Aus dem Neurowissenschaftlichen Forschungszentrum
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DISSERTATION

Assessing the effect of autoimmune receptor autoantibodies on synaptic function and behaviour

Bewertung der Auswirkungen von Autoimmune rezeptor Autoantikörpern auf synaptische Funktion und Verhalten

zur Erlangung des akademischen Grades
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List of abbreviations

AE	-	autoimmune encephalitis
AMPAR	-	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AQP4	-	aquaporin-4
BBB	-	blood-brain-barrier
CASPR2	-	contactin-associated protein-like 2
CNS	-	Central nervous System
CSF	-	cerebral spinal fluid
EPSCs	-	excitatory post-synaptic currents
$GABA_AR$/$GABA_BR$	-	γ-aminobutyric acid A and B receptor
IPSC	-	inhibitory postsynaptic currents
LGI1	-	leucine-rich glioma inactivated-1
mIPSCs	-	miniature postsynaptic currents
NMDAR	-	N-methyl-D-aspartate receptor
VGAT	-	vesicular GABA transporter

Abstract

γ-aminobutyric acid A receptor ($GABA_AR$) and N-methyl-D-aspartate receptor (NMDAR) encephalitis are both autoimmune disorders characterized by autoantibodies against receptors in the brain. Due to the relatively new discovery of these disorders, much is still unknown. Some studies have explored the cerebral spinal fluid (CSF) of patients to see how these antibodies could influence receptor function, however due to the large mixture of antibodies often observed in patient's CSF, linking antibodies to their mechanistic properties has proven to be challenging. Due to this, this thesis investigates four patient derived monoclonal antibodies to study how they individually contribute to these disorders. First, two autoantibodies against the $GABA_AR$, one targeting the α1-subunit and one requiring both a α1 and γ2-subunit of the receptor, were investigated with the help of immunocytochemistry, electrophysiology and calcium imaging experiments. Interestingly, these antibodies seem to work through distinct mechanisms. For example, the α1-antibody influenced receptor distribution at longer time points (>24hrs), driving the receptors away from the synapse by internalization. In addition, at shorter time points (<4min) the α1-antibody could directly facilitate an antagonistic effect on the receptor. In contrast, the α1γ2-antibody did not have any impact on these receptors, neither influencing signal transduction of the receptor, nor causing receptor redistribution. However, once microglia were added to the cultures, the α1γ2-antibody was able to engage microglia leading to removal of synaptic receptors. Second, two antibodies against NMDAR, one germline and one maturated, were investigated through electrophysiological experiments. Germline antibodies have not been investigated before in the context of autoimmune encephalitis and are generally not thought to cause autoimmunity. Interestingly, this germline antibody did disrupt receptor functionality, similar to the maturated antibody, albeit at a five-fold higher dose. These findings imply that everyone could be at risk of developing autoimmune encephalitis and not only individuals that have an initial tumor/infection as previously postulated. All in all, these findings highlight the tremendous antibody diversity underlying autoimmune encephalitis, complicating the development of new treatment strategies for patients, necessitating a more holistic approach to these disorders.

Zusammenfassung

γ-Aminobuttersäure-A-Rezeptor- ($GABA_AR$) und N-Methyl-D-Aspartat-Rezeptor- (NMDAR) Enzephalitis sind beides Autoimmunerkrankungen, die durch Autoantikörper gegen Rezeptoren im Gehirn gekennzeichnet sind. Da diese Erkrankungen erst vor kurzem entdeckt wurden, ist noch vieles unbekannt. In einigen Studien wurde die zerebrale Rückenmarksflüssigkeit (CSF) von Patienten untersucht, um herauszufinden, wie diese Antikörper die Rezeptorfunktion beeinflussen könnten. Aufgrund der großen Mischung von Antikörpern, die häufig in der CSF von Patienten beobachtet wird, hat sich die Zuordnung von Antikörpern zu ihren mechanistischen Eigenschaften jedoch als schwierig erwiesen. Aus diesem Grund werden in dieser Arbeit vier von Patienten stammende monoklonale Antikörper untersucht, um herauszufinden, wie sie individuell zu diesen Störungen beitragen. Zunächst wurden zwei Autoantikörper gegen den GABAAR, einer gegen die α1-Untereinheit und einer, der sowohl eine α1- als auch eine γ2-Untereinheit des Rezeptors benötigt, mit Hilfe von Immunozytochemie, Elektrophysiologie und Calcium-Imaging-Experimenten untersucht. Interessanterweise scheinen diese Antikörper über unterschiedliche Mechanismen zu wirken. Zum Beispiel beeinflusste der α1-Antikörper die Rezeptorverteilung zu längeren Zeitpunkten (>24 Stunden), indem er die Rezeptoren durch Internalisierung von der Synapse wegtrieb. Darüber hinaus konnte der α1-Antikörper zu kürzeren Zeitpunkten (<4min) direkt eine antagonistische Wirkung auf den Rezeptor ausüben. Im Gegensatz dazu zeigte der α1γ2-Antikörper keine intrinsische Wirkung auf diese Rezeptoren und beeinflusste weder die Signaltransduktion des Rezeptors noch bewirkte er eine Umverteilung des Rezeptors. Sobald jedoch Mikroglia zu den Kulturen hinzugefügt wurden, war der α1γ2-Antikörper in der Lage, die Mikroglia zu aktivieren, was zur Entfernung der synaptischen Rezeptoren führte. Zweitens wurden zwei Antikörper gegen NMDAR, ein Keimbahn-Antikörper und ein reifer Antikörper, in elektrophysiologischen Experimenten untersucht. Keimbahn-Antikörper wurden bisher noch nicht im Zusammenhang mit Autoimmunenzephalitis untersucht, und es wird allgemein nicht angenommen, dass sie Autoimmunität verursachen. Interessanterweise hat dieser Keimbahn-Antikörper die Rezeptorfunktionalität ähnlich wie der reife Antikörper gestört, wenn auch in einer fünffach höheren Dosis. Diese Ergebnisse deuten darauf hin, dass jeder Mensch ein Risiko für die Entwicklung einer Autoimmunenzephalitis haben könnte und nicht nur Personen, die einen Tumor oder eine Infektion haben, wie bisher angenommen wurde.

Alles in allem verdeutlichen diese Ergebnisse die enorme Vielfalt der Antikörper, die der Autoimmunenzephalitis zugrunde liegen, was die Entwicklung neuer Behandlungsstrategien für Patienten erschwert und einen ganzheitlicheren Ansatz für diese Erkrankungen erforderlich macht.

1 Introduction

1.1 Autoimmune encephalitis as a global health burden

Autoimmune encephalitis is an umbrella term for a clusterof disorders in which the immune system generates antibodies against the body's own neuronal proteins in the brain (1). The nature of these proteins varies wildly from Aquaporin-4 (AQP4), Contactin-associated protein-like 2 (CASPR2), Leucine-rich glioma inactivated-1 (LGI1), , N-methyl-D-aspartate receptor (NMDAR), to γ-aminobutyric acid A and B receptors ($GABA_AR$/$GABA_BR$) (2-4). The incidence and prevalence of this disease is currently difficult to estimate due to its relatively recent discovery (5), yet a population-based study published in 2018 found an incidence of 0.8/100,000 and a prevalence of 13.7/100,000 person-years, numbers that are similar to the incidence and prevalence of infectious encephalitis. It is not clear why people develop autoimmune encephalitis, but some subtypes are believed to be associated with certain cancers (30-60%) (6), whereas others are believed to be a cross-reactivity to a previous infection (7, 8). Even though the initial cause remains unknown, treatment plans are in place. The first line of treatment involves steroids, intravenous immune globulin, plasma exchange or a combination of all. When this treatment is not sufficient, immunosuppressants like rituximab can be given as well (9, 10). All treatments need to be administered chronically, which dramatically affects patient's quality of life.

1.2 Challenges in the field

As can be deducted from the wide variety of targets in the brain, autoimmune encephalitis is a very heterogeneous disease. Not only are there distinct differences between subtypes of the disease, e.g. NMDAR and $GABA_AR$ encephalitis, but there are also distinct differences within one subtype due to the many different varieties these autoantibodies can come in. Early studies of patients with auto-immune encephalitis utilized the cerebral spinal fluid (CSF) from patients to investigate how their associated antibodies affect neuronal function (11-14). One major drawback of this approach is that patient's CSF often contains a varietyof numerous autoantibodies, making it difficult to determine causal effects between specific antibodies and the etiology of a patient's phenotype. A major break-through in this emerging field was the molecular cloning of monoclonal antibodies from patient derived B-cells isolated from CSF (15-17). These

studies revealed while there are often dominant antibodies against one antigen, an array of antibodies of different types against different proteins and epitopes are also present within the same patient. With the isolation of monoclonal antibodies, it is now possible to study whether the ethology and pathophysiology of a patient's disease is defined by the repertoire of antibodies expressed and how mechanistically they alter receptor and/or neuronal function.

1.3 The aim of this work

This work sets out to answer two questions. The first is to understand how the variation in a patient's antibody repertoire contributes to auto-immune encephalitis. At present, antibodies are known to have three main mechanisms by which they can exert their effect. First, they can act directly on a given receptor, exerting agonistic, modulatory, or antagonistic effects (Fig. 1.1). Second, they can alter surface protein expression by influencing their rate of internalization, e.g. via receptor crosslinking (Fig. 1.2). Thirdly, antibodies might exert their effects by activating other actors within the immune system, e.g., via complement or Fc gamma receptors on immune cells (Fig.1.3). To gain a better understanding of which of these mechanisms contribute to autoimmune encephalitis, this thesis project focused on defining the modes of actions of two distinct $GABA_AR$ autoantibodies: one recognizing epitopes on an α1 subunit and the second recognizing an epitope shared between the α1 and γ2 subunit of these receptors.

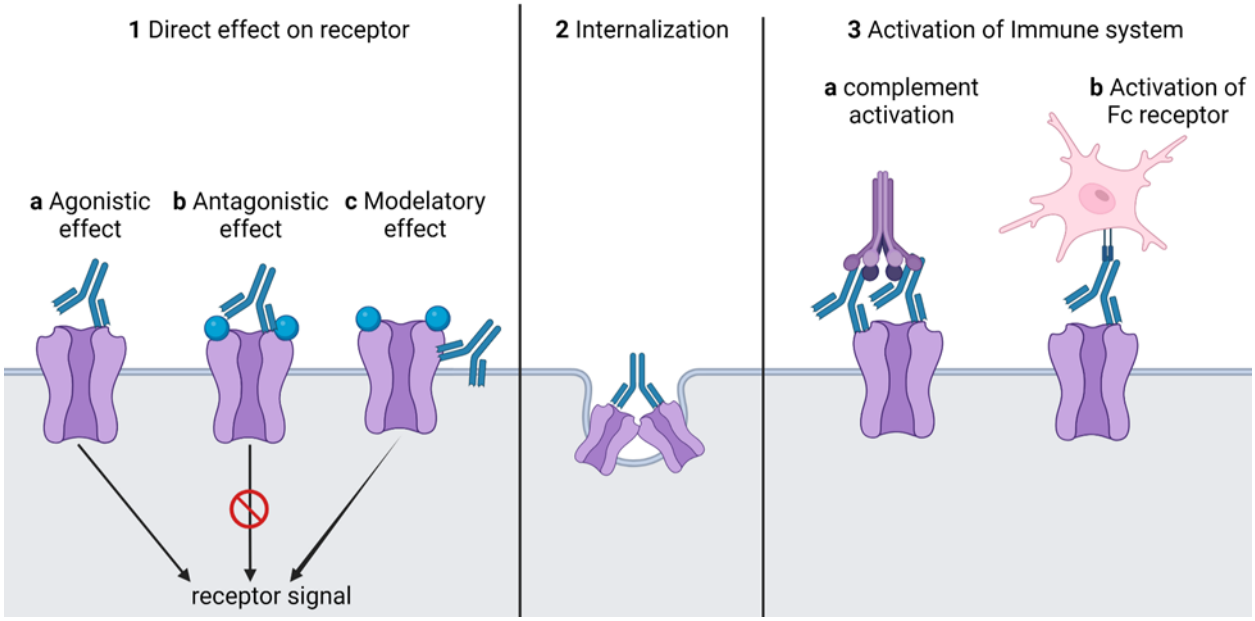

Figure 1: mechanisms by which antibodies exert their function. Adopted from (8).

The second aim of this work is to investigate if germline antibodies can influence signal transduction, in and of themselves, before they undergo somatic hypermutation and maturation. B-cells undergo negative selection in the bone marrow to prevent self-reactivity (18). It is therefore thought that autoimmunity is often a result of cross-reactive epitopes from a pathogen to self after somatic hypermutation. A hint that germline antibodies are not necessarily harmless, arose when we isolated an unmutated antibody from a patient that was diagnosed with NMDAR encephalitis along with several maturated NMDAR autoantibodies in their CSF (15). In this thesis, we have compared the impact of two of these NMDAR autoantibodies, one germline and one maturated, to explore whether both equally negatively influence signal transduction of the NMDAR and thus potentially cause disease.

1.4 $GABA_AR$ encephalitis

$GABA_AR$ encephalitis is a subtype of autoimmune encephalitis in which the body generates autoantibodies against the $GABA_AR$, first described by Petit-Pedrol et al. (13). $GABA_AR$ encephalitis affects both children and adults, all genders equally, and is associated with tumors in 40% of the cases (19, 20). Unlike other autoimmune encephalitis subtypes, $GABA_AR$ encephalitis often presents with a distinctive MRI pattern with multifocal cortical and subcortical lesions (20, 21) and symptoms include, but are not limited to, epilepsy, hallucinations, abnormal movement, and alterations in cognition, behavior, and levels of consciousness (12-14).

$GABA_ARs$ are ionotropic chloride channels that facilitate both fast inhibitory neurotransmission between neurons as well as tonic inhibitory tone. Each receptor is comprised of five different subunits, from an array of different subunit types; α (1-6), β (1-3), γ (1-3), ρ (1-3), δ, ε, θ, and π (22-24) with certain subunits having different expression patterns throughout the brain (25). Nonetheless, the most frequently expressed receptor isoform is α1γ2β2α1β2, arranged counterclockwise as seen from outside the cell (26). Intriguingly, these receptors also have many modulatory sites, of which the benzodiazepine site is one. Benzodiazepine acts as a positive modulator on the receptor, increasing its affinity for GABA, leading to increased receptor opening times (26). Benzodiazepine does not interact with all receptor types equally, but has a higher affinity for receptors that express a combination of α1 and γ2 subunits, or a combination of γ2 with an α2, α3, or α5, all with a higher sensitivity to benzodiazepine (26).

Previous research into $GABA_AR$ encephalitis has shown that cells treated with CSF for 24 hours or longer leads to internalization of the $GABA_AR$ at synapses, but not along dendrites (13). In addition, whole-cell patch-clamp experiments show a decrease in the amplitude and frequency of miniature postsynaptic currents (mIPSCs) after exposure to patient's CSF, hinting towards a possible removal of the receptors from the synapse (12). This idea is consistent with Western blot experiments showing reductions in surface expression of $GABA_ARs$ (14). To date, several antibodies against different subunits of the $GABA_AR$ have been discovered, with the predominant targets being the α1, β3, and γ2 subunits of the receptor (12-14). However as mentioned before, CSF of affected patients often contains a mixture of several autoantibodies with targets against several subunits of the $GABA_AR$. It is estimated from the literature that the mammalian central nervous system expresses as many as 11 distinct and functional receptor isotypes with varying modulatory sites, indicating that CSF with autoantibodies could set in motion several inactivation mechanisms at once, depending on the receptor epitope. This work sets out to elucidate whether indeed variation in antibody-driven mechanisms contributes to auto-immune encephalitis.

1.5 NMDAR encephalitis

NMDAR encephalitis was one of the first subtypes of autoimmune encephalitis to be discovered and it's therefore one of the most commonly studied and diagnosed subtypes of autoimmune encephalitis (15). This disease is often associated with ovarian teratomas, making the patients affected by NMDAR encephalitis more likely to be female. Symptoms often include psychiatric or memory problems, epileptic seizures, amnesia, and autonomic instability (27).

The NMDAR is an ionotropic glutamate receptor that is important for synaptic plasticity, memory and learning (28). It is a hetero-tetramer that can be formed by a combination of GluN1-3 subunits (29). Every NMDAR consists of two GluN1 subunits and requires either another pair of GluN2 or GluN3 subunits to form a functional receptor (29). However, the receptor is often composed of two GluN1 and two GluN2 subunits (30), making this receptor less variable compared to $GABA_ARs$.

Since its discovery in 2007, many studies have looked into how these autoantibodies contribute to disease pathology. To date, research has shown that the presence of autoantibodies leads to a decrease in NMDARs in the brain. This mechanism is believed to work through cross-linking receptors by these antibodies, triggering their internaliza-

tion (11). This theory seems to be confirmed by Fab-fragment experiments, which renders the antibody incapable of cross-linking, in which no such internalization is observed (31, 32) . Experiments outlined in this thesis have focused on assessing the impact of both germline and maturated NMDAR-autoantibodies, that bind to the GluN1 subunit of the NMDAR, on receptor function. Germline in this case refers to naïve autoimmune antibodies with few or no mutations. Due to the lack of hypermutations, such autoantibodies are predicted to bind weaker to the NMDAR than maturated autoantibodies (33). This thesis explores whether such germline antibodies can nonetheless adversely affect NMDAR function, a situation that alone could contribute to disease progression.

1.6 Objectives and aims

Autoimmune encephalitis is a complex disorder that is characterized by autoantibodies against many different targets in the brain. This dissertation aimed to highlight how variations in antibody driven mechanisms contribute to auto-immune encephalitis. To this end, we characterized in detail several patient derived monoclonal antibodies against ionotropic receptors, including antibodies against the α1-subunit or α1γ2-subunits of the $GABA_AR$ as well as against GluN1 subunits of the NMDAR. Our experiments examined possible direct effects of these antibodies on receptor function, distribution and impact on neuronal network, using immunocytochemical, electrophysiological, and calcium imaging techniques. The overall aim of this thesis was accomplished with help of the following objectives:

1. Characterize whether the cloned $GABA_AR$ autoantibodies not only bind to these receptors, but also induce functional changes to $GABA_ARs$.
2. Characterize how both α1-and α1γ2-$GABA_AR$ autoantibodies differ in the mechanism by which these antibodies affect $GABA_ARs$.
3. Assess whether germline as maturated NMDAR autoantibodies can alter receptor signal transduction.

2 Supplementary methods

All methods are described in their corresponding paper 1 (34) 2 (35) and 3 (33). This section will only describe the methodology of unpublished data.

Animals

Animal material was collected conform the Charité Medical University animal welfare committee's and the Berlin state government's regulations. Brain material was collected from male and female wildtype mice (RRID:IMSR_JAX:000664).

Microglia cultures

Microglia were isolated from the cortices of WT P0-2 mouse pups. For this purpose cortices were dissected out and digested in 0.05% Trypsin-EDTA (Gibco) for 20 min at 37°C. Digestion was stopped by replacing the trypsin-EDTA with DMEM-complete (10% FBS, 5% PenStrep) and cortices were subsequently triturated by pipetting them up and down 10x with a 1000µL pipet tip. Afterwards one cortex per T75 flask was cultured for 14 days in DMEM-complete media. Microglia were isolated just before addition to neurons by slapping the flask multiple times over 5 minutes followed by collecting the media.

Microglia staining experiments

Cortical-striatal co-cultures, prepared as described in (35), were incubated with 5µg/mL of the α1γ2-antibody for 1 hour at 37°C. Afterwards the cells were washed once with media to remove any unbound antibody. Immediately after washing, microglia were added to the cultures, in a ratio of 1 microglia per 3 neurons, and incubated for 6 hours at 37°C. At 6 hours, the cells were fixed for 4 min in 4% PFA and quenched in 25mM Glycine in PBS for 20 minutes. Blocking serum was used in all following steps (2% BSA, and 5% normal goat serum in PBS) unless described otherwise. Cells were permeabilized in 0.2% triton for 1 hour and incubated with a secondary alexafluor-594-anti-human antibody (1:1000, Jackson #109-585-003). After a washing step, a primary MAP2 antibody was added to the neurons for 1 hour (1:2000, Millipore Cat# AB5543, RRID:AB_571049). Cells were washed again and incubated for 1 hour with a secondary alexafluor-405-anti-chicken (Abcam Cat# ab175674, RRID:AB_2890171) or alexafluor-488-anti-chicken (Thermo Fisher Scientific Cat# A-11039, RRID:AB_2534096). Finally,

coverslips were washed one last time and mounted in Mowiol (10 mM Mowiol 4-88, roth #0713.2; 3.6 M glycerol; 0.2 M Tris in distilled water, pH 8.5).

Cells were imaged using a Nikon Spinning Disk Confocal CSU-X microscope that was under control of the NIS-Elements software (Nikon) at the Charité AMBIO facility. For image acquisition neurons were visualized with a 40X objective and z-stacks of 10 x 5 µm thick stacks were taken with a 561 and a 488/ 405 laser. Exposure time and gain were selected for each experiment individually but were kept consistent across all conditions per experiment. Per condition, 8 neurons were selected for imaging based on their MAP2 signal alone. To determine the number of α1γ2-puncta per dendrite length, images were analyzed in ImageJ. First, a secondary dendrite was selected via the MAP2 channel in each quadrant of the Image. Second, the segmented line tool was used to trace the dendrite in question to measure the length in µm. Third, the plugin 'Time Series Analyzer V3' was used to select all antibody puncta. For this, the auto ROI properties were set to 6 pixels and each puncta was manually selected. The plugin automatically stores all selected ROIs in the ROI manager. When all puncta are selected the Measure option of the ROI manager gave the number of puncta and the intensity of those ROIs across all channels. All data was collected in Excel (Microsoft) where the number of puncta per dendrite length could be calculated and subsequently plotted in Prism V7 to visualize and statistically test the data.

3. Results

Study 1 'Encephalitis patient-derived monoclonal $GABA_A$ receptor antibodies cause epileptic seizures' (34)

Initially after isolation of $GABA_AR$ autoantibodies from a patient by my collaborators, my first objective was to assess whether they influenced $GABA_AR$ functionality. For this, I selected two out of five autoantibodies isolated from an eight-year-old girl, one targeting the α1-subunit and the second targeting both an α1 and a γ2 subunit of these receptors (34). To assess whether these antibodies could influence the functionality of $GABA_ARs$, whole-cell patch-clamp experiments were performed. For this, striatal autapse cultures were incubated for 24-hours with 1µg/mL of the α1-antibody, the α1γ2-antibody, or a control-antibody (alemtuzumab). Autapses are special neuronal cultures where one neuron is grown on an astrocyte micro-island (36, 37), forcing the neuron to make synapses with itself.

My recordings showed that the α1-antibody leads to a strong reduction in all GABA-mediated currents as can be seen in the ~70% reduction in evoked inhibitory postsynaptic currents (IPSC) ($P=0.0001$, Kruskal-Wallis; untreated 1 ± 0.12, control-antibody 0.9 ± 0.17, α1-antibody 0.3 ± 0.10) (Fig. 2B, D) and causes the post-synaptic response to last longer by increasing the decay time ($P=0.0205$, Kruskal-Wallis; untreated 118 ± 6 ms, control-antibody 135 ± 15 ms, and α1-antibody 186 ± 18 ms) (Fig. 2E). The α1-antibody also caused a ~50% reduction in total GABA-mediated currents, which includes both synaptic and extrasynaptic receptors ($P=0.0003$, Kruskal-Wallis; untreated 1 ± 0.09, control-antibody 1 ± 0.07, α1-antibody 0.5 ± 0.06) (Fig 2C, F). In contrast, the α1γ2-antibody did not have any significant effect on any of the GABA-mediated currents, following a 24-hour antibody incubation period (Fig. 2B-F). Additionally, both antibodies had no effect on glutamatergic receptors, as illustrated by the lack of change in NMDA and Kainate-mediated currents (Fig. 2G-H).

Interestingly, in animal experiments, performed by my collaborators, both the α1 and α1γ2-autoantibodies induced seizures in rats when infused into the ventricular space for more than 24 hours (34). Together with my results, these data suggest the α1 and α1γ2-autoantibodies influence the $GABA_AR$ function but possibly via different mechanisms.

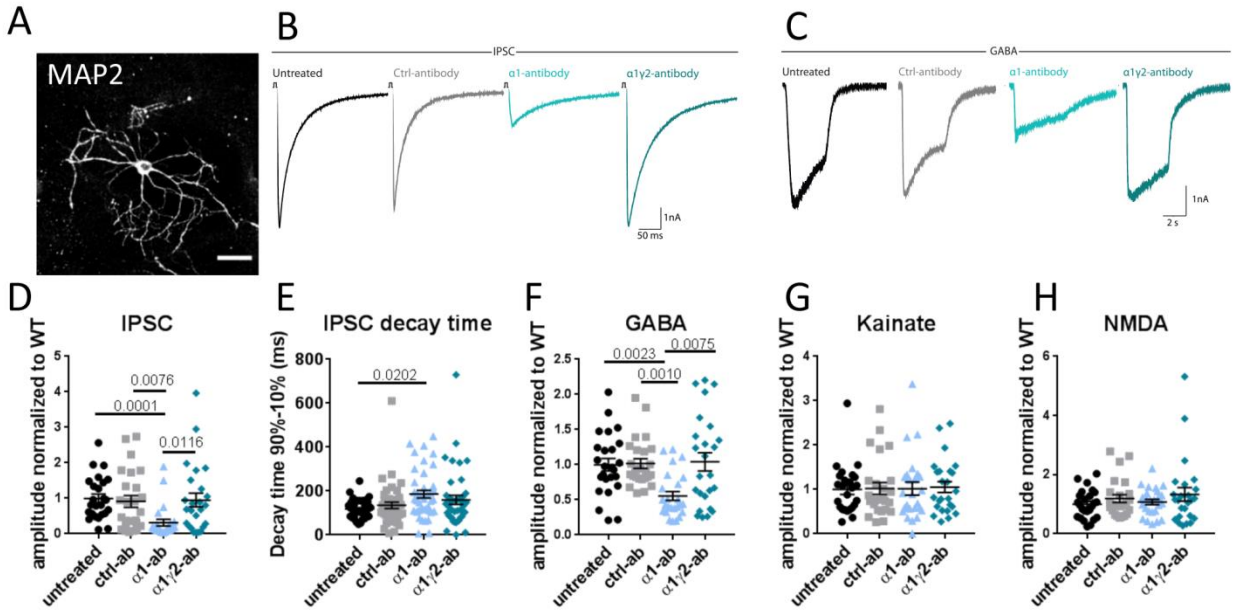

Figure 2: striatal autaptic whole-cell patch clamp recordings after 24-hour incubation with $GABA_AR$ encephalitis antibodies. A) MAP2 staining of a striatal autapse on an astrocyte micro-island. **B)** Example traces of IPSCs and **C)** GABA-mediated currents. Analysis of IPSC amplitude **(D)** (H(3)=20.51, P=0.0001, K-W; untreated 1 ± 0.12 vs. α1-antibody 0.3 ± 0.10 p=0.0001, α1- vs. control-antibody 0.9 ± 0.17 p=0.0076, α1- vs. α1γ2-antibody 0.9 ± 0.19 p=0.0116), IPSC decay-time **(E)** (H(3)=9.79, P=0.0205, K-W; untreated 118.4 ± 5.93 ms vs. α1-antibody 186.3 ± 17.68 ms, p=0.0202), GABA-mediated currents **(F)** (H(3)=18.8, P=0.0003, K-W; untreated 1 ± 0.09 vs. α1-antibody 0.5 ± 0.06 p=0.0023, α1- vs. control-antibody 1 ± 0.07 p=0.0010, α1- vs. α1γ2-antibody 1 ± 0.13 p=0.0075), Kainate-mediated currents **(G)** (H(3)=0.45, P=0.9288, K-W), and NMDA-mediated currents **(H)** (H(3)=0.75, P=0.8610, K-W). K-W=Kruskal-Wallis. Averages ± S.E.M. Each data point represents one neuron. Figures adjusted from (34) figure 3A-F and (35) Fig 3A, D.

Study 2 *'Differential effect of α1- and α1γ2- patient derived $GABA_AR$ encephalitis associated autoantibodies' (35)*

Although the α1- and α1γ2-antibodies promote epileptic activity in animals (34), it remains unclear whether they operated via similar or distinct mechanisms. Based on previous research detailing the mechanisms by which antibodies can cause disease (8), it is expected that there are three main pathways thought to contribute to autoimmune encephalitis: 1) direct actions on the receptor, 2) receptor internalization 3) immune cell engagement (Fig. 1), though the importance of each is not well understood. To provide clarity to these issues, my second objective was to get a better understanding of how the α1-and α1γ2-$GABA_AR$ autoantibodies differ in their mechanism by which these antibodies affect $GABA_ARs$. My initial data (Study 1) suggests that these antibodies might indeed increase network excitability via different mechanisms, as the α1-antibody was found to have dramatic effects on the GABA-mediated currents following a 24-hours incubation, whereas little or no effect on GABA-mediated currents were detected with the α1γ2-antibody. To explore whether this was due to weaker binding, I performed ad-

ditional experiments at both low and high concentrations of the α1γ2-antibody in a neuronal network.

This was accomplished by incubating cortical-striatal co-cultures with 1µg/mL of the α1- or control-antibody, and with 1µg/mL or 5µg/mL of the α1γ2-antibody for 24 hours. When looking at calcium currents in the network with the use of a calcium indicator jRCamP1b (under the synapsin promoter) (38), it became immediately apparent that in the presence of the α1 antibody the frequency of spiking activity of the cells in the network increases (Fig. 3A). After analysis of a 2-minute-long recording of the network, I observed that the frequency almost doubled for the α1-antibody compared to control. However, the α1γ2-antibody did not affect spiking activity at 1µg/mL nor at 5µg/mL (Fig. 3H) ($P<0.0001$, Kruskal-Wallis; untreated 9.58 ± 0.47 Hz, control 9.68 ± 0.53 Hz, α1-antibody 14.03 ± 0.66 Hz $p<0.0001$, α1γ2-antibody 10.68 ± 0.79 Hz, α1γ2-antibody 5µg 9.79 ± 0.54). To assess the degree of network inhibition, bicuculline was added to these networks to block most of the remaining GABA-mediated inhibition. For all conditions, I saw that the spiking activity became very synchronized and that most cells spiked at the same time (Fig. 3B). When I compared the spiking activity of each individual cell, before and after the addition of bicuculline, neurons increased their spiking activity when bicuculline was present in the untreated, control-antibody and at both high and low α1γ2-antibody concentrations (Fig. 3C-D, F-G). However, in the presence of the α1-antibody, individual neurons sometimes increased their spiking activity, while other either did not change their activity or decreased their activity (Fig. 3E) ($p=0.0024$, paired t-test; α1-antibody 13.76 ± 0.58 Hz, bicuculline 17.05 ± 0.69 Hz). When the average spiking frequency between conditions were compared, the addition of bicuculine was found to increase the average frequency for all conditions, though that increase was much smaller in the presence of the α1-antibody (Fig. 3I) ($p<0.0001$, ANOVA; untreated 123.5 ± 15.01%, control 136.6 ± 16.16%, α1-antibody 42.7 ± 9.66%, α1γ2-antibody 155 ± 16.01%, α1γ2-antibody 5µg 160.8 ± 20.02%). This suggests that the α1-antibody effectively blocks most GABA-mediated currents, with fewer functional $GABA_AR$ remaining in that network, while the α1γ2-antibody has little direct impact on these receptors.

These data strongly suggest that these antibodies elicit changes in network activity *in vivo*, by different mechanisms. To explore these, two strategies were taken. The first involved examining how the α1-antibody directly affects $GABA_AR$ function. The second focused on possible indirect effects of the α1γ2-antibody such as its impact on modula-

tors of the $GABA_AR$s and/or engagement of the immune system to clear antibody/receptor complexes.

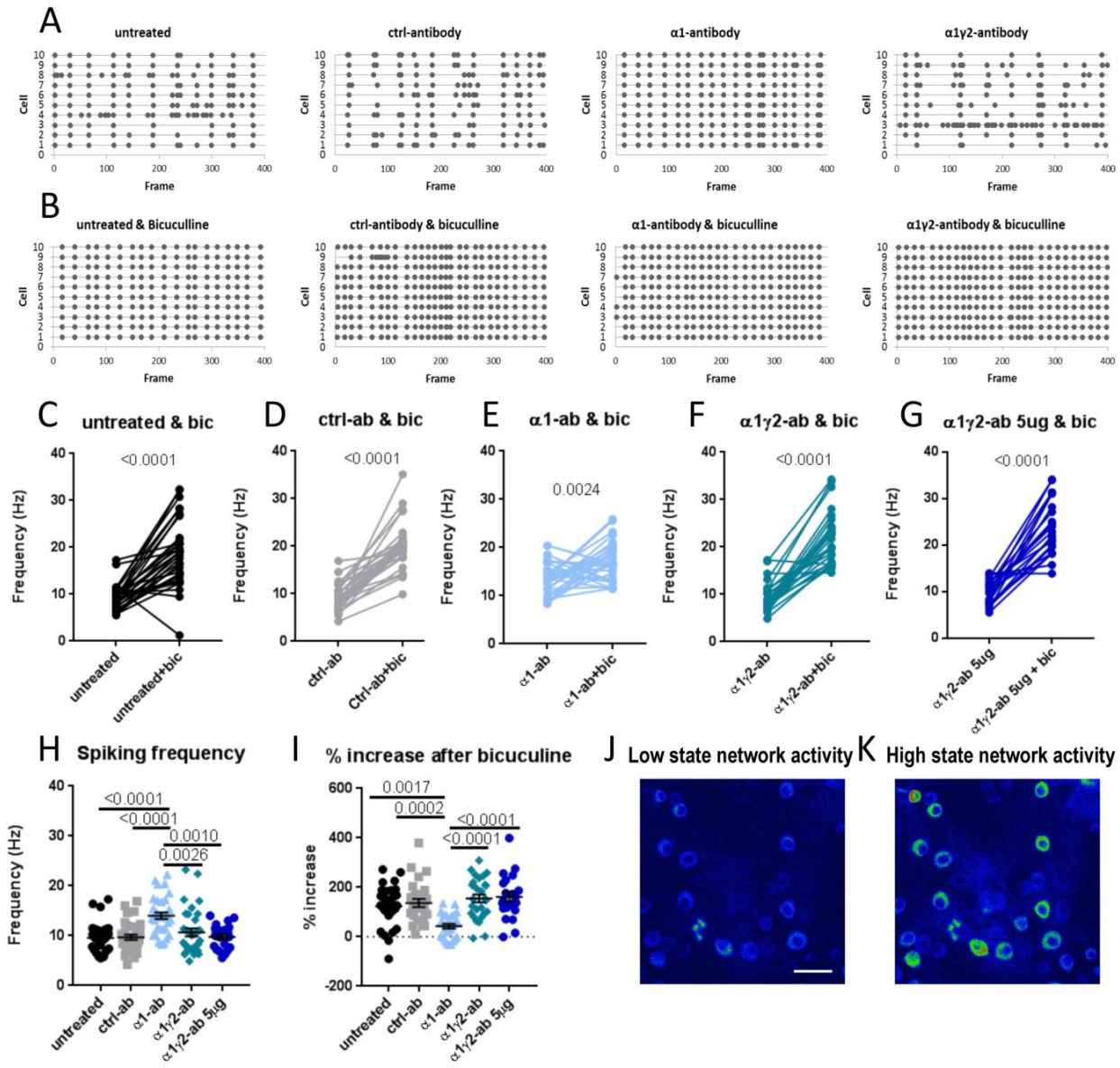

Figure 3: network spiking activity after 24-hour incubation with α1-antibody and a high and low concentration of α1γ2-antibody. A) Example spike plots of neuronal networks treated with autoantibodies for 24 hours. **B)** Example spike plots of neuronal networks after addition of bicuculine. Spike frequency of individual neurons before and after addition of bicuculine for all conditions **(C-G)** (untreated 9.30 ± 0.47 Hz vs. bicuculline 18.86 ± 1.22 Hz, t(32)=7.64, p<0.0001 control-antibody 9.17 ± 0.55 Hz vs. bicuculline 20.18 ± 0.99 Hz, t(26)=10.34, p<0.0001; α1-antibody 13.76 ± 0.58 Hz vs. bicuculline 17.05 ± 0.69, Hz t(32)=3.29, p=0.0024; α1γ2-antibody 9.30 ± 7.61 Hz vs. bicuculline 21.91 ± 1.08 Hz, t(26)=11.04, p<0.0001; α1γ2-antibody 5μg 9.79 ± 0.54 Hz vs. bicuculline 23.9 ± 1.31 Hz, t(20)=9.80, p<0.0001; all paired t-tests. Average spiking frequency per condition before **(H)** (H(4)=29.62, P<0.0001, Kruskal-Wallis; untreated 9.58 ± 0.47 Hz vs. α1-antibody 14.03 ± 0.66 Hz p<0.0001, control 9.68 ± 0.53 Hz vs. α1-antibody p<0.0001, α1-antibody vs. α1γ2-antibody 10.68 ± 0.79 Hz p=0.0010, α1-antibody vs. α1γ2-antibody 5μg 9.79 ± 0.54 p=0.0026) and after bicuculine addition **(I)**(F(4, 127)=9.46, p<0.0001, ANOVA; untreated 123.5 ± 15.01% vs. α1-antibody 42.7 ± 9.66% p=0.0017, control 136.6 ± 16.16% vs. α1-antibody p=0.0002, α1-antibody vs. α1γ2-antibody 155 ± 16.01% p<0.0001, α1-antibody vs. α1γ2-antibody 5μg 160.8 ± 20.02% p<0.0001). Example image of a neuronal network in a low spike state **(J)** and a high spike state **(K)**, scale bar 40μm. Averages ± S.E.M. Each data point in A-B represents one spike in calcium currents in one neuron. Each data point in C-I represents the average spiking activity of one ROI. Figure taken from (35) figure 4.

Exploring α1-antibody mediated mechanisms.

The dramatic decrease in GABA-mediated currents in striatal autaptic neurons following 24-hour incubation with the α1-antibody suggests possible direct effects and/or internalization of the receptors. To rule out any changes at the pre-synapse, I recorded mIPSCs and sucrose responses in striatal autaptic neurons. The former allows direct measurement of synaptic $GABA_AR$ responses to the release of neurotransmitter from a single synaptic vesicle (SV), while the sucrose response is a measure of both presynaptic release probability and synaptic vesical number per synapse. When mIPSCs were recorded, I observed a significant reduction in the frequency of these events (P=0.0149, Kruskal-Wallis; untreated 0.89 ± 0.20 Hz, α1-antibody 0.46 ± 0.14 Hz, control-antibody 1.42 ± 0.32 Hz) (Fig. 4A). Moreover, the amplitude of the post-synaptic current showed a trend towards decrease in the presence of the α1-antibody (untreated 34 ± 3 pA, control-antibody 36 ± 3 pA, α1-antibody 25 ± 3 pA) (Fig. 4B). These results suggest (1) fewer synaptic vesicles, reducing the probability that one is spontaneously released, (2) fewer synapses, (3) the amplitude of the mIPSCs fell under detectable

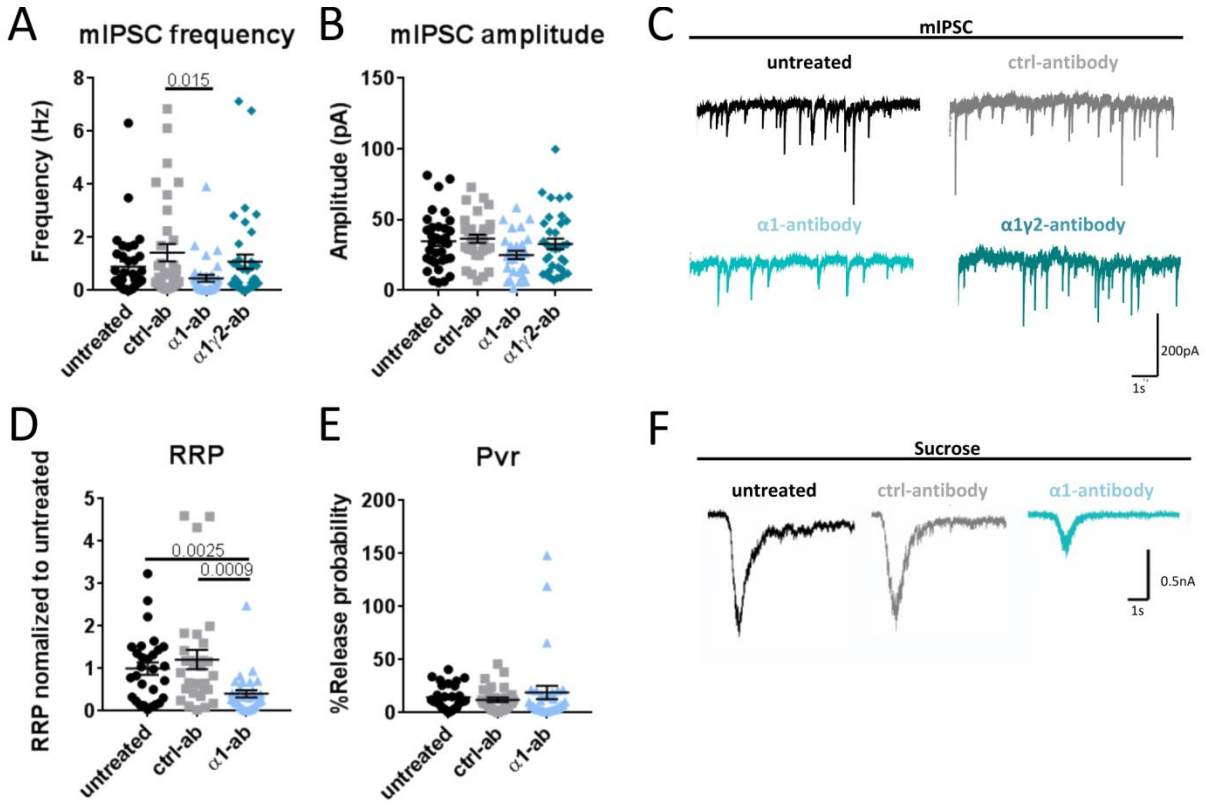

Figure 4: miniature post-synaptic currents after 24 hour of $GABA_AR$ incubation. Analysis of mIPSC frequency **(A)** (H(3)=10.49, P=0.0149, K-W; control 1.42 ± 0.32 Hz vs. α1-antibody 0.46 ± 0.14 Hz p=0.0151) and amplitude **(B)** (H(3)=6.94, P=0.0737, K-W). **C)** Example traces of mIPSCs per condition. **D)** Analysis of ready releasable pool (H(3)=16.2, P=0.0003, K-W; untreated 1 ± 0.15 vs. α1-antibody 0.40 ± 0.08 p=0.0025, control 1.21 ± 0.22 vs. α1-antibody p=0.0009) and **(E)** release probability (H(3)=1.59, P=0.4506, K-W) with accompanying example sucrose traces **(F)**. K-W=Kruskal-Wallis. Averages ± S.E.M. Each data point represents one neuron. Figure adjusted from (35) figure 3H-M.

levels due to fewer receptors at the post-synapse. To assess whether the decline in mIPSC frequency is due to lower release probability of presynaptic vesicles or fewer post-synaptic receptors, I triggered the release of all docked synaptic vesicles with 0.5mM sucrose. Here, it became clear that neurons treated with the α1-antibody have smaller post-synaptic current (P=0.0003, Kruskal-Wallis; untreated 1 ± 0.15, α1-antibody 0.40 ± 0.08, and control-antibody 1.21 ± 0.22) (Fig. 4D, F), but that the release probability is not changed (Fig. 4E), arguing that the α1-antibody has primarily a post-synaptic effect on the $GABA_A$Rs.

One possible mechanism underlying the reduction of GABA-mediated currents is that the antibody triggers receptor internalization. To explore this hypothesis, I performed immunocytochemical experiments to monitor the redistribution of receptors due to the presence of this antibody. This was accomplished by incubating cortical-striatal co-cultures with the α1-antibody for 1 hour at 15°C and 24 hours at 37°C. Labeling experiments performed for shorter times at reduced temperature permit antibody binding, while slowing down their internalization, providing an estimation of receptor internalization/redistribution due to the antibody over a 24-hour window. To monitor synaptic loss, cells were stained, post fixation, for the vesicular GABA transporter (VGAT), an inhibitory pre-synapse marker as well as for the α1-antibody (Fig. 5A, B).

An analysis of the fraction of α1-antibody positive puncta co-localizing with VGAT puncta revealed that initially ~80% of all VGAT puncta were positive for α1-antibody puncta. However after 24 hours, this degree of colocalization dropped to ~50% (Fig. 5C) (p<0.0001, Welch´s corrected t-test, 81 ± 2%, 51 ± 4%). Additionally, of all the α1-antibody puncta ~65% were initially located at synaptic sites, whereas ~35% were located at non-synaptic sites (Fig. 5D). These data indicate that the α1-antibody promotes the redistribution of receptors away from synapses. This concept was supported by analysis of puncta intensity. Here, I observed that the average intensity of the α1-antibody decreased over time (Fig. 5E) (p<0.0001, Welch's t-test; 1-hour 1 ± 0.04, 24-hour 0.73 ± 0.05) at both synaptic (Fig. 5F) (p=0.0003, Welch's t-test; 1-hour 1 ± 0.04, 24-hour 0.74 ± 0.06) and extrasynaptic sites (Fig. 5G) (p=0.0214, Welch's t-test, 1-hour 1 ± 0.04, 24-hour 0.84 ± 0.05). Interestingly, VGAT puncta intensity was also decreased over time (Fig. 5H) (p=0.0142, Welch's t-test; 1-hour 1 ± 0.03, 24-hour 0.82 ± 0.049) which could hint at a reduction in synapse size due to receptor removal from the post-synapse.

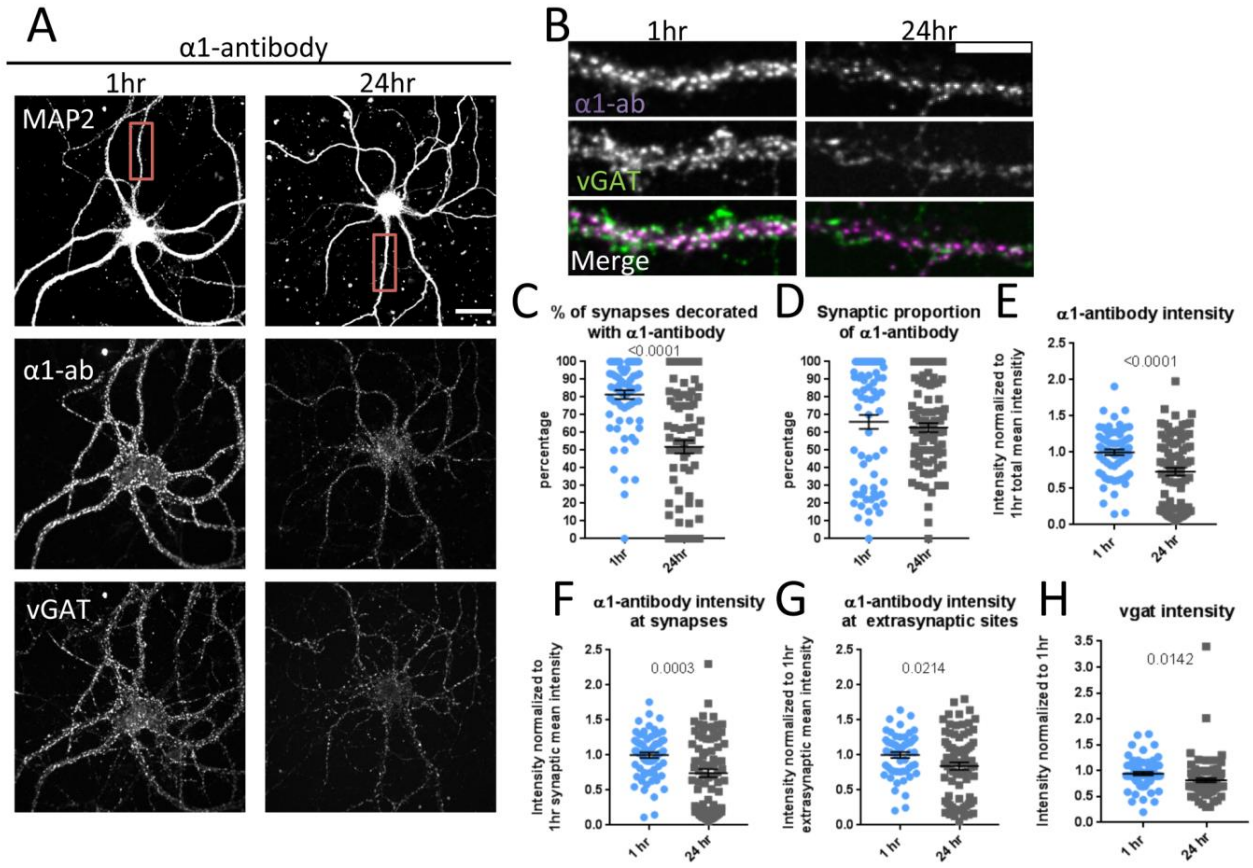

Figure 5: Immunocytochemical staining of cortical-striatal co-cultures with α1-antibody at 1 and 24 hours. A) Example images of a neuron stained with MAP2, the α1-antibody, and vGAT, scale bar 20µm. **B)** zoom in showing the overlay of vGAT (green) and α1-antibody (magenta), scale bar 10 µm. **C)** the percentage of synapses that are decorated with the α1-antibody over time ($t(146.4)=6.664$, $p<0.0001$, Welch´s t-test). **D)** the percentage of α1-antibody puncta that are located at synapses over time ($t(121)=0.691$, $p=0.4907$, Welch's t-test). **E)** Difference in α1-antibody intensity over time ($t(148.5)=4.11$, $p<0.0001$, Welch's t-test). Intensity of the α1-antibody at synapses **(F)** ($t(136.4)=3.701$, $p=0.0003$, Welch's t-test) and at extra-synaptic sites **(G)** ($t(129.8)=2.329$, $p=0.0214$, Welch´s t-test). Intensity of vGAT puncta at 1 hour and 24 hour of α1-antibody treatment **(H)** ($t(156.1)=2.479$, $p=0.0142$, Welch´s t-test). Averages ± S.E.M. Each data point represents one ROI. Figure adjusted from (35) Figure 1.

Mechanistically, these data suggest that the α1-antibody could reduce GABA-mediated currents, during 24-hour incubation, through the redistribution/internalization of these receptors. However, it is also possible that it could have faster direct effects on these receptors. To investigate this possibility, I incubated cortical-striatal co-cultures with 1µg/mL of the α1-antibody for 1 hour and looked at the effect on mIPSCs. This analysis showed that the α1-antibody not only reduced the frequency (Fig. 6A, C) ($p=0.0004$, ANOVA; untreated 4.62 ± 0.41 Hz, control 3.90 ± 0.50 Hz, α1-antibody 2.10 ± 0.41 Hz), but also the amplitude of mIPSC responses compared to controls (Fig. 6B,D) ($p=0.0010$, ANOVA; untreated 42.97 ± 3.24 pA, control-antibody 44.03 ± 3.87 pA, α1-antibody 27.45 ± 2.71 pA). No change in the kinetics of these responses was detected (Fig. 6F-H). These data imply that the α1-antibody may indeed act directly on $GABA_AR$s on a faster time scale. The reduction in GABA-mediated currents was

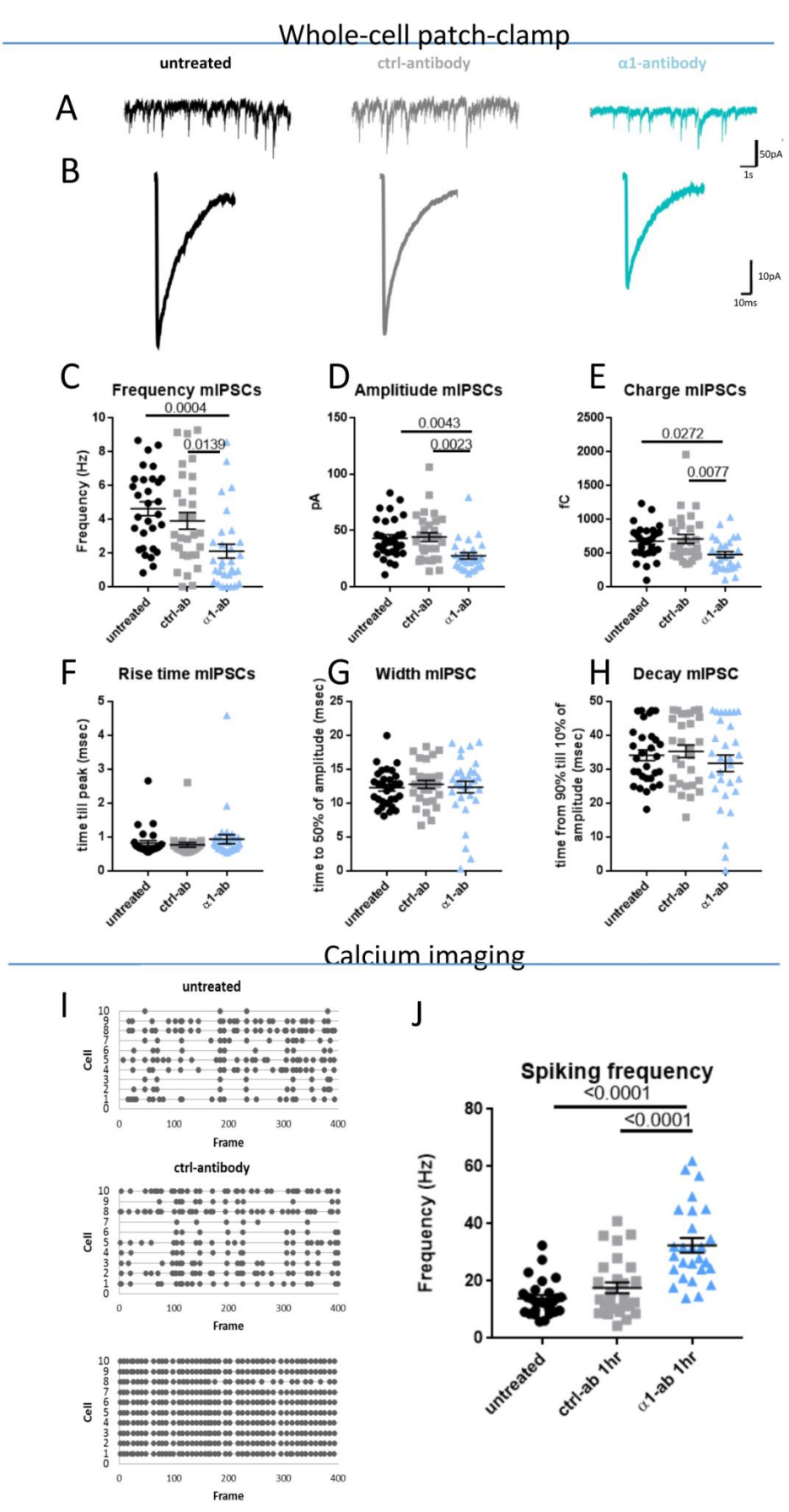

Figure 6: mIPSC recordings and calcium imaging after 1-hour of α1-antibody. A) Example traces of mIPSC frequency. **B)** Example of mIPSC amplitude. **C)** average mIPSC frequency per neuron per condition ($F(2, 87)=8.596$, $p=0.0004$, ANOVA; untreated 4.62 ± 0.41 Hz vs. α1-antibody 2.10 ± 0.41 Hz $p=0.0004$, control 3.90 ± 0.50 Hz vs. α1-antibody $p=0.0139$). **D)** Average mIPSC amplitude per neuron per condition ($F(2, 83)=7.505$ $p=0.0010$, ANOVA; untreated 42.97 ± 3.24 pA vs. α1-antibody 27.45 ± 2.71 pA $p=0.0043$, control 44.03 ± 3.87 pA vs. α1-antibody $p=0.0023$). **E)** average mIPSC current per neuron ($F(2, 83)=5.47$, $p=0.0058$, ANOVA; untreated 671.5 ± 44.21 fC vs. α1-antibody 475.5 ± 45.19 fC $p=0.0272$, control-antibody 707.3 ± 64.83 fC vs. α1-antibody $p=0.0077$). **F)** Average mIPSC rise time per neuron ($F(2, 86)=0.7636$ $p=0.4691$, ANOVA). **G)** Average half width per neuron ($F(2, 86)= 0.1598$ $p=0.8526$, ANOVA). **H)** Average mIPSC decay time per neuron ($F(2, 86)=0.8043$ $p=0.4508$, ANOVA). **I)** Example spike plots of networks after 1-hour of α1-antibody incubation where each dot represents a spike in calcium currents in one cell. **J)** Analysis of average spiking activity per condition where each dot represents the average spiking frequency of neurons in one field of view

(H(2)=31.18, P<0.0001, Kruskal-Wallis; untreated 13.98 ± 1.21 Hz vs. α1-antibody 32.46 ± 2.58 Hz p<0.0001, control 17.64 ± 1.93 Hz vs. α1-antibody p<0.0001). Averages ± S.E.M. Figure adjusted from (35) figure 5 and 6.

sufficient to increase network spiking activity in cortical-striatal co-cultures following a 1hr addition of the α1-antibody. Here, I detected a ~2-fold increase in spiking activity with the α1-antibody compared to control groups (Fig. 6I, J) (P<0.0001, Kruskal-Wallis; untreated 13.98 ± 1.21 Hz, control-antibody 17.64 ± 1.93 Hz, α1-antibody 32.46 ± 2.58 Hz).

The dramatic change in neuronal spiking activity and GABA-mediated currents after only 1 hour suggests that the α1-antibody might have a direct antagonistic effect on these receptors. However, this time scale cannot rule out indirect mechanisms such as the rapid redistribution or internalization of these receptors. I thus used calcium imaging and the rapid addition of antibody to gain clues to the speed of this inhibition. This was

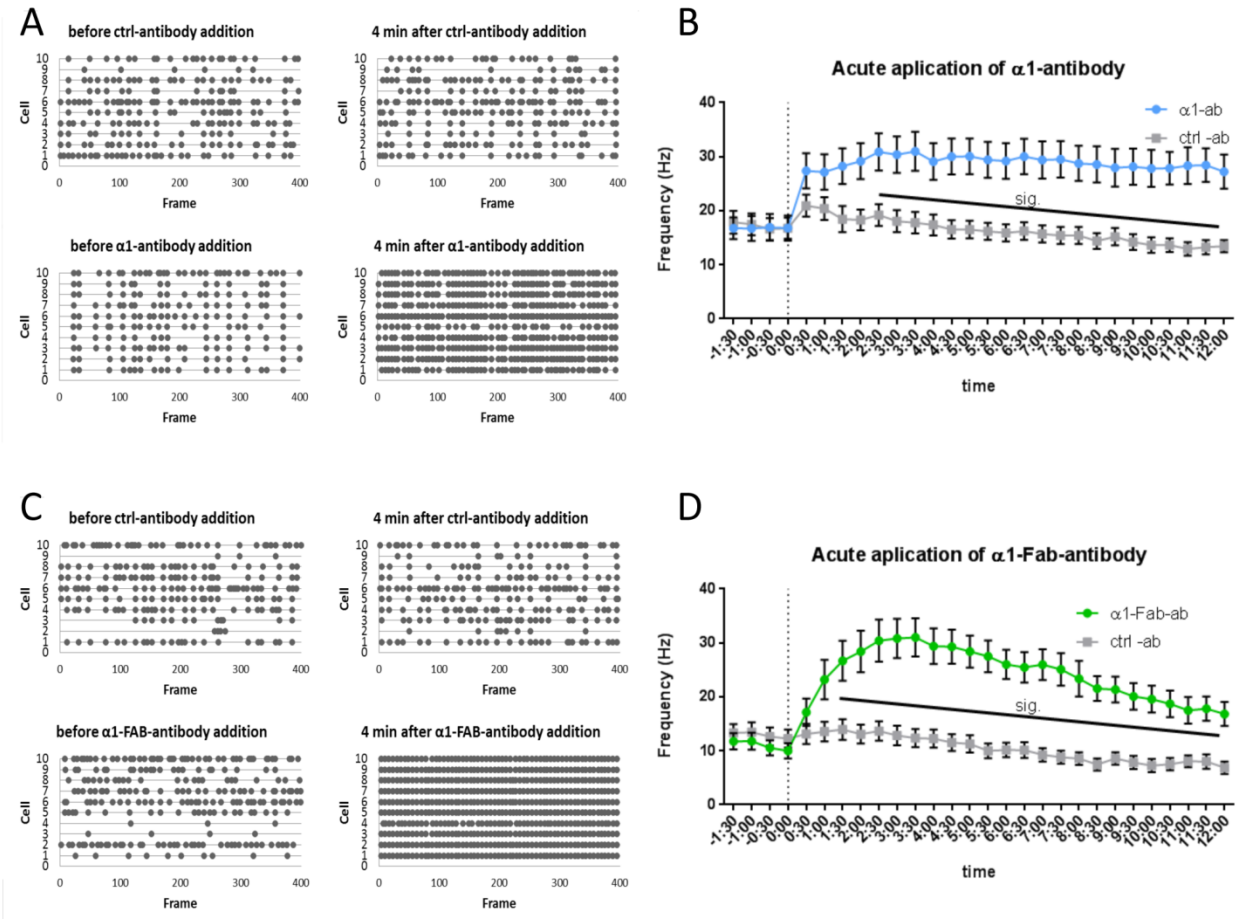

Figure 7: Calcium imaging experiments of acute addition of α1-antibody. A) Example spike plots of networks before and directly after addition of full-length α1-antibody where one dot represents one cell. **B)** Average spiking frequency of all cells in a field of view before and after antibody addition (F(1, 29)=10.82, p=0.0026, repeated measures two-way ANOVA). **C)** Example spike plots of a field of view before and after addition of the α1-antibody fab-fragment where one dot represents one cell. **D)** Average spiking frequency of all cells in a field of view before and after the addition of the fab-fragment (F(1, 33)=18.27, p=0.0002, repeated measures two-way ANOVA). Averages ± S.E.M. Each data point in A and C represents one calcium current in one neuron. Line in B and D represents the average spiking activity of all fields of view. Figure adjusted from (35) figure 6.

accomplished by monitory spiking activity before and immediately after the addition of the α1-antibody for an additional 12 minutes. Here, it was observed that spiking activity of the network increased in frequency immediately after addition of the α1-antibody, becoming significantly different from the control group after only 2 minutes (Fig. 7 A, B) ($p=0.0026$, repeated measures two-way ANOVA), which support direct real-time effects of this antibody on these receptors. To rule out antibody-induced internalization as a mechanism, the experiment was repeated with a Fab-fragment of the α1-antibody. Importantly, Fab-antibodies consist only of the antigen binding region of IgG-antibodies that operate as a monomer due to the absence of their Fc-regions. As such, they retain antigen binding, but cannot induce receptor cross-linking-mediated internalization (31, 39). When I added the Fab fragment of the α1-antibody to these cortical-striatal co-cultures, I observed an immediate increase in spiking activity that became significantly compared to control after 1.5 minutes (Fig. 7 C,D) ($p=0.0002$, repeated measures two-way ANOVA). These data indicate that the α1-antibody can rapidly alter GABA-mediated currents in a manner that does not rely on redistribution of the receptor away from the synapse.

The α1γ2-antibody

Surprisingly, the α1γ2-antibody elicited no effects on $GABA_AR$ functionality, in either my electrophysiological or calcium imaging experiments, yet was capable of inducing seizures when delivered cerebroventricular in a rodent model. This led me to hypothesize that this antibody might mechanistically work via mechanisms that are not readily present in my cortical-striatal co-culture system. Two such mechanisms include actions of these antibodies to physically block binding of modulators of $GABA_ARs$ and/or recruitment of cellular components of the immune system, such as microglia. A third yet less likely mechanism is one that involves an antibody-induced redistribution of α1γ2-containing receptors. The impact of the latter could depend on the abundance and distribution of this $GABA_AR$ subtype.

Addressing this latter mechanism first, I used immunocytochemistry to monitor the distribution of receptors bound by the α1γ2-antibody in my cortical-striatal culture model. Here again, I used antibodies against VGAT to identify inhibitory synapses in culture treated with 1µg/mL of the α1γ2-antibody for 1 hour at 15°C or 24 hours at 37°C (Fig. 8

A, B). When comparing how many of the VGAT puncta colocalize with α1γ2-antibody puncta over time, there was no significant difference (Fig. 8C). Interestingly, the α1γ2-antibody primarily decorated receptors situated at synaptic (~70%), compared to extra-synaptic sites. This proportion did not change over time (Fig. 8D). Instead, I observed that α1γ2-antibody puncta intensity, both at synaptic (Fig. 8F) (1-hour 1 ± 0.05, 24-hour 1.58 ± 0.12, p<0.0001, Welch's t-test) and extrasynaptic sites (Fig. 8G) (1-hour 1 ± 0.05, 24-hour 1.57 ± 0.17, p=0.0017, Welch´s t-test), increased over time (Fig. 8E) (1-hour 1 ± 0.40, 24-hour 1.45 ± 0.12, p=0.0003, Welch´s t-test). Why this occurs is unclear but could be due to a low affinity of this antibody, slowing its accumulation on synaptic receptors. Resolving these possibilities will require further investigation. Interestingly, VGAT intensity remained stable over time (Fig. 8H).

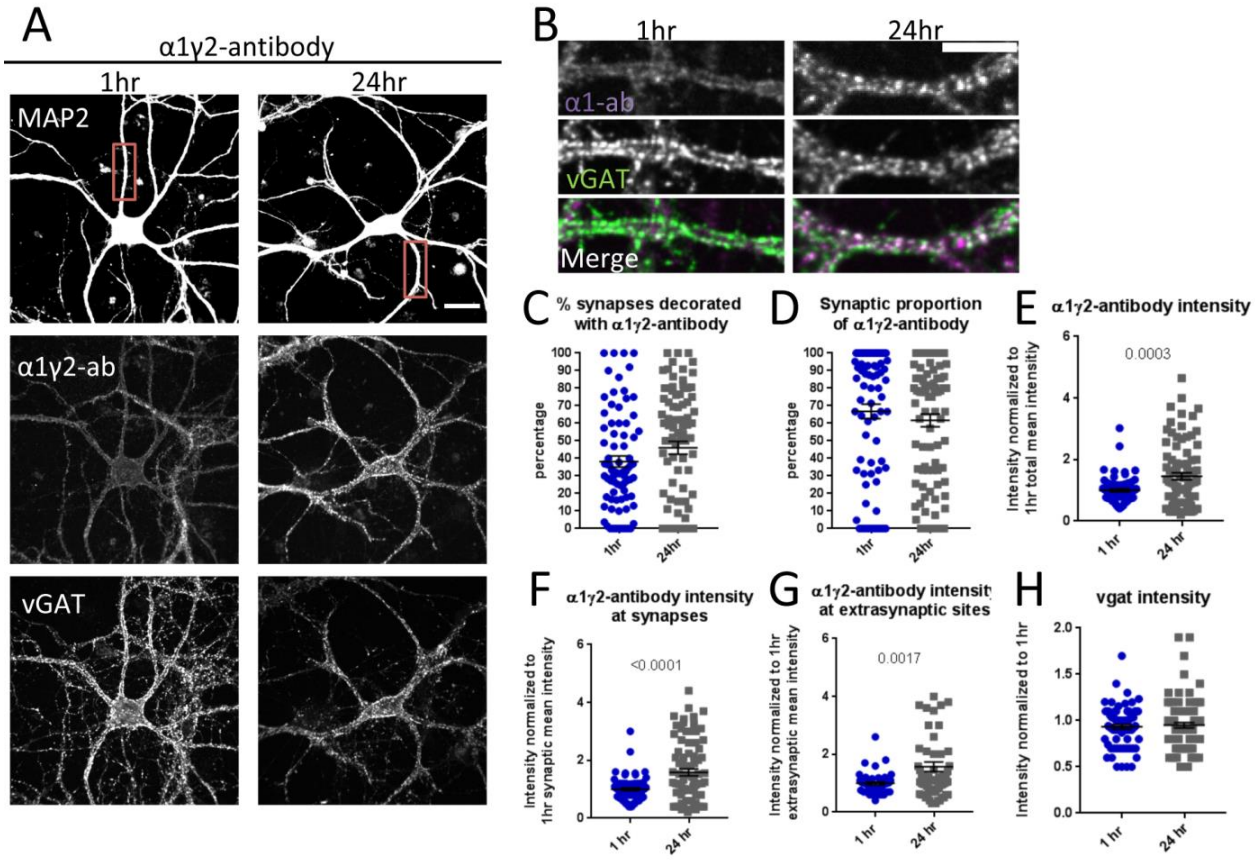

Figure 8: Immunocytochemical staining of cortical-striatal mass-cultures with α1γ2-antibody. A) Example images of neurons incubated for 1 hour at 15°C and for 24 hours at 37°C with the α1γ2-antibody and co-stained with anti-MAP2 and anti-vGAT antibodies, scale bar 20µm. **B)** Zoom in of the area marked with a box in A that shows the antibody and vGAT puncta in more detail, scale bar 10 µm. **C)** The percentage of synapses that are decorated with the α1γ2-antibody over time (t(163)=1.62, p=0.1072, unpaired t-test). **D)** The percentage of α1γ2-antibody puncta that are located at synapses over time (t(161)=0.9327 p=0.3524, unpaired t-test). **E)** Difference in α1γ2-antibody intensity over time (t(109.8)=3.702, p=0.0003, Welch´s t-test). **(F)** Intensity of the α1γ2-antibody at synapses (t(100.7)=4.442, p<0.0001, Welch's t-test) **(G)** and at extra-synaptic sites (t(81.46)=3.243, p=0.0017, Welch´s t-test). **(H)** Intensity of vGAT puncta at 1 hour and 24 hour of α1γ2-antibody treatment (t(158.4)=0.4497, p=0.6535, Welch´s t-test). Averages ± S.E.M. Each data point represents one ROI. Figure adjusted from (35) figure 2.

The ability of the α1γ2-antibody to decorate inhibitory synapses indicates that its lack of efficacy is not due to its inability to bind receptors. This is consistent with immunocytochemical experiments in which these antibodies readily decorated HEK293 cells expressing α1γ2, but not α3γ2 containing receptors (data not shown). These initial data also rule out a redistribution mechanism, as this antibody failed to promote the synaptic loss of these receptors over time.

Intriguingly, it is notable that α1γ2-antibody binding requires the presence of two subunits: α1 and γ2, which are known to constitute the binding site of benzodiazepine, a modulator of $GABA_AR$ function that enhances the agonist effects of GABA (40). Importantly, the brain contains naturally occurring benzo-like compounds called “endozepines”, which could in principle be antagonized by this antibody *in vivo* but not *in vitro* (41). This logic led me to hypothesize that mechanistically the α1γ2-antibody could operate by antagonizing the potentiating effect of endozepines *in vivo*.

To test this hypothesis, I performed electrophysiology experiments to examine whether the α1γ2-antibody could block the potentiating effect of the benzodiazepine, diazepam. In these experiments, cortical-striatal co-cultures were incubated with the α1γ2-antibody for 1 hour. After which mIPSCs were recorded before and after the addi-

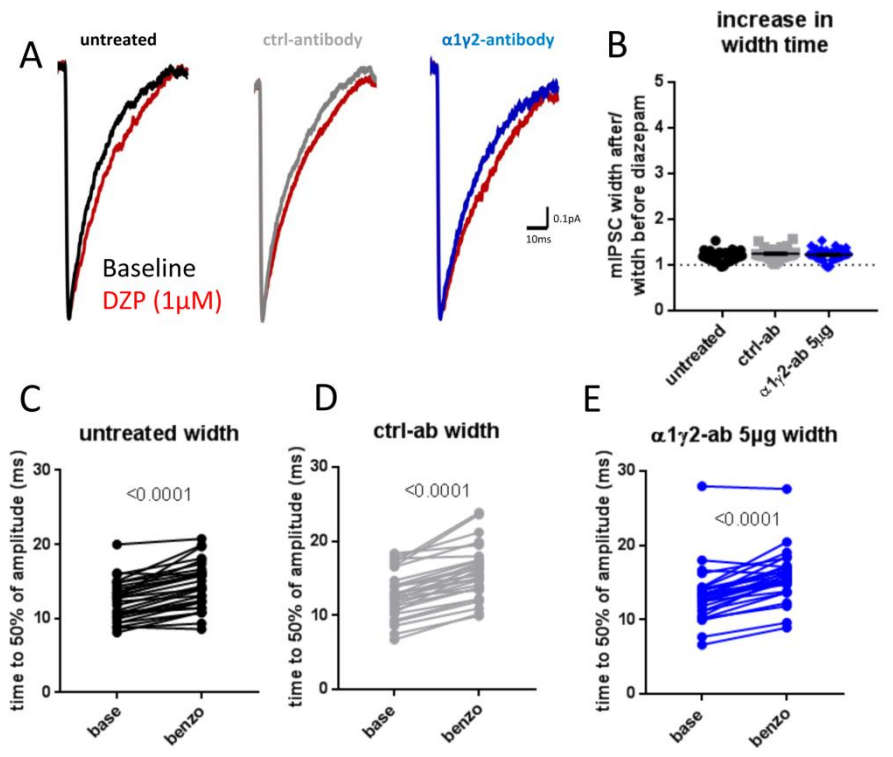

Figure 9: recoding of mIPSC half width time after 1-hour α1γ2-antibody incubation in the presence and absence of diazepam. A) Example traces of mIPSC half width in the presence and absence of diazepam. **B)** Quantification of the increase in half width in the presence of diazepam compared to without (calculated as half width with diazepam/half width baseline) ($F(2.86)=1.782$, $p=0.1744$, ANOVA; untreated 1.18 ± 0.02, control-antibody 1.25 ± 0.02, α1γ2-antibody 1.22 ± 0.02). Graphs showing the average half width time of mIPSCs for each individual neuron. The lines connect the half width time before and after addition of diazepam for each cell for untreated neurons **(C)** ($t(29)=7.991$, $p<0.0001$, paired t-test; untreated base 12.27 ± 0.49ms, untreated benzo 14.46 ± 0.57ms), neurons with control-antibody **(D)** ($t(28)=10.32$, $p<0.0001$, paired t-test; control-antibody base 12.77 ± 0.57ms, control-antibody benzo 15.74 ± 0.66ms), and neurons with the α1γ2-antibody (E) ($t(29)=8.73$, $p<0.0001$, paired t-test; α1γ2-antibody base 13.14 ± 0.67ms, α1γ2-antibody benzo 15.8 ± 0.61ms). Averages ± S.E.M. Each data point represents one neuron. Figure adjusted from (35) figure 7.

tion of 1µM diazepam. The data were subsequently analyzed for half-width time of each response, which increases in the presence of diazepam (26). When looking at the traces (Fig. 9A), one can see that the presence of diazepam increased the half-width time of mIPSCs for untreated neurons (Fig. 9C) ($p<0.0001$, paired t-test; untreated base 12.27 ± 0.49ms, untreated benzo 14.46 ± 0.57ms), neurons treated with control-antibody (Fig. 9D) ($p<0.0001$, paired t-test; control-antibody base 12.77 ± 0.57ms, control-antibody benzo 15.74 ± 0.66ms), as well as neurons incubated with the α1γ2-antibody (Fig. 9E) ($p<0.0001$, paired t-test; α1γ2-antibody base 13.14 ± 0.67ms, α1γ2-antibody benzo 15.8 ± 0.61ms). When the half-width time after diazepam was divided by the half-width time before diazepam (Fig. 9B), there was no detectable difference between groups in their response to diazepam, indicating that the α1γ2-antibody has no pronounced effect on receptor modulation by diazepam. One caveat of these mIPSC experiments is that cortical-striatal neurons also express other benzo-sensitive subunits (42), which could mask the effect of the α1γ2-antibody in these assays. To over-come this limitation, I also examined the effect of the α1γ2-antibody and diazepam at the network level using calcium imaging to determine the frequency of neuronal spiking (Fig. 10A). Here, I observed in untreated cultures that diazepam triggers a dramatic reduction in spiking activity (Fig. 10B), which increases after bicuculline is added (Fig. 10C-F) (untreated (D): $p<0.0001$, repeated measures ANOVA; base 13.94 ± 1.22 Hz, benzo 2.03 ± 0.53 Hz, bic 38.93 ± 3.04 Hz; control-antibody (E): $p<0.0001$ repeated measures ANOVA; base 17.64 ± 1.93 Hz, benzo 5.78 ± 1.44 Hz, bic 43.08 ± 3.70 Hz; α1γ2-antibody 5µg (F): $p<0.0001$, repeated measures ANOVA; base 12.87 ± 1.16 Hz, benzo 4.64 ± 1.06 Hz, bic 39.38 ± 3.34 Hz). When I calculate the decrease in spiking activity caused by diazepam compared to spiking activity in baseline conditions, I saw that all networks, including those treated with the α1γ2-antibody, reduced their activity by ~80% (Fig. 10H). When we compare the activity of baseline networks to activity after the addition of bicuculline, I observed a 200% increase in activity even in cultures treated with α1γ2-antibody (Fig. 10I). Together with the electrophysiological data, these network data indicate that the α1γ2-antibody does not alter neuronal excitability by altering the function of the benzodiazepine site, a conclusion that will need to be confirmed by more detailed biophysical recordings.

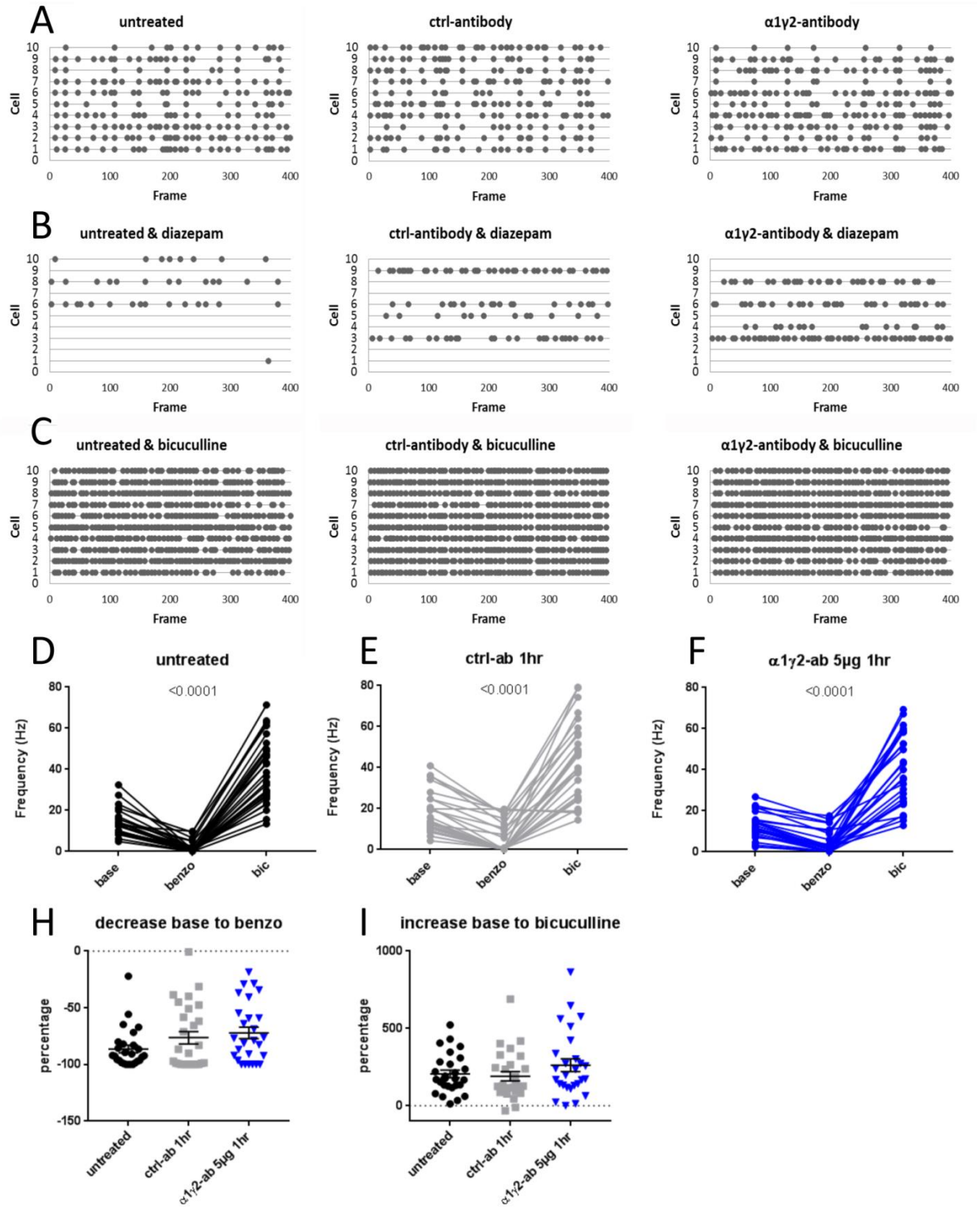

Figure 10: effect of diazepam on network spiking activity not affected by α1γ2-antibody. A) Example spike plots of network spiking after 1-hour of antibody incubation. **(B)** Example spike plots of network spiking activity after 1µM diazepam was added to the network. **(C)** Example spike plots of network spiking activity after the addition of 30µM bicuculline to the network. **(D)** Average spiking activity per field of view of untreated neurons in baseline, diazepam, and bicuculline conditions ($F(2, 52)=129.8$, $p<0.0001$, rmA; base 13.94 ± 1.22 Hz vs. benzo 2.03 ± 0.53 Hz $p<0.0001$, base vs. bic 38.93 ± 3.04 Hz $p<0.0001$, benzo vs. bic <0.0001). **(E)** Average spiking activity per field of view in the presence of control-antibody in baseline, diazepam, and bicuculline conditions ($F(2, 52)=91.27$, $p<0.0001$ rmA; base 17.64 ± 1.93 Hz vs. benzo 5.78 ± 1.44 Hz $p=0.0003$, base vs. bic 43.08 ± 3.70 Hz $p<0.0001$, benzo vs. bic $p<0.0001$). **(F)** Average spiking activity per field of view in the presence of α1γ2-antibody in baseline, diazepam, and bicuculline conditions ($F(2, 52)=94.32$, $p<0.0001$, rmA; base 12.87 ± 1.16 Hz vs. benzo 4.64 ± 1.06 Hz $p=0.0084$,

base vs. bic 39.38 ± 3.34 Hz p<0.0001, benzo vs. bic p<0.0001). **(H)** Average decrease in spiking activity per field of view from baseline to diazepam conditions (H(2)=2.554, P=0.2789, K-W). **(I)** Average increase in spiking activity per field of view from baseline to bicuculline conditions (H(2)=2.302, P=0.3164, K-W). K-W=Kruskal-Wallis, rmA=repeated measures ANOVA. Averages ± S.E.M. Figure adjusted from (35) figure 8.

Another hypothesis that I wanted to test was whether the apparent silent binding of the α1γ2-antibody could promote disease-causing phenotypes via the engagement of microglia, which could actively strip antibody/$GABA_AR$ complexes from neurons, reducing inhibitory drive. Initial staining experiments showed that when the α1γ2-antibody is added for one hour to cortical-striatal mass-cultures, and the unbound antibody is removed by a media exchange before adding microglia for 6 hours, the presence of microglia led to fewer antibody puncta on the neuron compared to neurons only treated with the antibody (Fig. 10A, B) (p=0.0017, Welch´s t-test; without microglia 21 ± 1.25 puncta per 100 µm dendrite, with microglia 16 ± 0.93 puncta per 100 µm dendrite). These data indicate that microglia could indeed facilitate the removal of the antibody/receptor complexes from neurons and synapses, a condition that could lead to increased network excitability.

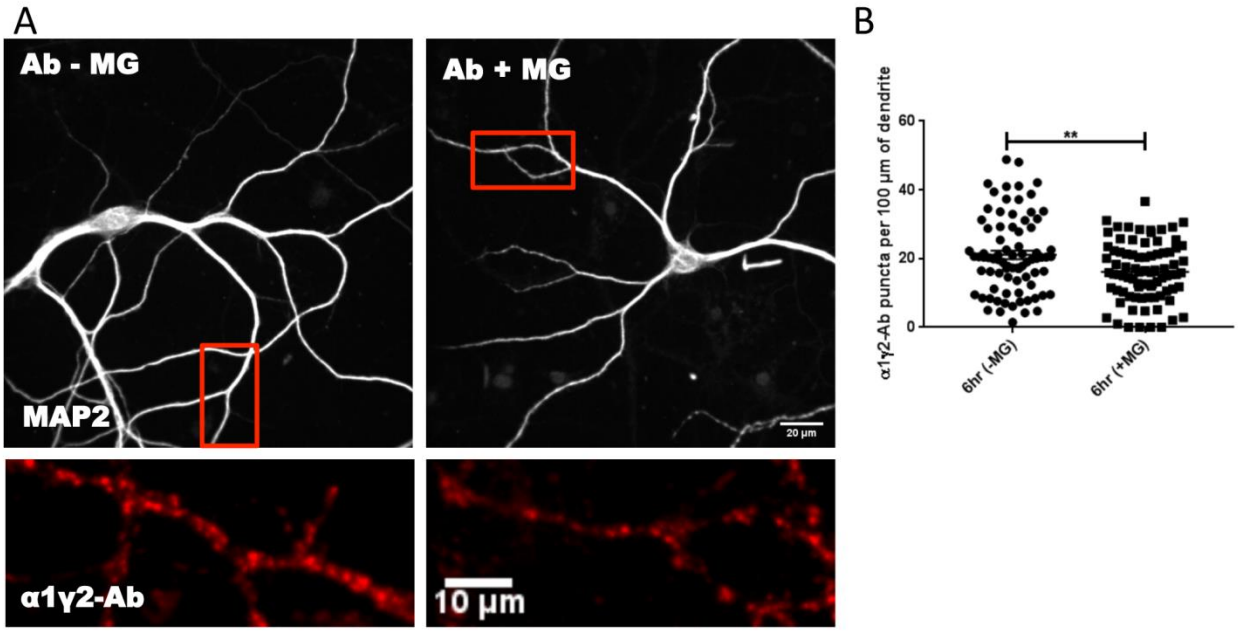

Figure 11 : Microglia-mediated α1γ2-antibody eating. A) Example images of neurons stained with the α1γ2 antibody in the absence and presence of microglia. **B)** Quantification of the number of α1γ2-antibody puncta per unit length of dendrite (t(161)=3.184, p=0.0017, Welch´s t-test; without microglia 21 ± 1.25 puncta per 100 µm dendrite, with microglia 16 ± 0.93 puncta per 100 µm dendrite) . Averages ± S.E.M. Each data point represents one dendrite. Unpublished data.

Study 3 *'N-methyl-D-aspartate receptor dysfunction by unmutated human antibodies against the NR1 subunit' (33)*

In addition to studying how autoantibody epitopes influence the mechanisms involved in autoimmune encephalitis, this work also wanted to examine whether autoantibody maturation plays a role in autoimmune encephalitis. More specifically, I wanted to address whether germline antibodies against NMDARs could in and of themselves alter NMDAR function. As discussed above, it has previously been reported that mature NMDAR-antibodies can decrease NMDA-mediated receptor currents after 24-hour incubation (15). Intriguingly, during the search for patient autoantibodies against the NMDAR, my collaborators isolated from the same patient a germline autoantibody (33), raising the question whether this immature antibody can also contribute to the etiology of this patient´s symptoms. To test this concept, I performed whole cell patch recording of hippocampal autapses incubated with 5µg/mL of the germline antibody and 1µg/mL of a maturated antibody for 24 hours. When excitatory post-synaptic currents (EPSCs) were recorded there was no apparent difference between the germline, maturated, and control antibody (Fig. 12C). However, as the majority of the EPSC response is mediated by the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), EPSCs were next recorded in the presence of NBQX, an AMPAR blocker to isolate the NMDAR-mediated component of the EPSC (Fig. 12A, D). These experiments revealed that the germline antibody as well as the maturated-ab could indeed decrease evoked NMDA-mediated currents (Fig. 12A,D) ($P=0.0002$, Kruskal-Wallis; mGO ctrl-ab 1 ± 0.10, germline-ab 0.5 ± 0.09, maturated-ab 0.5 ± 0.10) as well as postsynaptic currents after bath application of NMDA (Fig. 12B, E) ($P<0.0001$, Kruskal-Wallis; mGO ctrl-ab 1 ± 0.07, germline-ab 0.6 ± 0.07, maturated-ab 0.6 ± 0.05). The antibodies were highly specific as they did not influence kainate and GABA currents (Fig. 12F-G). However when the germline antibody was added to the neurons at 1µg/mL the antibody did not have any effect (See Fig. 3F-J in (33)) indicating that the germline antibody can influence signal transduction, but needs a 5-fold higher concentration than the maturated antibody to cause a similar effect.

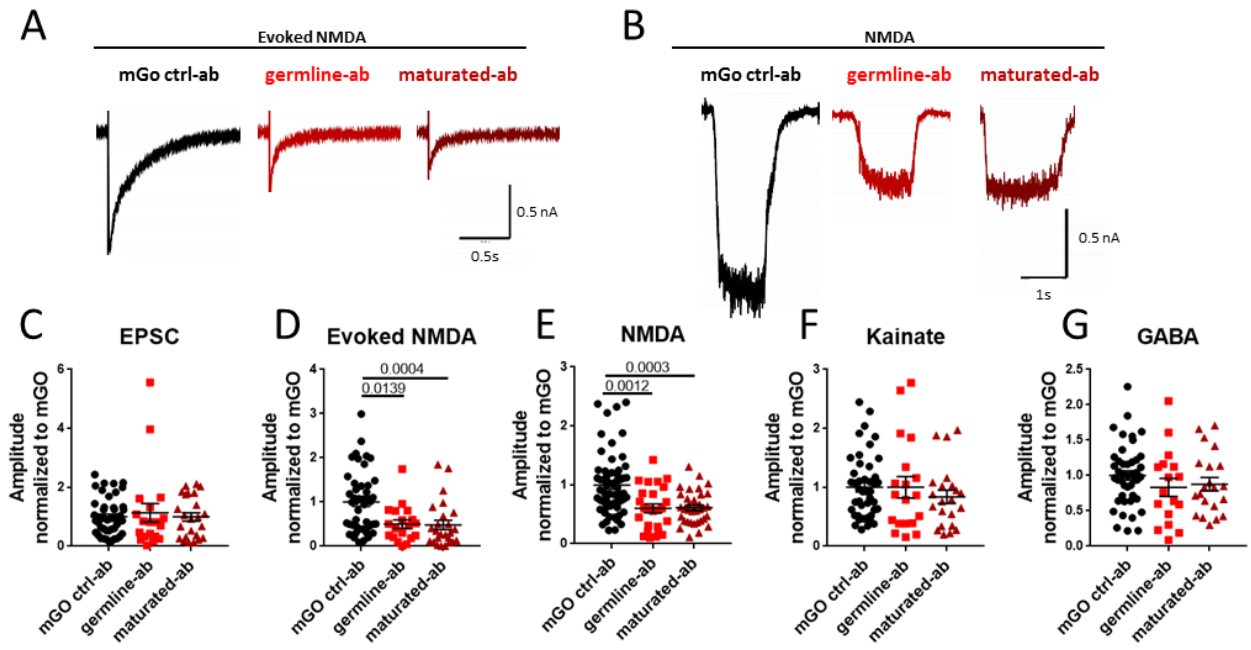

Figure 12: whole-cell patch-clamp recordings from hippocampal neurons incubated with a germline or maturated NMDA antibody for 24 hours. A) Example traces of synaptic NMDA responses. **B)** Example traces of the average postsynaptic response after bath application of NMDA. **C)** EPSC Amplitude normalized to mGO condition (H(2)=0.805, P=0.6687, K-W; mGO ctrl-ab 1 ± 0.09, germline-ab 1.14 ± 0.32, maturated-ab 0.99 ± 0.14). **D)** Average decrease in synaptic NMDA responses (H(2)=17.37, P=0.0002, K-W; mGO ctrl-ab 1 ± 0.10 vs. germline-ab 0.5 ± 0.09 p=0.0139, mGO ctrl-ab vs. maturated-ab 0.5 ± 0.10 p=0.0004). **E)** Average decrease after bath application of NMDA (H(2)=20.79, P<0.0001, K-W; mGO ctrl-ab 1 ± 0.07 vs. germline-ab 0.6 ± 0.07 p=0.0012, mGO cltr-ab vs. maturated-ab 0.6 ± 0.05 p=0.0003). **F)** Response after bath application of Kainate (F(2,83)=0.6151, p=0.5430, ANOVA; mGO ctrl-ab 1 ± 0.08, germline-ab 1 ± 0.18, maturated-ab 0.8 ± 0.11). **G)** Response after bath application of GABA (F(2,79)=1.041 p=0.3578, ANOVA; mGO ctrl-ab 1 ± 0.07, germline-ab 0.8 ± 0.13, maturated-ab 0.9 ± 0.09). K-W=Kruskal-Wallis. Averages ± S.E.M. Each data point represents the response of one neuron. Unpublished data.

Literature shows that NMDA-antibodies trigger internalization that occurs between 4-6 hours after incubation (11). To explore whether the germline antibody could work via direct effects as seen with the $GABA_AR$ antibody or via internalization as seen with the maturated NMDAR antibodies, I repeated the patch-clamp experiments after 3-hour antibody incubation. This time-point is predicted to fall before the internalization becomes apparent. As before, EPSCs were not affected by either antibody (Fig. 13C). However, neither evoked-NMDA (Fig. 13A,D) nor bath applied NMDA currents (Fig. 13B,E) were affected by the antibodies contrary to the effects I saw after 24-hour incubation. In addition, kainate (Fig. 13F) and GABA currents (Fig. 13G) were also not affected. These data indicate that both the mature and germline antibody do not seem to have any direct effects on the receptor and might be working through internalization as reported in the literature.

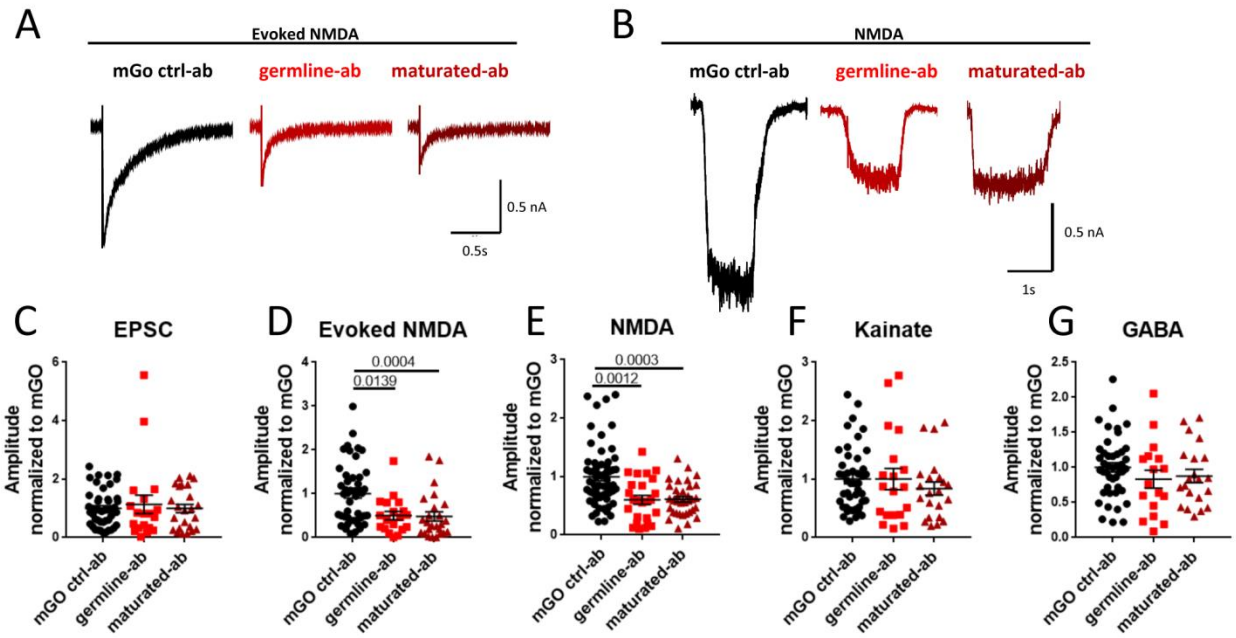

Figure 13: whole-cell patch-clamp recordings from hippocampal neurons incubated with a germline or maturated NMDA antibody for 3 hours. A) Example traces of synaptic NMDA responses. **B)** Example traces of the average postsynaptic response after bath application of NMDA. **C)** EPSC Amplitude normalized to mGO condition (F(2, 61)=1.428, p=0.2476, ANOVA; mGO ctrl-ab 1 ± 0.13, germline-ab 0.7 ± 0.12, maturated-ab 0.9 ± 0.12). **D)** Average decrease in synaptic NMDA responses (F(2, 59)=1.916, p=0.1563, ANOVA; mGO ctrl-ab 1 ± 0.12, germline-ab 0.9 ± 0.29, maturated-ab 0.5 ± 0.08). **E)** Average decrease after bath application of NMDA (H(2)=5.722, P=0.0572, K-W; mGO ctrl-ab 1 ± 0.10, germline-ab 1 ± 0.22, maturated-ab 0.7 ± 0.12). **F)** Response after bath application of Kainate (H(2)=5.667, P=0.0588, K-W; mGO ctrl-ab 1 ± 0.09, germline-ab 0.8 ± 0.07, maturated-ab 1.1 ± 0.13). **G)** Response after bath application of GABA (F(2,62)=0.3603, p=0.6989, ANOVA; mGO ctrl-ab 1 ± 0.08, germline-ab 1 ± 0.11, maturated-ab 0.9 ± 0.12). K-W=Kruskal-Wallis. Averages ± S.E.M. Each data point represents the response of one neuron. Figure adjusted from (33) figure 3K-O expanded with unpublished maturated-antibody data.

4. Discussion

Autoimmune encephalitis is a relatively new and devastating disorder. A better understanding of the mechanisms underlying autoimmune encephalitis could improve treatment outcomes for patients. Therefore, in this thesis, I have used a collection of patient-derived monoclonal antibodies to explore whether these disease-associated autoantibodies could contribute to symptom progression by promoting direct effects on receptor function, dampen receptor function by promoting their internalization, and/or promote receptor removal by engaging phagocytic immune cells. My studies reveal that autoimmune antibodies can disrupt receptor function through all three of these mechanisms. The α1-$GABA_AR$ and NMDAR-antibodies can disrupt receptor function by triggering their internalization, however the α1-antibody can also directly inhibit GABAergic receptor function. Surprisingly, I found that neutral/silent autoantibodies (α1γ2) can contribute to the loss of $GABA_AR$ function by engaging microglia. Furthermore, my work revealed that germline antibodies can also engage receptors, altering their function, laying the foundation for the expansion and maturation of these antibodies during disease progression.

4.1 Mechanism underlying the α1-antibody

The α1-antibody showed both short and long-term effects on the $GABA_AR$. Short term, the α1-antibody had a strong antagonistic effect on mIPSCs frequency, amplitude, and current (~ 1hr following antibody addition), with no effects on mIPSC kinetics (Fig. 6). In addition, calcium imaging revealed that neuronal spiking activity was increased following the 1-hour addition of the α1-antibody (Fig. 6). Intriguingly, increased spiking could be induced in as little as 2 minutes after the acute addition of the α1-antibody (Fig. 7). This rapid increase was also observed following the addition of a Fab-fragment of the α1-antibody (Fig. 7). As the Fc region of IgGs is known to promote receptor cross-linking and internalization (31, 39), the Fab data indicate that these increases in neuronal spiking activity occur independent of antibody-mediated cross-linking of the receptors and may be due to direct antagonistic effects of the α1-antibody on $GABA_AR$ function. Furthermore, the lack of changes in mIPSC kinetics implies that the antibody does not directly modify receptor opening properties nor necessarily cause postsynaptic receptor isotype switching. Of note, the time scale of the reduction in signal transduction

indicates that the α1-antibody can possibly directly blocks receptor conductance. Of course, other direct effects like conformational changes to the receptor that trigger fast internalization cannot be ruled out without more in-depth biophysical studies.

Long-term, the α1-antibody reduced GABA-mediated receptor currents during whole-cell patch-clamp experiments (Fig. 2) that were due to changes at the post-synapse (Fig. 4). This effect was very specific and did not affect any excitatory signal transduction (Fig. 2). Consistent with the reduction in GABA-mediated currents, the α1-antibody induced increases in calcium-spiking activity in neuronal mass-cultures (Fig. 3). When the location of the α1-antibody puncta was compared to VGAT-labeled inhibitory presynaptic boutons, I found that the α1-antibody puncta co-localized less frequently with this pre-synaptic marker overtime (Fig. 5), indicating that the α1-antibody labeled $GABA_A$Rs were either redistributed to the extra-synaptic space or internalized.

Taken together, these data suggest that the α1-antibody can operate via at least two molecular mechanisms: one that disrupts GABA-mediated signaling through a direct antagonistic effect on the receptor, and the second that causes the loss of synaptic receptors via their redistribution or internalization. It is not clear if the antibody actively contributes to both mechanisms or whether the long-term effects are merely caused by compensatory mechanisms in response to the short-term effects. In terms of autoantibodies, importantly, direct effects of autoantibodies on receptors have been reported before for both glycine and $GABA_B$ receptors (43, 44), yet are otherwise uncommon, whereas autoantibody cross-linking and receptor redistribution have been described as a common mechanism underlying autoimmune encephalitis (45) including antibodies present in the CSF of $GABA_A$RE patients (12-14).

4.2 Mechanism underlying the α1γ2-antibody

The α1γ2-antibody, unlike the α1-antibody, had little or no effect on GABA-mediated currents. This result was very surprising since animal studies showed that the α1γ2-antibody can induce seizures in rats (34). My immunocytochemical staining ruled out that the lack of effect was due to a lack of receptor binding, as ~40% of all inhibitory synapses were decorated by the α1γ2-antibody (Fig. 8). Interestingly, ~80% of the α1γ2-antibody puncta co-localized with synapses, which is expected, as the γ2-subunit functions as an anchoring subunit for the $GABA_A$Rs to the membrane-associated cytoskeletal scaffolding assembled at the post-synapse (46). The observed lack of effect

was also not mitigated by increasing the α1γ2-antibody concentration 5-fold, as illustrated by the unaffected network activity in our 24-hour calcium imaging experiments (Fig. 3). These observations led me to wonder what could possibly explain the discrepancy between the results in dissociated cell-culture and *in vivo* animal studies.

One hypothesis I developed was that the α1γ2-antibody could be interfering with the benzodiazepine site of the $GABA_AR$, blocking the actions of endozepines in animals. Endozepines are known to regulate GABA transmission and play and important role in the inhibitory/excitatory (I/E) balance (40, 41). Interference in this regulatory mechanism by the α1γ2-antibody could thus promote seizures. This hypothesis is supported by research that shows that decreased endozepine levels are associated with epilepsy in patients (47). During our experiments, diazepam increased, as expected, the half-width time of mIPSCs in untreated cultures. Surprisingly, the presence of the α1γ2-antibody did not interfere with the ability of diazepam to modulate the GABA response, making it unlikely that this antibody influences the accessibility and functionality of the benzodiazepine binding site (Fig 9,10).

An alternative hypothesis whereby the α1γ2-antibody could affect neuronal excitability *in vivo*, but not in culture, is through its engagement of central nervous system (CNS) localized immune cells via the Fc region of the antibody. Consistent with this hypothesis, when microglia were added to my cultures, they were able to strip synaptic α1γ2-antibody puncta along the length of the neuronal dendrites (Fig. 11), indicating that the α1γ2-antibody can engage immune cells via their Fc region. These results give the first indication that cellular immunity might play a bigger role in autoimmune encephalitis than previously thought. Only a few studies have looked at this issue. For example, in neuromyelitis optica complement activation can lead to microglia activation (48, 49) (50), while maternal transfer of autoimmune antibodies can lead to activated microglia and reductions in synapse number in their offspring (51). However, while relatively new in autoimmune encephalitis, the role of microglia has been extensively studied in other diseases, such as Alzheimer's disease (52) and rheumatoid arthritis (53). It is therefore not surprising that microglia could be important in autoimmune encephalitis (AE).

Together, these data indicate that the α1γ2-antibody might bind silently to α1γ2-containing $GABA_ARs$, where it facilitates receptor removal via activation of microglia. Mechanistically, this could lead to a loss of neuronal $GABA_ARs$, a shift in the E/I balance (54), increasing seizure activity seen *in vivo* in animal experiments and individuals with $GABA_AR$ encephalitis. In parallel, the activation of microglia might lead to the secretion

of cytokines leading to B and T-cell activation further down the line. The involvement of these latter players is likely since T-cell activation often results in lesions, an observation often seen in $GABA_AR$ encephalitis patients (10, 55). However, many open questions remain regarding the involvement of cellular immunity in AE. For example, are microglia a universal player in all AE? Do the antibodies come first, or does the cellular immune system? Does one drive the other? Exploring such questions should lead to a better mechanistic understanding of AE.

It is important to recognize that possible direct effects of the α1γ2-antibody on $GABA_AR$ could have been missed in my assays, as α1γ2 subunits are only present in a subset of receptors in a cell-type/region-specific manner (56), necessitating experiments with defined subunit compositions in heterologous cells. My assays would also fail to detect antibody mediated disruption of α1γ2-receptor complexes with for example post-synaptic anchoring proteins including the tetraspanin superfamily of transmembrane proteins also called Lipoma HMGIC fusion partner-like 4 (LHFPL-4). These are known to promote the synaptic clustering of γ2-containing $GABA_ARs$ through its interaction with Neuroligin-2 (46, 57). Of note, LHFPL-4 only clusters these receptors at inhibitory synapses on excitatory neurons primarily in the hippocampus and cerebellum (46, 57). Such action of the α1γ2-antibody could cause seizures in animals, but not increase network excitability in our cortical-striatal cultures, which consist solely of inhibitory neurons. Similar results have also been observed for NMDAR encephalitis, where the same antibody had different effects in different brain regions (58).

4.3 Mechanism underlying the NMDAR antibodies

This study also investigated the actions of two NMDAR autoantibodies, one germline and one maturated via whole-cell patch-clamp recordings. Interestingly, the naturally occurring germline antibody was able to selectively inhibit NMDAR-mediated currents only when delivered at a 5-fold higher concentration than a maturated NMDAR-antibody. These findings suggest that germline antibodies can affect NMDAR function, albeit to a lesser extent than fully maturated antibodies, possibly due to their lower affinity. Interestingly, even germline antibodies seem rather specific as illustrated by the lack of any effect on inhibitory and even excitatory, kainate-mediated, currents (Fig. 12 and 13).

Mechanistically, several NMDAR-antibodies, in other studies, were found to reduce NMDAR currents by triggering internalization ~4-6 hours after antibody incubation (11).

To explore whether germline antibodies utilize internalization and/or can illicit direct effects, patch-clamp experiments were repeated after a 3-hour antibody incubation, which is predicted to predate the internalization timeframe. During these experiments no effects on NMDAR mediated currents were observed for either germline or maturated antibodies (Fig 13). These data confirm previous observations that maturated NMDAR-antibodies require longer timeframes to illicit their effects (11). They also hint that germline antibodies might work via a similar internalization mechanism, which should be confirmed with cellular internalization assays. Of interest is whether, as observed for the α1γ2-antibody, microglia recognize and mediate clearance of germline NMDAR antibodies. One important issue for consideration is that in these experiments the variable region of the germline antibody was recombinantly fused to the Fc region of the IgG1 class before purification. However, germline antibodies normally exist as IgM isotypes *in vivo.* This could have profound consequences for future in depth research into the mechanisms underlying this antibody, as complement activation favors IgM-mediated activation and Fc-gamma receptors on microglia primarily recognize specific sequences in IgG1 and IgG3 (59-62). It would thus make sense to repeat electrophysiological and microglia experiments with the germline NMDAR-antibody's variable sequences incorporated as both IgG1 and IgM isotype antibodies.

With regards to the effect of the germline antibody, their role in healthy people has always been a conundrum. Some research has shown that germline antibodies might be beneficial for the regulation of immune responses by reducing inflammation and promoting the clearance of debris (63). Our own results not only verify the presence of such antibodies in patients, but also that they can functionally affect receptor function, perhaps even in the context of autoimmune encephalitis (63). This fact could have widespread consequences, as the literature shows that ~10% of the population might have germline antibodies against the NMDAR (64, 65). Fortunately, most healthy individuals have an intact blood brain barrier (BBB) that will keep these antibodies outside the brain. However, in people with compromised BBB, i.e., due to viral or bacterial infection (66, 67), such germline antibodies might cause disease or could contribute to disease progression. Future research should help to resolve these issues.

5. Conclusions

A growing number of studies have sought to understand the mechanisms whereby pathogenic antibodies cause disease. These include direct effects on receptor function, promotion of receptor internalization, and/or the recruitment of other immune actors. For some types of autoimmune encephalitis, such as NMDAR encephalitis, antibody induced receptor internalization appears to be the preferred mechanism (11, 27, 31, 68). Others such as AQP4 encephalitis engage a complement cascade (49, 69), whereas others seem to be a mixture of several mechanisms e.g., $GABA_AR$ encephalitis (34, 35, 70, 71), and/or eliciting distinct effects in different brain regions (56, 58, 72). The location of each antibody epitope appears to be a primary determinant of which mechanism is used. For example, if the epitope is on a ligand binding site, direct effects might occur, while others can promote receptor cross-linking and their internalization. The least understood mechanism involves the activation and recruitment of immune cells that either strip receptors or kill cells. This diversity creates both opportunities and challenges when trying to develop better therapeutic strategies for patients with these devastating disorders.

Implications for future research

Due to the tremendous variability in how antibodies mechanistically drive autoimmune encephalitis, developing effective therapies will remain a major challenge, with trade-offs between broad non-specific immune suppressive drugs and biologics with great specificity, but which cannot address complex antibody repertoires. Some emerging therapies, based on cellular therapies hold some promise, but could be costly. These include a new generation of Chimeric AutoAntibody Receptor T-cell (CAAR-Ts) used to eliminate specific antibody producing B-cells (73, 74). However, this strategy might only be feasible when a patient's antibodies are acting on a relatively limited number of receptors/epitopes (75, 76). Alternatively, drugs or biologics that could dampen antibody mediated microglia activation might prevent disease progression by blocking the down-stream engagement of the peripheral immune system. Finally, strategies aimed at restoring immune-cell tolerance to specific autoantigens could provide a long-term suppression of these devastating auto-immune disorders (77, 78).

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Statutory Declaration

“I, Adriana van Casteren, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic “Assessing the effect of autoimmune receptor autoantibodies on synaptic function and behaviour” “Bewertung der Auswirkungen von Autoimmune rezeptor Autoantikörpern auf synaptische Funktion und Verhalten”, independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; http://www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

Declaration of your own contribution to the publications

Adriana van Casteren contributed the following to the below listed publications:

Publication 1: Kreye J, Wright SK, van Casteren A, Stöffler L, Machule ML, Reincke SM, Nikolaus M, van Hoof S, Sanchez-Sendin E, Homeyer MA, Cordero Gómez C, Kornau HC, Schmitz D, Kaindl AM, Boehm-Sturm P, Mueller S, Wilson MA, Upadhya MA, Dhangar DR, Greenhill S, Woodhall G, Turko P, Vida I, Garner CC, Wickel J, Geis C, Fukata Y, Fukata M, Prüss H, Encephalitis patient-derived monoclonal GABAA receptor antibodies cause epileptic seizures, Journal of experimental medicine, 2021
Contribution (please set out in detail):

Execution of experiments:
Whole-cell patch-clamp recordings of autaptic neurons (Fig. 3A-F)
Data analysis and visualization:
Analysis and visualization of autaptic recordings (Fig. 3A-F)
Writing and editing of the manuscript:
Results, methods of autaptic recordings (Fig. 3A-F)

Publication 2: van Casteren ACM, Ackermann F, Rahman KA, Andrzejak E, Rosenmund C, Kreye J, Prüss H, Garner CC, Ichkova A, Differential Modes of Action of α1- and α1γ2-Autoantibodies Derived from Patients with GABAAR Encephalitis, eNeuro, 2022
Contribution (please set out in detail):

Planning of the experiments:
Adriana van Casteren, Frauke Ackermann, Kazi Rahman, Ewa Andrzejak, Craig Garner, Christian Rosenmund, Aleksandra Ichkova
Execution of experiments:
Adriana van Casteren
Data analysis:
Adriana van Casteren except for mIPSC data (Fig. 3K-M) which was analyzed by Aleksandra Ichkova
Data visualization:
Adriana van Casteren
Writing and editing of the manuscript:
First draft of the paper by Adriana van Casteren, editing by Craig Garner and Aleksandra Ichkova with inputs from all the authors.

Publication 3: Wenke, N.K., Kreye, J., Andrzejak, E., van Casteren, A., Leubner, J., Murgueitio, M.S., Reincke, S.M., Secker, C., Schmidl, L., Geis, C., Ackermann, F., Nikolaus, M., Garner, C.C., Wardemann, H., Wolber, G. and Prüss, H, N-methyl-D-aspartate receptor dysfunction by unmutated human antibodies against the NR1 subunit, Annals of neurology, 2019

Contribution (please set out in detail):

Execution of experiments:

Whole-cell patch-clamp recordings of autaptic neurons (Fig. 3A-J) together with Ewa Andrzejak, (Fig. 3K-O) by Adriana van Casteren.

Data analysis and visualization:

Analysis and visualization of autaptic recordings (Fig. 3)

Writing and editing of the manuscript:

Methods and results of patch-clamp experiments (Fig. 3)

_____

Signature, date and stamp of first supervising university professor / lecturer

_____

Signature of doctoral candidate

Excerpt from Journal Summary List -1-

Journal Data Filtered By: **Selected JCR Year: 2019** Selected Editions: SCIE,SSCI
Selected Categories: **"MEDICINE, RESEARCH and EXPERIMENTAL"**
Selected Category Scheme: WoS
Gesamtanzahl: 138 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE MEDICINE	85,220	36.130	0.168730
2	Science Translational Medicine	34,479	16.304	0.116030
3	JOURNAL OF CLINICAL INVESTIGATION	109,020	11.864	0.125830
4	JOURNAL OF EXPERIMENTAL MEDICINE	63,562	11.743	0.067350
5	TRENDS IN MOLECULAR MEDICINE	10,618	11.099	0.018720
6	Annual Review of Medicine	6,267	9.716	0.009390
7	MOLECULAR ASPECTS OF MEDICINE	6,207	9.577	0.005750
8	MOLECULAR THERAPY	17,977	8.986	0.030980
9	EMBO Molecular Medicine	8,366	8.821	0.022770
10	Theranostics	12,995	8.579	0.029740
11	Clinical and Translational Medicine	1,349	7.919	0.003280
12	Wiley Interdisciplinary Reviews-Nanomedicine and Nanobiotechnology	2,819	7.689	0.004240
13	Molecular Therapy-Nucleic Acids	5,024	7.032	0.013550
14	JCI Insight	7,697	6.205	0.034400
15	Cold Spring Harbor Perspectives in Medicine	7,647	6.000	0.016800
16	ALTEX-Alternatives to Animal Experimentation	1,413	5.787	0.002210
17	JOURNAL OF BIOMEDICAL SCIENCE	4,499	5.762	0.006380
18	EBioMedicine	8,133	5.736	0.029330
19	npj Vaccines	596	5.699	0.002400
20	Cancer Biology & Medicine	1,389	5.432	0.003640

Printing copy of the publication -1-

https://doi.org/ 10.1084/jem.20210012

Excerpt from Journal Summary List -2-

Journal Data Filtered By: **Selected JCR Year: 2020** Selected Editions: SCIE,SSCI
Selected Categories: **"NEUROSCIENCES"** Selected Category Scheme: WoS
Gesamtanzahl: 273 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS NEUROSCIENCE	49,897	34.870	0.048890
2	NATURE NEUROSCIENCE	73,709	24.884	0.128020
3	TRENDS IN COGNITIVE SCIENCES	33,482	20.229	0.036270
4	NEURON	111,115	17.173	0.175220
5	ACTA NEUROPATHOLOGICA	28,031	17.088	0.036970
6	MOLECULAR PSYCHIATRY	28,622	15.992	0.046220
7	Molecular Neurodegeneration	6,772	14.195	0.011650
8	TRENDS IN NEUROSCIENCES	22,858	13.837	0.019470
9	Nature Human Behaviour	5,549	13.663	0.023120
10	BRAIN	64,627	13.501	0.061550
11	BIOLOGICAL PSYCHIATRY	50,155	13.382	0.045540
12	JOURNAL OF PINEAL RESEARCH	12,492	13.007	0.008170
13	BEHAVIORAL AND BRAIN SCIENCES	11,610	12.579	0.007760
14	Annual Review of Neuroscience	14,699	12.449	0.010490
15	PROGRESS IN NEUROBIOLOGY	15,161	11.685	0.010300
16	SLEEP MEDICINE REVIEWS	11,218	11.609	0.014840
17	ANNALS OF NEUROLOGY	43,728	10.422	0.039960
18	NEUROSCIENCE AND BIOBEHAVIORAL REVIEWS	36,525	8.989	0.048970
19	Brain Stimulation	9,206	8.955	0.015960
20	npj Parkinsons Disease	1,093	8.651	0.003040
21	FRONTIERS IN NEUROENDOCRINOLOGY	5,338	8.606	0.005050

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
22	Neurology-Neuroimmunology & Neuroinflammation	3,863	8.485	0.008390
23	Journal of Neuroinflammation	19,657	8.322	0.027070
24	NEUROPATHOLOGY AND APPLIED NEUROBIOLOGY	4,791	8.090	0.004640
25	NEURAL NETWORKS	18,837	8.050	0.019420
26	Translational Neurodegeneration	1,759	8.014	0.003160
27	NEUROPSYCHOPHARMACOLOGY	30,856	7.853	0.034600
28	Acta Neuropathologica Communications	6,580	7.801	0.016320
29	Fluids and Barriers of the CNS	1,956	7.662	0.002170
30	Neurotherapeutics	6,764	7.620	0.009400
31	NEUROSCIENTIST	5,949	7.519	0.005010
32	Molecular Autism	3,579	7.509	0.007400
33	GLIA	17,858	7.452	0.016000
34	NEUROPSYCHOLOGY REVIEW	3,941	7.444	0.003460
35	Current Neuropharmacology	6,080	7.363	0.007730
36	JOURNAL OF HEADACHE AND PAIN	5,400	7.277	0.008140
37	BRAIN BEHAVIOR AND IMMUNITY	24,161	7.217	0.026930
38	Alzheimers Research & Therapy	5,593	6.982	0.011680
39	PAIN	45,325	6.961	0.031030
40	Translational Stroke Research	3,377	6.829	0.003920
41	BIPOLAR DISORDERS	6,185	6.744	0.007510
42	CURRENT OPINION IN NEUROBIOLOGY	17,009	6.627	0.025180
43	NEUROIMAGE	119,618	6.556	0.105820

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
44	BRAIN PATHOLOGY	6,559	6.508	0.006220
45	Developmental Cognitive Neuroscience	4,477	6.464	0.011160
46	Annual Review of Vision Science	935	6.422	0.004560
47	Multiple Sclerosis Journal	15,551	6.312	0.016680
48	CEPHALALGIA	12,756	6.292	0.011940
49	Biological Psychiatry-Cognitive Neuroscience and Neuroimaging	2,193	6.204	0.007120
50	JOURNAL OF CEREBRAL BLOOD FLOW AND METABOLISM	22,732	6.200	0.019640
51	JOURNAL OF PSYCHIATRY & NEUROSCIENCE	4,100	6.186	0.004200
52	JOURNAL OF NEUROSCIENCE	186,015	6.167	0.130970
53	EUROPEAN JOURNAL OF NEUROLOGY	14,490	6.089	0.016730
54	NEUROBIOLOGY OF DISEASE	21,360	5.996	0.020680
55	Dialogues in Clinical Neuroscience	5,272	5.986	0.005200
56	SLEEP	28,688	5.849	0.023920
57	JOURNAL OF PAIN	13,655	5.820	0.014690
58	Frontiers in Aging Neuroscience	13,654	5.750	0.025540
59	CURRENT OPINION IN NEUROLOGY	6,723	5.710	0.008480
60	Frontiers in Molecular Neuroscience	10,570	5.639	0.022450
61	MOLECULAR NEUROBIOLOGY	20,795	5.590	0.033020
62	Journal of Parkinsons Disease	3,562	5.568	0.006390
63	Frontiers in Cellular Neuroscience	17,299	5.505	0.033870
64	Neurobiology of Stress	1,628	5.441	0.004280
65	Cognitive Computation	2,407	5.418	0.002870

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
66	Journal of Neural Engineering	9,572	5.379	0.012750
67	JOURNAL OF NEUROCHEMISTRY	40,281	5.372	0.019170
68	CEREBRAL CORTEX	34,416	5.357	0.047520
69	Nature and Science of Sleep	1,240	5.346	0.002290
70	EXPERIMENTAL NEUROLOGY	24,022	5.330	0.015650
71	JOURNAL OF NEUROTRAUMA	19,004	5.269	0.018210
72	NEUROPHARMACOLOGY	26,426	5.250	0.027960
73	CNS Neuroscience & Therapeutics	5,113	5.243	0.005790
74	Neuroscience Bulletin	3,348	5.203	0.004590
75	PSYCHIATRY AND CLINICAL NEUROSCIENCES	5,454	5.188	0.004700
76	JOURNAL OF PHYSIOLOGY-LONDON	58,028	5.182	0.034600
77	INTERNATIONAL JOURNAL OF NEUROPSYCHOPHARMACOLOGY	7,865	5.176	0.008440
78	Neural Regeneration Research	7,769	5.135	0.010800
79	Cognitive Neurodynamics	1,435	5.082	0.001570
80	Current Neurology and Neuroscience Reports	4,549	5.081	0.007300
81	PROGRESS IN NEURO-PSYCHOPHARMACOLOGY & BIOLOGICAL PSYCHIATRY	13,777	5.067	0.013440
82	CELLULAR AND MOLECULAR NEUROBIOLOGY	6,220	5.046	0.005990
83	HUMAN BRAIN MAPPING	27,538	5.038	0.035480
84	NUTRITIONAL NEUROSCIENCE	2,828	4.994	0.002960
85	NEUROENDOCRINOLOGY	6,227	4.914	0.006300
86	PSYCHONEUROENDOCRINOLOGY	22,335	4.905	0.025020
87	Frontiers in Neuroscience	27,452	4.677	0.057310

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
88	NEUROBIOLOGY OF AGING	27,226	4.673	0.026480
89	Network Neuroscience	613	4.625	0.002110
90	EUROPEAN NEUROPSYCHOPHARMACOLOGY	8,999	4.600	0.011190
91	PSYCHOPHARMACOLOGY	26,451	4.530	0.017630
92	Annals of Clinical and Translational Neurology	4,188	4.511	0.012270
93	Frontiers in Synaptic Neuroscience	990	4.506	0.002000
94	JOURNAL OF ALZHEIMERS DISEASE	30,802	4.472	0.046550
95	Current Opinion in Behavioral Sciences	3,724	4.466	0.015020
96	CLINICAL AUTONOMIC RESEARCH	2,164	4.435	0.002530
97	ACS Chemical Neuroscience	10,120	4.418	0.015000
98	CNS & Neurological Disorders-Drug Targets	4,004	4.388	0.002850
99	REVIEWS IN THE NEUROSCIENCES	2,759	4.353	0.002840
100	npj Science of Learning	318	4.350	0.001270
101	MOLECULAR AND CELLULAR NEUROSCIENCE	7,486	4.314	0.005270
102	NEUROMUSCULAR DISORDERS	6,588	4.296	0.007410
103	NEUROTOXICOLOGY	8,569	4.294	0.006950
104	Journal of NeuroEngineering and Rehabilitation	7,017	4.262	0.007590
105	JOURNAL OF NEUROSCIENCE RESEARCH	15,264	4.164	0.009390
106	JOURNAL OF PSYCHOPHARMACOLOGY	8,158	4.153	0.010010
107	Journal of Neuroimmune Pharmacology	3,445	4.147	0.002860
108	ASN Neuro	1,176	4.146	0.001120
109	NEUROINFORMATICS	1,780	4.085	0.002900

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
110	Frontiers in Neuroinformatics	3,340	4.081	0.005750
110	eNeuro	5,122	4.081	0.019030
112	BRAIN RESEARCH BULLETIN	11,823	4.077	0.008440
113	Molecular Brain	3,782	4.041	0.005750
114	CORTEX	13,326	4.027	0.021450
115	Journal of Neurodevelopmental Disorders	1,819	4.025	0.002850
116	PSYCHOPHYSIOLOGY	17,106	4.016	0.011490
117	Frontiers in Neurology	18,626	4.003	0.040660
118	NEUROCHEMICAL RESEARCH	12,688	3.996	0.010650
119	JOURNAL OF SLEEP RESEARCH	8,023	3.981	0.007750
120	Developmental Neurobiology	3,458	3.964	0.004950
121	EUROPEAN JOURNAL OF PAIN	9,204	3.931	0.009110
122	NEUROCHEMISTRY INTERNATIONAL	10,823	3.921	0.008090
123	NEUROTOXICITY RESEARCH	4,578	3.911	0.004170
124	HIPPOCAMPUS	9,540	3.899	0.008390
125	Frontiers in Neuroanatomy	4,556	3.856	0.009800
126	CEREBELLUM	3,968	3.847	0.005100
127	NEUROMOLECULAR MEDICINE	2,423	3.843	0.001780
128	Neural Development	1,135	3.842	0.001290
129	NEUROLOGIC CLINICS	3,097	3.806	0.003060
130	Purinergic Signalling	2,490	3.765	0.002020
131	NEUROTOXICOLOGY AND TERATOLOGY	4,139	3.763	0.002750

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https://doi.org/10.1523/ENEURO.0369-22.2022

Excerpt from Journal Summary List -3-

Journal Data Filtered By: **Selected JCR Year: 2016** Selected Editions: SCIE,SSCI
Selected Categories: **"NEUROSCIENCES"** Selected Category Scheme: WoS
Gesamtanzahl: 258 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS NEUROSCIENCE	36,952	28.880	0.071380
2	NATURE NEUROSCIENCE	54,399	17.839	0.160740
3	Annual Review of Neuroscience	13,211	15.630	0.020660
4	TRENDS IN COGNITIVE SCIENCES	23,273	15.402	0.046360
5	BEHAVIORAL AND BRAIN SCIENCES	8,195	14.200	0.010940
6	NEURON	82,253	14.024	0.227070
7	PROGRESS IN NEUROBIOLOGY	12,163	13.217	0.018020
8	MOLECULAR PSYCHIATRY	17,452	13.204	0.049670
9	ACTA NEUROPATHOLOGICA	16,462	12.213	0.037060
10	BIOLOGICAL PSYCHIATRY	41,859	11.412	0.067400
11	TRENDS IN NEUROSCIENCES	19,178	11.124	0.029690
12	JOURNAL OF PINEAL RESEARCH	7,278	10.391	0.008040
13	BRAIN	48,061	10.292	0.077590
14	ANNALS OF NEUROLOGY	34,215	9.890	0.057310
15	FRONTIERS IN NEUROENDOCRINOLOGY	3,516	9.425	0.006600
16	SLEEP MEDICINE REVIEWS	4,980	8.958	0.009730
17	NEUROSCIENCE AND BIOBEHAVIORAL REVIEWS	20,452	8.299	0.047230
18	NEUROSCIENTIST	4,325	7.391	0.009890
19	Molecular Neurodegeneration	2,946	6.780	0.009540
20	CEREBRAL CORTEX	27,496	6.559	0.063240
21	NEUROPSYCHOPHARMACOLOGY	23,920	6.403	0.046670
22	NEUROPSYCHOLOGY REVIEW	2,478	6.352	0.004650
23	GLIA	12,781	6.200	0.021920
24	Alzheimers Research & Therapy	1,699	6.196	0.007180
25	MOLECULAR NEUROBIOLOGY	7,338	6.190	0.017440
26	NEUROSIGNALS	653	6.143	0.000670
27	CURRENT OPINION IN NEUROBIOLOGY	13,188	6.133	0.036730
28	Brain Stimulation	3,905	6.078	0.013020
29	JOURNAL OF NEUROSCIENCE	171,800	5.988	0.319910
30	BRAIN BEHAVIOR AND IMMUNITY	10,719	5.964	0.026460
31	NEUROIMAGE	85,630	5.835	0.173210
32	PAIN	35,333	5.445	0.044460
33	NEUROPATHOLOGY AND APPLIED NEUROBIOLOGY	3,413	5.347	0.006400
34	NEURAL NETWORKS	8,741	5.287	0.010250
35	BRAIN PATHOLOGY	4,580	5.272	0.008450
36	JOURNAL OF NEUROTRAUMA	12,787	5.190	0.021640
37	Neurotherapeutics	3,451	5.166	0.008220
38	JOURNAL OF PSYCHIATRY & NEUROSCIENCE	2,759	5.165	0.004970
39	NEUROBIOLOGY OF AGING	20,010	5.117	0.046250

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BRIEF COMMUNICATION

N-Methyl-D-Aspartate Receptor Dysfunction by Unmutated Human Antibodies Against the NR1 Subunit

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Anti–N-methyl-D-aspartate receptor (NMDAR) encephalitis is the most common autoimmune encephalitis related to autoantibody-mediated synaptic dysfunction. Cerebrospinal fluid–derived human monoclonal NR1 autoantibodies showed low numbers of somatic hypermutations or were unmutated. These unexpected germline-configured antibodies showed weaker binding to the NMDAR than matured antibodies from the same patient. In primary hippocampal neurons, germline NR1 autoantibodies strongly and specifically reduced total and synaptic NMDAR currents in a dose- and time-dependent manner. The findings suggest that functional NMDAR antibodies are part of the human naïve B cell repertoire. Given their effects on synaptic function, they might contribute to a broad spectrum of neuropsychiatric symptoms.

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Autoantibodies against the aminoterminal domain (ATD) of the NR1 subunit of the N-methyl-D-aspartate (NMDA) receptor (NMDAR) are the hallmark of NMDAR encephalitis, the most common autoimmune encephalitis presenting with psychosis, epileptic seizures, amnesia, and autonomic instability.[1] The disease can be triggered by NMDAR-expressing teratomas[2] and occur secondarily to viral encephalitis;[3,4] however, in most cases, the initiating events remain unclear. Intracerebroventricular injection of cerebrospinal fluid (CSF), as well as a single recombinant monoclonal NR1 immunoglobulin G1 (IgG1) antibody obtained from clonally expanded intrathecal plasma cells of a patient with NMDAR encephalitis into mice, led to transient behavioral changes compatible with human disease symptoms.[5,6]

We could recently generate a panel of human monoclonal NMDAR autoantibodies from antibody-secreting cells in CSF of patients with NMDAR encephalitis.[7] Unexpectedly, several NR1-reactive autoantibodies from different patients were unmutated, suggesting that they had not been selected for high affinity during germinal center reactions and instead were derived from activated naïve B cells. We therefore determined whether these germline NR1 antibodies showed functional effects similar to affinity-matured NR1 autoantibodies leading to synaptic dysfunction.

Materials and Methods

Recombinant Monoclonal NMDAR Antibodies

Recombinant monoclonal human NR1 IgG autoantibodies were generated as described.[7,8] The study was approved by the Charité University hospital Review Board, and informed consent was obtained from each subject. The control antibody (mGo53) is a nonreactive isotype-matched human antibody.[9] Immunostaining, using primary hippocampal neurons, unfixed mouse brain sections, HEK cell-expressed NR1 N368Q mutants, and brain sections after intravenous antibody injection, followed our established protocols.[7] We generated germline counterpart versions from maturated monoclonal NR1 antibodies with best matching variable V(D)J genes and elimination of somatic hypermutations.[10] Relative affinity curves were calculated based on concentration-dependent antibody binding to hippocampal sections, adapted from previous work using HEK cells transfected with the NR1 subunit.[11] For bilateral intracerebroventricular injection, 200 μg of antibodies were infused over 14 days using osmotic minipumps.[12]

Super-Resolution Imaging

Direct stochastic reconstruction microscopy (dSTORM) was performed in primary hippocampal neurons (DIV 14) as described.[12]

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Human NR1 autoantibody (clone #003-109; 4 μg/ml) and polyclonal guinea pig anti-Homer-1 (1:300; Synaptic Systems GmbH, Goettingen, Germany) were used as primary antibodies followed by AlexaFluor-647 goat/anti-human (1:200; Life Technologies, Carlsbad, CA) and CF-568 donkey/anti–guinea pig (1:200; Biotium, Fremont, CA) as secondary antibodies.

Electrophysiological Recordings

Autaptic murine hippocampal neurons (DIV 14-17) were incubated with 1 or 5 μg/ml human NR1 (#003-109) or control antibody at 37°C for 3 or 24 hours. Data were acquired as described.[13] Cells were recorded in standard intra- and extracellular solutions, except for chemically induced NMDA responses, measured in extracellular solution containing 0 mM of Mg^{2+}, 0.2 mM of $CaCl_2$, and 10 μM of glycine, and evoked NMDA responses, measured in extracellular solution containing 0 mM of Mg^{2+}, 2 mM of $CaCl_2$, 10 μM of glycine, and 10 μM of NBQX (Tocris Bioscience, Bristol, UK). For kinetics of synaptic NMDA responses, non-silent traces from each cell were averaged and rise time and decay time constant (τ) measured from 10% to 90% or 90% to 10% of the peak, respectively. Decays were fitted with a double exponential and decay time constants for each of the fits converted to a weighted decay.

Homology Modeling

The homology model of the ATD of the human NMDA receptor was generated using the crystal structure of the rat NMDA receptor subunit, zeta-1. The homology modeling application of MOE 2014.09 ("Molecular Operating Environment [MOE], 2014.09", 2015) was used with 10 main chain models, each with one side chain. Samples were built using the amber12 force field.[14]

Results

Germline NR1 Autoantibodies Target the NMDAR in vitro and in vivo

The CSF autoantibody repertoire in NMDAR encephalitis contains NR1-binding and non-NR1-binding antibodies.[7] Across all 8 patients, NR1-binding antibodies had significantly lower numbers of somatic hypermutations (SHM) in the Ig heavy (5.1 ± 4.0 versus 11.9 ± 8.3) and corresponding Ig light chains (3.9 ± 4.8 versus 7.2 ± 5.4)

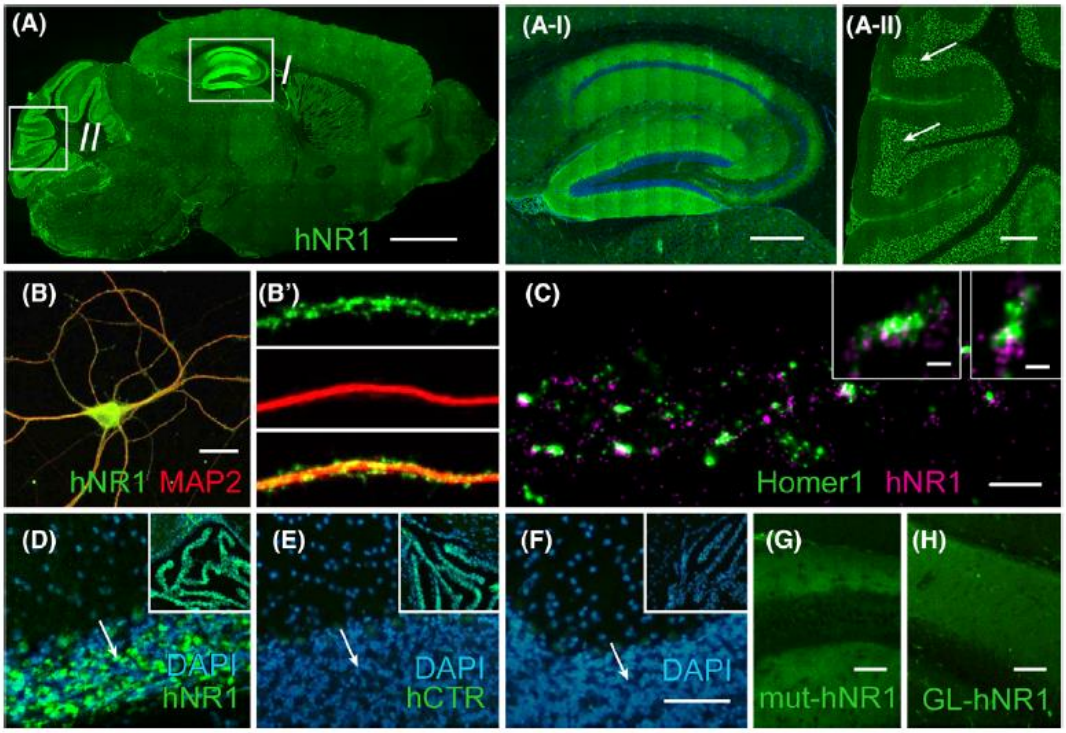

FIGURE 1: Target specificity of the germline NR1 autoantibody #003-109. Immunofluorescence staining showed the characteristic NR1 pattern in the brain (A), in particular of hippocampal neuropil (A-I) and cerebellar granule cells (A-II, arrows). NMDAR-expressing synaptic clusters were specifically labeled on primary hippocampal neurons (B; green: NR1, red: MAP2). dSTORM imaging confirmed NR1 expression in the synapse (C; purple: NR1, green: Homer1). Germline NR1 antibodies (D), but not isotype control antibodies (E), bound to cerebellar granule cells (arrows) 24 hours after intravenous injection together with lipopolysaccharide. Antibodies were present in the circulation as confirmed with stainings of the choroid plexus (D,E, inserts), in contrast to control brains of untreated mice (F). Intracerebroventricular injection of maturated (G) and germline (H) NR1 antibodies showed similar neuropil binding in the hippocampus. Scale bars: A = 2 mm; A-I/A-II = 500 μm; B = 20 μm; C = 1 μm (inserts: 200 nm); F = 100 μm (for D–F); G,H = 100 μm. DAPI = 4′,6-diamidino-2-phenylindole; MAP2, microtubule-associated protein 2; NMDAR = N-methyl-D-aspartate receptor.

than non-NR1-binding antibodies (total, 9.0 ± 7.9 versus 19.1 ± 12.6 mean ± SD; p = 0.018, unpaired t test). Individual NR1 antibodies were even completely unmutated (#003-109) or contained only silent SHM (#007-142, #007-169).[7]

The germline antibody, #003-109, accounted for 1 of 41 (2.4%) of antibody-secreting cells analyzed of this patient and showed the characteristic NR1 pattern on unfixed mouse brain sections (Fig 1A) and the NMDAR cluster distribution on primary hippocampal mouse neurons previously observed for mutated NR1 antibodies[7] (Fig 1B). dSTORM of hippocampal neurons demonstrated NMDAR distribution at synapses, opposed to Homer1-positive postsynaptic densities (Fig C). Intravenous injection of #003-109 resulted in binding to cerebellar granule cells *in vivo* (Fig 1D), which was not detectable with the isotype control (Fig 1E). Intracerebroventricular injection of maturated (Fig 1G) and germline (Fig 1H) NR1 antibodies showed similar neuropil binding in the hippocampus.

Binding of Mutated, Germline, and Reverted Antibodies to NR1

Binding of #003-109 was prevented by the single-amino-acid mutation, N368Q, in the ATD of NR1 (Fig 2A,B).[7] To model the possible antibody/ATD interaction *in silico*, protein-protein docking was performed against ATD using ClusPro (Fig 2C). ATD residues N368 and G369 are

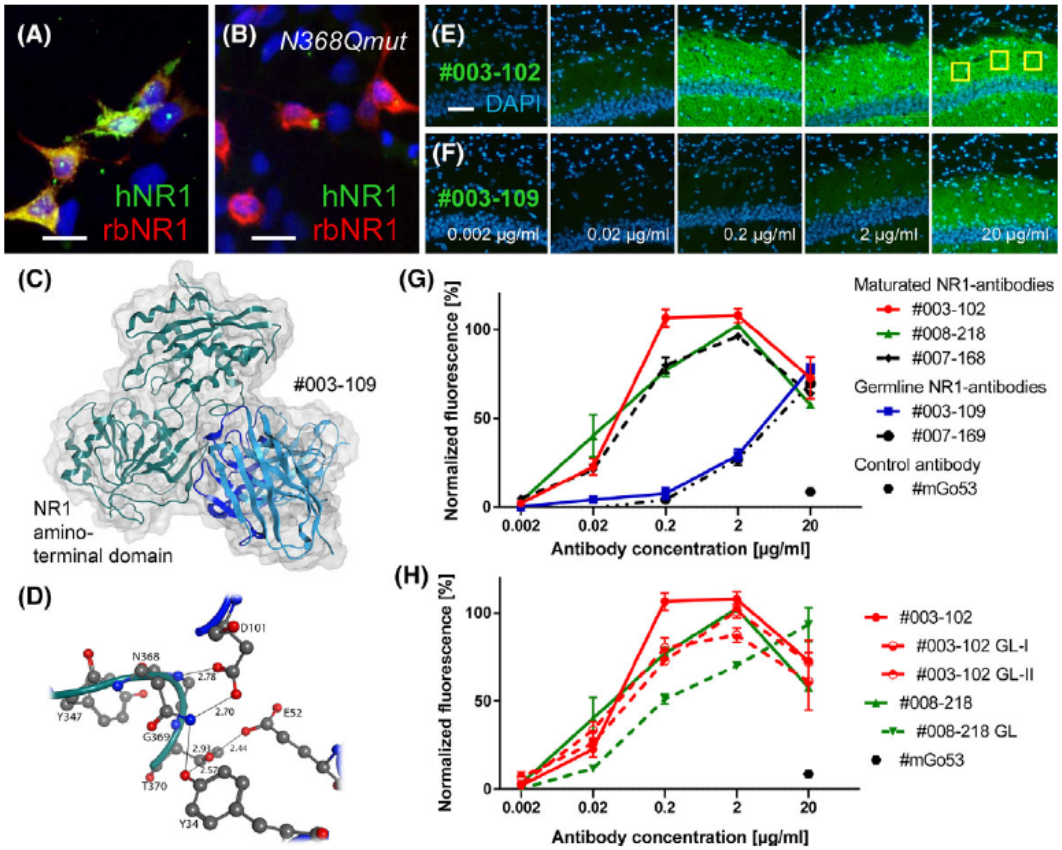

FIGURE 2: Interactions between germline antibodies and the NMDAR. Germline antibodies strongly bound to NR1 protein in transiently transfected HEK cells (A). In contrast, NR1 N368Q mutation completely omitted human antibody binding (B). Predicted binding pose of antibody #003-109 (blue, with complementarity determining regions in dark blue) to the ATD (green), the 3D model of the antibody, was generated by the antibody modeling tool of MOE2014.09 (C). Interaction of the key residues N368 and G369 in the H-bond network is illustrated in ball and stick mode; interactions are shown as black lines with the molecule distance in Å (D). Binding strengths with increasing concentrations of NR1 antibodies were determined by fluorescence intensities of hippocampal brain sections, exemplarily shown for a mutated (E) and germline antibody (F). Plotting these binding strengths against antibody concentrations showed the range of relative affinity curves of monoclonal patient-derived NR1 autoantibodies with weaker binding of the germline antibodies (G). "Back-mutation" of the high-affinity NR1 antibodies to germline configuration (*GL* = germline; two possible germline antibodies for #003-102) showed only minimal reduction of the binding strengths (H). Data are mean ± SEM, n = 3 independent stainings (G,H) each representing the mean of three hippocampal areas (yellow rectangles in E) per antibody/concentration. Scale bars: A,B = 20 µm; E = 100 µm (for E,F). DAPI = 4′,6-diamidino-2-phenylindole; hNR1/rbNR1 = human/rabbit NR1 antibody; NMDAR = N-methyl-D-aspartate receptor.

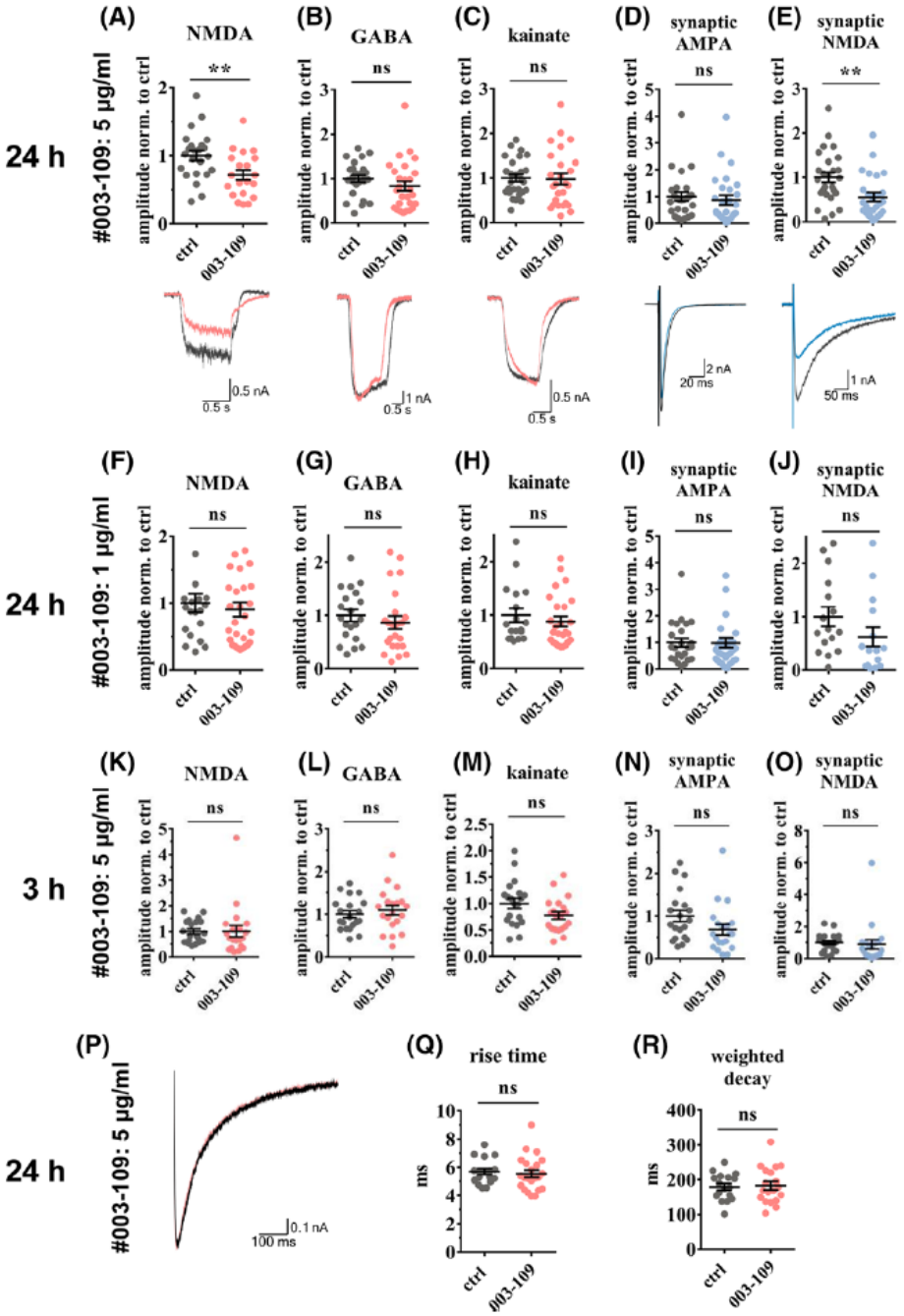

FIGURE 3: Germline antibody #003-109 reduced total and synaptic NMDA currents. Patch clamp whole-cell recordings of autaptic murine neuronal cultures showed that 24 hours of incubation with antibody #003-109 (5 µg/ml) selectively reduced total NMDA currents by 30% (A, $p = 0.009$), but not GABA or kainate currents (B,C), which were evoked by a 1-second bath application of 10 µM of NMDA (A), 5 µM of GABA (B), or 20 µM of kainate (C), respectively. Synaptic NMDA currents, evoked in the presence of 10 µM of glycine, 10 µM of NBQX, and $0Mg^{2+}$, showed 45% reduction (E, $p = 0.003$) whereas synaptic AMPA currents remained unchanged (D). The selective effect on NMDA currents was abolished at lower concentrations of antibody #003-109 (1 µg/ml; F–J). Total GABA and kainate and synaptic AMPA currents were not affected (G–I), while a trend toward reduced synaptic NMDA currents persisted at this antibody concentration (J, $p = 0.152$). Established concentrations of antibody #003-109 (5 µg/ml), but shorter incubation of 3 hours, were not sufficient to cause reduced NMDA currents (K–O). Averaged traces of exemplary cells incubated for 24 hours with germline and control antibodies (5 µg/ml) were scaled to 1 nA for easier comparison (P) and showed equal rise time (Q) and weighted decay (R) of synaptic NMDA currents. Data are mean ± SEM, Student's *t* test, n = 20 to 28 (A–E), n = 15 to 32 (F–J), or n = 20 (K–O) cells per group from four independent experiments. AMPA = alpha-amino propionic acid; ctrl = control; GABA = gamma-aminobutyric acid; NMDA = N-methyl-D-aspartate; ns = not significant.

embedded in the protein-protein interface forming a network of H-bonds with the antibody residues (Fig 2D). N368 is the only amino acid on the receptor side that stabilizes the binding to both, the antibody heavy and the light chains (Fig 2D). Binding curves of patient-derived germline NR1 antibodies showed generally lower relative affinity to hippocampal sections than mutated antibodies (Fig 2E–G). However, reverting mutated patient antibodies to germline changed the binding strengths only minimally, suggesting a similar functional role already of the naïve antibodies (Fig 2H).

Unmutated NR1 Antibodies Selectively Reduced Total and Synaptic NMDAR Currents

We expected smaller electrophysiological changes induced by antibody #003-109 compared to mutated NR1 antibodies, given the lower binding to murine brain (Fig 2E,F). Indeed, incubation of autaptic mouse hippocampal neurons with 5 μg/ml of germline antibody #003-109 for 24 hours resulted in ~30% reduction of the total NMDA currents compared to the isotype control antibody (Fig 3A). The antibody effect was specific, given that the total gamma-aminobutyric acid (GABA)- and kainate-mediated currents remained unaffected after application of 5 μM of GABA or 20 μM of kainate, respectively (Fig 3B,C). Measuring synaptic responses in the presence of 10 μM of glycine,10 μM of NBQX, and 0 mM of Mg^{2+}, NR1 antibody treatment also reduced synaptic NMDA currents by ~45%, while synaptic alpha-amino propionic acid (AMPA) currents did not differ from controls (Fig 3D,E). In contrast to higher-affinity mutated NR1 antibodies,[7] the effects were not detectable with lower antibody concentrations (1 μg/ml of #003-109; Fig 3F–J). In addition, shorter incubation for 3 hours with germline antibody was not sufficient to reduce synaptic or whole-cell NMDA currents (Fig 3K–O). The kinetics of synaptic NMDA responses were not altered by NR1 antibody treatment (Fig 3P–R).

Discussion

The present study followed the unexpected observation that human NR1 autoantibodies have low numbers of somatic hypermutations and that even germline-encoded, unmutated NR1 autoantibodies are found in patients with NMDAR encephalitis. Patient-derived germline antibodies had lower binding compared to mutated NR1 antibodies, but were also functional in selectively reducing synaptic NMDAR currents in a dose- and time-dependent manner. They should be present in the patient's CSF as clone #003-109 derived from a CSF plasma cell, which is believed to continuously produce several thousand IgG molecules per second.[15,16]

The finding of germline-configured functional NMDAR autoantibodies in the human repertoire might explain the mysterious observation of the high frequency of serum NMDAR antibodies in different diseases and blood donors.[17] Generally, B cells carrying high-affinity autoreactive antibodies undergo negative selection, while low-affinity antibodies might remain in the repertoire.[18] Thus, the here identified NR1 autoantibodies likely did not see their antigen during B cell development, or were of sufficiently low affinity to remain part of the naïve B cell repertoire,[19] and might therefore be present in every individual. The important role of naïve B cells in NMDAR encephalitis was recently suggested, although the experimental approach did not allow information on antibody mutations.[20] Likewise, autoreactive naïve B cells were recently observed in a related antibody-mediated disease, neuromyelitis optica.[21] It is still an open question how NMDAR-expressing tumors that might contain germinal center-like structures,[2,20,22,23] viral brain infections,[3,4] or additional factors lead to the maturation and expansion of NR1 antibody-producing cells in relatively rare cases, ultimately resulting in NMDAR encephalitis. In ovarian teratomas, tumor-intrinsic abnormalities, such as organized dysplastic neurons, may facilitate the development of NMDAR autoimmunity.[22] No clear distinction between the here examined germline and mutated antibodies was noted in patients without a tumor (#007, #008) compared to a patient with an ovarian carcinoma (#003).

Distinct unmutated ("naturally occurring") autoantibodies are innate-like components of the immune system that facilitate the clearance of invading pathogens, induce apoptosis in cancer cells, promote remyelination, or delay disease progression in murine models of inflammation and neurodegeneration.[24–26] May unmutated NMDAR autoantibodies have been similarly selected because of evolutionary importance (eg, for neutralization of released NMDAR protein), thereby preventing dysfunctional immune stimulation? Indeed, preexisting NMDAR antibodies were associated with smaller lesion size after stroke in one study, possibly related to reduction of glutamate-mediated excitotoxicity.[27] Also, there are examples of other germline antibodies that are reactive to commensal bacteria at mucosal barriers, but at the expense of pathogenic reactivity to self-proteins.[28]

Future studies should examine antibody effects beyond receptor internalization[29] and clarify under which conditions NR1 (and potentially further) autoantibody-producing cells escape negative selection and expand to cause encephalitis. They should also address whether and at which concentrations functional NMDAR autoantibodies are part of the healthy human naïve B cell repertoire and

may thus contribute to a broader spectrum of neuropsychiatric symptoms than previously assumed.

Acknowledgment

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Author Contributions

N.K.W., J.K., H.W., G.W., and H.P. contributed to the conception and design of the study. N.K.W., J.K., E.A., A.v.C., J.L., M.S.M., S.M.R., C.S., L.S., C.G., F.A., M.N., C.C.G., H.W., G.W., and H.P. contributed to the acquisition and analysis of data. N.K.W., J.K., E.A., A.v.C., M.S.M., S.M.R., L.S., C.G., H.W., G.W., and H.P. contributed to drafting the text and preparing the figures.

Potential Conflicts of Interest

Nothing to report.

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Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

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