *in vitro* compared to control antibody (unpublished data). All in all, this supports the hypothesis that the variable disease picture of NMDAR

encephalitis might be accompanied with symptoms arising from diverse autoantibodies.

Furthermore, the detection of other autoantibodies gives rise to the question if only NR1 autoreactivity is causal for the initiation of the disease or if a general disturbance of central tolerance leads to the generation of several autoantibodies besides NR1 specific autoantibodies that are able to cause diverse clinical symptoms.

4. Conclusion

Autoimmune diseases can develop dependent on the appearance of diverse genetic and environmental factors. In NMDAR encephalitis, humoral autoimmunity is thought to play a major role.

In this study, we highlighted the pathophysiological role of NMDAR autoantibodies and the B cell repertoire of NMDAR encephalitis patients. Analyzing the antibody repertoire of eight patients, we detected NR1 autoantibodies with germline configuration, so called NABs, as well as NR1 autoantibodies displaying somatic hypermutation and high affinity binding to the NMDAR. All autoantibodies targeting the NR1 subunit, including NABs, were physiologically functional, as they were able to efficiently bind the NMDAR and subsequently alter synaptic currents. Taken together, NMDAR autoantibodies are highly functional and are probably causal for major symptoms of NMDAR encephalitis patients as they are able to elicit drastic physiological alterations in neurons.

Nonetheless, other autoantibodies have been detected in the autoantibody repertoire of NMDAR encephalitis patients, raising the question to what extend they contribute to the quite variable disease picture. Additionally, NMDAR encephalitis might not only be an isolated autoimmune reaction to the NMDAR, but a broader autoimmune response with severe disruptions in central tolerance. Here, further studies are necessary to illuminate what pathophysiological changes these non-NMDAR autoantibodies are able to induce. All in all, we could show strong pathogenic effects of NMDAR autoantibodies and were able to contribute to a better understanding of the pathomechanism of NMDAR encephalitis.

5. Appendix

5.1. References

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5.2. List of Figures

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5.3. Abbreviations

AID	activation-induced cytidine deaminase
AIE	autoimmune encephalitis
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ASC	antibody-secreting cells
ATD	amino-terminal domain
ABD	agonist binding domain
BCR	B cell receptor
$Ca2^{+}$	calcium
Caspr2	contactin-associated protein-like 2
CD	cluster of differentiation
CDRs	complementarity-determining regions
CSR	class switch-recombination
CTD	carboxyl-terminal domain
D	diversity
D1	upper lobe
D2	lower lobe
DC	dendritic cells
DIV	days in vitro

DMEM	Dulbecco’s modified Eagle medium
DMSO	dimethylsulphoxide
CNS	central nervous system
CSF	cerebrospinal fluid
FACS	fluorescence-activated cell sorting
FBS	foetal bovine serum
FCS	forward scatter
FDC	follicular dendritic cells
GABAB	γ-aminobutyric acid class B
GC	germinal center
GFAP	glial fibrillary acidic protein
HEK293T	human embryonic kidney
hNR1	human monoclonal NR1
HSE	herpes simplex virus encephalitis
ICU	intensive care unit
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IgK	immunoglobulin kappa chain
IgL	immunoglobulin lambda chain
ITPR1	inositol 1,4,5-trisphosphate receptor type 1
J	joining
K^+	potassium
LGI1	leucine-rich glioma inactivated 1
LTD	long-term depression
LTP	long-term potentiation
MBC	memory B cells
$Mg2^+$	magnesium
MHC	major histocompatibility complex
msNR1	mouse NR1
N368Q	NR1 subunit construct with amino acid 368 mutated
Na+	sodium
NABs	natural occurring autoantibodies
NAM	negative allosteric modulators
NMDA	N-methyl-D-aspartate

NMDAR	N-methyl-D-aspartate receptor
NR1	GluN1
NR2	GluN2
PAM	positive allosteric modulators
PBS	phosphate-buffered saline
PCP	phencyclidine
PCR	polymerase chain reaction
PEI	polyethylenimine
PLL	poly-L-lysine
rbNR1	rabbit NR1
RT	reverse transcriptase
RYR2	ryanodine receptor 2
SHM	somatic hypermutation
SSC	side scatter
TCR	T cell receptor
TFH	T follicular helper cell
TMD	transmembrane domain
V	variable
Zn^{2+}	zinc ion

5.4. Statement of Contribution

The present study was conducted as a collaborative scientific project. In the following I state my contributions to the published data.

In my doctoral thesis I stained cells of patients' CSF samples and single cell sorted them with FACS and performed reverse transcriptase PCR of patient's number AI ENC 004, 007, 008 and 010. I analyzed and interpreted the sequence data for the repertoire analysis, immunoglobulin classes were determined, analysis of VDJ segments and hypermutation were compared to germline configuration. In addition to the previous mentioned patient samples, also AI ENC 035 and 002 were amplified with PCR to sequence the immunoglobulin sequences of heavy, lambda and kappa chains. Subsequent cloning, purification and transfection in HEK293T cells were accomplished. The supernatants of the cell culture samples were measured with Enzyme-linked Immunosorbent Assay (ELISA). I established a protocol for non-fixated mouse brain section and staining of all of the 170 generated monoclonal antibodies. In addition, I tested all 170 monoclonal antibodies on transfected HEK293T cells expressing the NR1 subunit of the NMDAR. Subcloning of N368Q mutant NR1, expression in HEK293T cells and subsequent staining of all nine monoclonal NMDAR autoantibodies. Furthermore, I established a protocol for large scale production and purification of human monoclonal antibodies and provided the significant amount of antibody needed for the functional assays.

Injection of the monoclonal autoantibody intravenously was executed by PD Dr. med. Harald Prüß and subsequent staining of *in vivo* injected mice 3, 3 - diaminobenzidine (DAB) staining was performed by myself.

I performed all further analysis to identify the additional autoantibodies. I established different protocols for immunoprecipitation of the monoclonal autoantibodies with Western Blot analysis, subsequent enzymatic digestion, mass spectrometric determination and following data analysis. The mass spectrometry facilities were operated by Dr. Caroline May and Dr. Oliver Klein.

All experiments underlying figures Fig.1A-D, Fig. 2 A-D, L-M, N-Q, Fig. 3, Fig. 4 B, C, E, F, of the article published in BRAIN of Oxford Academic were designed, conducted and analyzed by me. Together with Dr. med. Jakob Kreye, I provided

experimental data and processed it for the following figures and tables: Fig. 1F, Fig. S1, Fig. S2, Table S1, S3.

Experimental contributions to this data by others are stated in the subsequent:

PD Dr. med. Harald Prüß and Dr. med. Jakob Kreye collected the patients' samples.

Dr. med. Jakob Kreye performed FACS stain and reverse transcriptase on patient samples AI ENC 003, 011, 035, 002 and data analysis. The specific PCR, amplification and cloning was conducted of AI ENC 003, 011.

Electrophysiology experiments were performed by Dr. med. Nikolaus Maier, PhD Benjamin R. Rost, Alexander Stumpf (Fig. 5 D, E).

Neurons were stained with the monoclonal autoantibody and the calcium influx imaging experiments and NMDAR cluster counting was executed by Mariya Chayka (Fig. 2 E-F, H-K, Fig. 5 A-C, F-H).
Some additional ELISA analysis and FACS cell staining's with the N368Q mutant and NR1 transfected HEK293T cells were conducted by Lam-Thanh (Fig. 2 G, Fig. 4 H, G).
The initial draft of the publication was written in a cooperative manner with PD Dr. med. Harald Prüß, Dr. med. Jakob Kreye and myself. Helpful discussions and advice were given by Dr. Rajagopal Murugan, Prof. Dr. med. Ulrich Dirnagl, Prof. Dr. Craig Garner, Prof. Dr. Dietmar Schmitz and Prof. Dr. Hedda Wardemann.

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Thank you all for your support, it means a lot to me.

5.6. Curriculum Vitae

The online version does not contain the CV due to data privacy protection reasons.

5.7. Statement of Authorship

I hereby declare that the work presented in this thesis has been written independently and without inappropriate support. All sources of information are referenced. I hereby declare that this thesis has not been submitted, either in the same or in a different form, to this or any other university for a degree.

Freising, den 24.09.2018

Nina Kerstin Wenke