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POUR OBTENIR LE GRADE DE

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Par Daniel HUNTER

**A CHARACTERISATION OF HIPPOCAMPAL
PATHOPHYSIOLOGY IN AUTOIMMUNE ENCEPHALITIS:
FROM SYNAPSE TO CIRCUIT**

Sous la direction de : Laurent GROC

Soutenue le 11 octobre 2023

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PATHOPHYSIOLOGY IN AUTOIMMUNE
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FROM SYNAPSE TO CIRCUIT

Submitted for the Degree of Doctor of Philosophy

On Wednesday 11th October 2023

by

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A

ABSTRACTS & SUMMARIES

Abstract

NMDAR and GABA_AR encephalitides are immune-mediated neurological syndromes, in which the expression of pathogenic autoantibodies (Abs), directed against either NMDARs or GABA_ARs respectively, results in complex disease presenting with seizures and neuropsychiatric complications. Molecular investigations have delineated the actions of patient-derived Abs on their target antigens, whereby NMDAR and GABA_AR Abs ultimately drive respective hypofunctions of major excitatory or inhibitory ion channels. Paradoxically however, despite this converse action of disease-causing Abs, preclinical investigations have demonstrated that both NMDAR and GABA_AR Abs induce hyperexcitation of neuronal networks. As such, we aimed to characterise a broader-scale functional impact of autoantibody actions, at synaptic, cellular and circuit-levels, to finely elucidate the mechanisms by which both NMDAR and GABA_AR Abs elicit a hyper-excitable seizure phenotype in clinical settings.

We observed that 24-hour autoantibody exposure, irrespective of the target antigen, reduced the amplitude of both spontaneous excitatory and inhibitory postsynaptic currents. Intriguingly, the magnitude of reduction in inhibition was consistently greater, suggesting that the synaptic input onto pyramidal cells becomes unbalanced in favour of hyperexcitation. Examination of spontaneous current kinetics, and immunocytochemistry experiments further suggest these alterations are underpinned by a displacement of both AMPARs and GABA_ARs from their respective postsynaptic compartments. Further, imaging and multiplicity analysis revealed that both pathogenic Abs drive a selective depletion of inhibitory synaptic scaffolding protein, gephyrin, in a phosphorylation-associated manner. However, excitatory synaptic scaffold, homer-1c, remained intact after exposure to Abs, further supporting a shift towards synaptic hyperexcitation, despite overall depression of synaptic inputs.

To understand how these aberrant synaptic inputs are functionally integrated into hippocampal network activity we subsequently characterised a range of intrinsic cellular properties of excitatory and inhibitory hippocampal populations. We observed that GABA_AR Abs uniquely disrupt the excitatory cell populations, driving an increase in the resting membrane potential and an increased action potential output in response to current injection. Further investigations suggested this increase in excitability may be underpinned by the loss of tonic GABAergic inhibition and/or modulation of axonal GABA_AR channel conductance. Additionally, we uncovered a unique impact of NMDAR Abs on the inhibitory cell populations, whereby intrinsic excitability is

significantly reduced. In this case, exposure to NMDAR Abs results in hyperpolarisation of interneuron resting potentials and reduced action potential output. This is in line with a classical disinhibitory model, in which potent inhibitory control over hippocampal networks is abolished, leading to destabilised hyperfunction of the local excitatory cell population. Cell-attached recordings and calcium imaging experiments further provided support of these findings, in which we observed hyperactivation of excitatory cell populations, after exposure to either pathogenic Ab, and a unique hypersynchronous state after NMDAR Ab exposure. Taken together, these findings are indicative of two discreet disease models whereby synaptic inputs are similarly dysregulated but differentially gated by shifts in intrinsic cellular properties, resulting in an overall similar hyperexcitable phenotype at the scale of hippocampal network function. Ultimately, our work has expanded on the current knowledge of pathological mechanisms involved in two autoimmune encephalitic diseases and helps to identify key nodes of convergence and divergence in these two conditions, lending some explanation towards the similar phenotypic presentations observed at the clinical scale.

Full Summary

NMDAR encephalitis and GABA_AR encephalitis are two autoimmune disorders that share similar clinical symptoms despite different underlying mechanisms. In NMDAR encephalitis, autoantibodies cause a reduction in the expression and localization of NMDARs, leading to a decrease in excitatory function and altered network connectivity. On the other hand, GABA_AR encephalitis involves the direct antagonism of GABA_ARs by autoantibodies, resulting in a decrease in inhibitory function. Despite these distinct mechanisms, both diseases exhibit common symptoms such as seizures, cognitive dysfunction, and autonomic instability. However, NMDAR encephalitis is also associated with neuropsychiatric symptoms resembling schizophrenia, which are rarely observed in GABA_AR encephalitis cases. The mechanisms underlying the neuropsychiatric symptoms in NMDAR encephalitis are not fully understood. It is hypothesized that the autoantibodies may have distinct impacts on inhibitory interneuron populations in the hippocampus, which play a crucial role in regulating overall circuit behaviour. As such, interneuron dysfunction may contribute to the psychotic symptoms observed in NMDAR encephalitis. Furthermore, interneurons in the hippocampus are more reliant on NMDARs for excitatory synaptic transmission compared to pyramidal cells. This higher reliance, along with the presence of specific NMDAR subunits, makes interneurons more susceptible to the effects of NMDAR autoantibodies. The disruption of excitatory drive onto inhibitory networks can lead to local disinhibition and widespread epileptiform activity. Overall, while NMDAR encephalitis and GABA_AR encephalitis have different underlying mechanisms, their clinical manifestations show considerable overlap. Understanding the shared and distinct mechanisms of these diseases is crucial for developing targeted therapeutic interventions.

The primary objective of this thesis is to understand the functional mechanisms behind hippocampal pathophysiology caused by NMDAR and GABA_AR autoantibodies. The study aims to investigate synaptic, cellular, and network deficits induced by autoantibody actions in hippocampal cell preparations. The first aim involves examining the synaptic phenotype by analysing excitatory and inhibitory synaptic activity using electrophysiology and immunohistochemistry. The second aim focuses on the cellular phenotype by studying the intrinsic excitability of CA1 hippocampal neurons and their response to NMDAR and GABA_AR autoantibodies. The third aim explores the network phenotype by assessing the overall network activity and synchronicity of cell activations in hippocampal circuits exposed to autoantibodies. The ultimate objective is to gain a comprehensive understanding of the impact of NMDAR and GABA_AR autoantibodies on hippocampal function, shedding light on the mechanisms underlying autoimmune encephalitis and its associated symptoms.

Early studies on the molecular pathogenicity of patient autoantibodies were limited by the availability of samples, such as serum and cerebrospinal fluid. Patient samples contain a combination of different autoantibodies with varying targets, making it challenging to determine the precise pathogenicity of specific autoantibodies. However, advances in recombinant monoclonal antibody production have allowed

researchers to generate patient-replicated monoclonal autoantibodies in unlimited quantities. Using these monoclonal antibodies avoids the presence of unknown factors in patient samples and allows for precise control of the concentration of pathogenic immunoglobulins. We have employed two pathogenic autoantibodies in our investigations: one targeting the NMDAR and the other targeting the GABA_AR. These monoclonal antibodies were derived from patient samples and extensively characterized in previous studies. The anti-NMDAR monoclonal antibody binds to the GluN1 subunit of the receptor, affecting its clustering at postsynaptic compartments and reducing NMDAR-mediated synaptic currents. The anti-GABA_AR monoclonal antibody binds to α 1-containing GABA_ARs and induces a reduction in channel function and synaptic displacement of the receptors. The use of these monoclonal autoantibodies in research allows for the replication of the disease state and provides a controlled environment for studying the pathogenic mechanisms of autoimmune encephalitis. While monoclonal antibodies may not fully replicate the complexity of polyclonal disease states, they offer greater replicability and precise characterization of the effects of autoantibodies on specific receptor targets. Ultimately, monoclonal autoantibodies serve as valuable tools for studying autoimmune encephalitis. They provide a controlled and reproducible system for investigating the pathogenic effects of autoantibodies on neuronal proteins, shedding light on disease mechanisms and potential therapeutic approaches.

In Chapter II we aimed to evaluate the quality and suitability of the human-derived anti-NMDAR monoclonal autoantibody, #003-102, for investigating antibody-mediated pathology in autoimmune encephalitis. We performed immunocytochemical and histochemical analyses to determine its reactivity to the neuronal surface, specificity for the target, and its impact on astrocytes and microglia in organotypic hippocampal cultures. The results showed that the antibody bound to hippocampal neurons, correlated with NMDAR expression, and met pre-set criteria for maintained binding and specificity. Further, we found that it did not cause immune activation of astrocytic and microglial cells in the organotypic culture system. However, we acknowledge that the possibility of cytokine release by autoantibodies without altering cell morphology and immune marker expression cannot be ruled out, suggesting the need for further investigation. Based on these findings, we determined that a concentration of 0.5 μ g/ml and a 24-hour exposure time of the antibody were appropriate for the planned experimental work in *in vitro* hippocampal preparations.

Given the array of homeostatic systems in place, each dependent on effective crosstalk between excitatory and inhibitory synaptic signalling, the mechanistic underpinnings of autoimmune epileptogenesis may reside in pathological co-regulation of excitatory and inhibitory neurotransmission. We would expect NMDAR and GABA_AR autoantibodies to elicit downstream perturbations across both neurotransmission systems, inhibitory and excitatory. However, the characterisation of this cross-synaptic disruption remains to be fully investigated. In Chapter III we hypothesise that antibody-mediated interference of NMDAR or GABA_AR organisation and function will metastasise into global dysregulation of ionotropic synaptic signalling, impacting glutamatergic

and GABAergic neurotransmission. Investigations into the co-regulation of glutamatergic and GABAergic systems, in the context of autoimmune encephalitis, may provide novel avenues for therapeutic development and intervention for these disease states. Moreover, identification of EI co-regulation in these diseases may further expand our knowledge regarding the interaction between excitatory and inhibitory systems in the maintenance of hippocampal homeostasis on a more fundamental level. In this Chapter we aimed to investigate the synaptic effects of NMDAR and GABA_AR autoantibodies on excitatory and inhibitory neurotransmission. This demonstrated that both types of autoantibodies led to a reduction in AMPAR-mediated currents, indicating a decrease in excitatory synaptic transmission. We also observed a moderate decrease in the localization of AMPARs at postsynaptic sites. Interestingly, the reduction in current magnitude was greater than the depletion of synaptic AMPARs, suggesting possible alterations in the nanoscale organization of these receptors. Additionally, both types of autoantibodies caused a substantial reduction in GABA_AR-mediated currents, indicating a decrease in inhibitory synaptic transmission. This reduction was expected in the case of GABA_AR autoantibodies but was also observed with NMDAR autoantibodies. The findings suggest that autoantibodies targeting single neuronal antigens can impact synaptic function through multiple mechanisms, affecting both excitatory and inhibitory synaptic activity.

Additionally, we further investigated the balance between spontaneous excitatory and inhibitory synaptic inputs, finding that the balance was shifted towards hyperexcitation despite the overall reduction in both excitation and inhibition. Moreover, we exposed CA1 principal cells to autoantibodies and recorded excitatory and inhibitory synaptic currents in the absence of action potential-driven activity. In these experiments we found that NMDAR autoantibodies reduced AMPAR-mediated excitatory inputs, while GABA_AR autoantibodies did not substantially alter miniature excitatory postsynaptic current (mEPSC) amplitudes. However, we observed a reduction in the frequency of miniature inhibitory postsynaptic current (mIPSC) events, after exposure to either pathogenic autoantibody samples. This data was used to model the synaptic multiplicity, as a measure of the degree of connectivity between principal cells, and interneurons-to-principal cells. This data suggested that neither type of autoantibody altered the density of excitatory synapses, but both caused a downregulation of inhibitory synapses on the CA1 pyramidal neurons. To investigate the molecular mechanisms underlying synaptic destabilization, we then examined the role of excitatory and inhibitory synaptic scaffolding proteins, homer-1c and gephyrin, respectively. We found no significant impact on the excitatory synaptic scaffolds; however, incubation with autoantibodies in the absence of astrocytes prevented the reduction of synaptically localised AMPARs, suggesting that astrocytes may play a role in the pathogenic alterations induced by autoantibodies. Additionally, we also observed a decrease in the density of inhibitory synaptic scaffolding protein, gephyrin, which was mediated by hyperphosphorylation of a specific serine residue, S270. Interestingly, similar levels of phosphorylation and degradation of inhibitory synaptic scaffolds

were observed with both GABA_AR and NMDAR autoantibodies, indicating a common substrate for pathogenicity in autoimmune encephalitis, regardless of the specific target antigens.

While we observed significant perturbations to both excitatory and inhibitory synaptic phenotypes, after incubation with NMDAR and GABA_AR autoantibodies, we hypothesised that such autoantibodies may impact the functional properties of extrasynaptic ion channels involved in neuronal excitability. While reductions in tonic GABAergic inhibition might be expected in conditions of reduced inhibitory synaptic activity, evidence suggests that loss of synaptic inhibition during status epilepticus either does not alter – or alternatively, can increase – tonic GABAergic conductance. Moreover, a large body of literature has demonstrated that GABA_ARs located at the axon initial segment can influence intrinsic neuronal function and action potential generation. Regarding NMDAR autoantibodies, they may reduce tonic excitatory currents, particularly in inhibitory neuronal populations. The presence of magnesium-insensitive GluN2D-containing NMDARs on inhibitory interneurons contributes to their reliance on tonically active channels. This disinhibitory mechanism has been implicated in psychotomimetic effects and rapid antidepressant effects. The text speculates that NMDAR autoantibodies may decrease tonic excitatory currents, potentially leading to both seizure and psychosis phenotypes. Together, providing a solid basis for the possibility that action of NMDAR and GABA_AR autoantibodies, targeting non-synaptic receptor populations, may strongly modulate the intrinsic excitability of hippocampal neurons. To investigate these ideas, in Chapter IV, we performed whole-cell current clamp and cell-attached voltage-clamp recordings on CA1 pyramidal neurons and inhibitory interneurons to measure action potential output, intrinsic excitability, and spontaneous activity without intracellular manipulation. Our results showed that GABA_AR autoantibodies increased the excitability of pyramidal neurons, leading to increased action potential output, depolarization of resting membrane potential, decreased rheobase and spike latency, and increased input resistance. In contrast, NMDAR autoantibodies did not have any significant effect on these parameters. The loss of tonic GABAergic inhibition on principal cells was observed, which may promote cellular hyperactivity and depolarization of the resting potential. Further, GABA_AR autoantibodies, uniquely, were found to alter action potential waveforms, reducing amplitude and prolonging duration, possibly by affecting the function of voltage-gated sodium and potassium channels. Conversely, NMDAR autoantibodies selectively reduced GABAergic neuronal excitability, leading to a negative shift of input-output plots, hyperpolarization of resting potential, and increased rheobase. The mechanism by which these autoantibodies exert their effects is not fully understood, but strongly suggests the modulation of extrasynaptic GABA_AR and NMDARs. Cell-attached voltage-clamp experiments further demonstrated that inhibitory cellular outputs were silenced after exposure to NMDAR autoantibodies, while excitatory neuron activity increased after exposure to either autoantibody. Taken together, these findings suggest that the combined effects of synaptic input alterations and changes in cellular excitability contribute to pyramidal cell hyperexcitation in hippocampal circuits.

Given our prior results, and the knowledge that seizure phenotypes are highly prevalent in both NMDAR and GABA_AR encephalitis, we hypothesised that autoantibody actions would converge to induce hyperactivity in hippocampal networks. To address this hypothesis in Chapter V, we conducted calcium imaging experiments on cultured hippocampal slices exposed to NMDAR and GABA_AR autoantibodies, aiming to examine their impact on network activity. Given the substantial role of the CA1 hippocampal subfield in memory processing and cognitive dysfunction, as well as its association with epilepsy, we focussed primarily on dysfunction within this subfield. We also note that the use of multi-electrode array (MEA) recording techniques to further study network activity has led to some disparity in previous reports, specifically regarding the impact of NMDAR autoantibodies on dissociated network activity. We suggest that both autoantibodies are likely to induce hyperexcitability at the network level, with NMDAR autoantibodies possibly affecting synchronicity through their specific impact on GABAergic interneurons. Our experiments using calcium imaging revealed that both autoantibodies lead to hyperactivity in CA1 pyramidal neurons, indicating a hyperactive network state. As such, we expect that this antibody-mediated hyperactivation is likely due to altered synaptic inputs favouring hyperexcitation and selective impacts on cellular excitability. We further propose that destabilization and hyperactivation of hippocampal networks could be a key factor in seizure generation in patients with autoimmune encephalitis. The hippocampus is widely associated with various cognitive functions, and hippocampal hyperactivation may result in deficits in cognitive processing. As such, we considered the importance of fine coordination in the activity of CA1 output cells for healthy cognitive processes. We uncovered a selective alteration by NMDAR autoantibodies, leading to a hypersynchronous network state. With this data, it is tempting to speculate that dysfunction in inhibitory interneurons and reduced feedback and lateral inhibition could be linked to the development of hypersynchronous hippocampal discharges. We further suggest that future work may aim to characterise the impact of autoantibodies on specific subpopulations of GABAergic interneurons. Additionally, we also performed MEA recordings on dissociated hippocampal cultures and found that NMDAR autoantibodies increased network activity, while GABA_AR autoantibodies did not have a noticeable effect. The lack of consistency between the results of calcium imaging and MEA recordings may be attributed to high degree of variability – inherent in MEA datasets – or may reflect hippocampal circuit architecture-dependent impacts of autoantibodies. Overall, we emphasize the role of NMDAR and GABA_AR autoantibodies in altering hippocampal network activity and highlight the potential implications for seizures, cognitive dysfunction, and synaptic plasticity.

Résumé

Les encéphalites à NMDAR et à GABAAR sont des syndromes neurologiques à médiation immunitaire qui se manifestent par des crises d'épilepsie et des complications neuropsychiatriques. Les études moléculaires ont mis en évidence une voie d'action des auto-anticorps dérivés des patients, par laquelle les NMDAR et les GABAAR sont transloqués hors des compartiments synaptiques, puis internalisés. Paradoxalement, cette diminution de l'expression de surface des canaux ioniques excitateurs ou inhibiteurs entraîne une hyperexcitation des réseaux neuronaux. Nous visons à caractériser l'impact fonctionnel à plus grande échelle de l'action des anticorps, aux niveaux synaptique, cellulaire et du circuit, afin d'élucider finement les mécanismes par lesquels les auto-anticorps NMDAR et GABAAR provoquent un phénotype de crise hyperexcitable dans des contextes cliniques.

En utilisant une série de techniques électrophysiologiques de patch-clamp, nous avons examiné les perturbations synaptiques induites par les actions indirectes des auto-anticorps NMDAR et GABAAR. Il s'agit d'un dysfonctionnement des entrées excitatrices et inhibitrices dans les cellules de l'hippocampe. En outre, nous avons élargi le modèle de pathogenèse de la maladie pour inclure l'altération de la fonctionnalité des interneurons GABAergiques dans les réseaux hippocampiques, en caractérisant les changements dans l'excitabilité cellulaire intrinsèque de populations discrètes de cellules hippocampiques.

Nous avons effectué des enregistrements électrophysiologiques par voltage-clamp à cellules entières de neurones pyramidaux CA1 dans des tranches organotypiques d'hippocampe afin d'étudier la neurotransmission spontanée au niveau des synapses excitatrices et inhibitrices. Nous avons démontré que l'exposition à des auto-anticorps NMDAR et GABAAR réduit l'amplitude des courants postsynaptiques médiés par AMPAR et GABAAR (sEPSC et sIPSC, respectivement). De manière intrigante, les réductions relatives observées dans les amplitudes des sIPSC des cellules exposées aux auto-anticorps NMDAR et GABAAR étaient plus importantes que celles observées dans les sEPSC. Les déficits plus prononcés de la neurotransmission inhibitrice suggèrent que, tout compte fait, les entrées synaptiques dans les cellules pyramidales sont déséquilibrées en faveur d'une hyperexcitation. Nous avons également examiné la cinétique de montée et de descente des sEPSC et sIPSC restants, ne révélant aucune altération de la forme d'onde des courants synaptiques, ce qui suggère une perte d'AMPA- et de GABAARs dans leurs compartiments postsynaptiques respectifs.

Pour comprendre comment ces entrées synaptiques aberrantes sont fonctionnellement intégrées dans l'activité du réseau hippocampique, nous avons ensuite étudié les populations de cellules hippocampiques à la recherche d'altérations de l'excitabilité intrinsèque. Ici, nous avons utilisé une technique de courant-clamp pour caractériser les altérations possibles à travers une gamme de propriétés cellulaires intrinsèques sur les populations excitatrices et inhibitrices. Nous avons montré que les auto-anticorps GABAAR perturbent

uniquement la population de cellules excitatrices, en provoquant une augmentation du potentiel de membrane au repos, ce qui entraîne une propension accrue à déclencher des potentiels d'action en réponse à l'injection de courant. Cette augmentation du potentiel de repos peut être étayée par la perte de l'inhibition tonique GABAergique sur ces cellules, connue pour contribuer à l'excitabilité intrinsèque dynamique, que nous avons également observée comme étant abolie après exposition aux auto-anticorps GABAAR. De plus, nous avons démontré un impact unique des anticorps NMDAR sur les populations de cellules inhibitrices, par lequel l'excitabilité intrinsèque est significativement réduite. Dans ce cas, l'exposition aux auto-anticorps NMDAR semble hyperpolariser le potentiel de membrane au repos des interneurons GABAergiques, réduisant ainsi leur potentiel d'action. Ce phénomène est conforme à un modèle désinhibiteur classique, dans lequel un contrôle inhibiteur puissant sur les réseaux hippocampiques est aboli, ce qui entraîne une activité hyperexcitable déstabilisée dans la population de cellules excitatrices.

L'ensemble de ces résultats indique l'existence de deux modèles pathologiques distincts dans lesquels les entrées synaptiques sont dérégulées et gérées de manière différentielle par des propriétés cellulaires intrinsèques à l'échelle de la fonction du réseau hippocampique. En fin de compte, notre travail a permis d'élargir les connaissances actuelles sur les mécanismes pathologiques impliqués dans deux maladies encéphaliques auto-immunes, et aide à identifier les principaux nœuds de convergence dans ces deux conditions, ce qui explique en partie les présentations phénotypiques similaires observées à l'échelle clinique.

Résumé complet de la thèse

L'encéphalite à récepteurs NMDA et l'encéphalite à récepteurs GABAA sont deux maladies auto-immunes qui présentent des symptômes cliniques similaires malgré des mécanismes sous-jacents différents. Dans l'encéphalite à récepteurs NMDA, les auto-anticorps provoquent une réduction de l'expression et de la localisation des récepteurs NMDA, ce qui entraîne une diminution de la fonction excitatrice et une altération de la connectivité du réseau. En revanche, l'encéphalite des récepteurs GABAA implique l'antagonisme direct des récepteurs GABAA par les auto-anticorps, ce qui entraîne une diminution de la fonction inhibitrice. Malgré ces mécanismes distincts, les deux maladies présentent des symptômes communs tels que des crises d'épilepsie, un dysfonctionnement cognitif et une instabilité autonome. Cependant, l'encéphalite des récepteurs NMDA est également associée à des symptômes neuropsychiatriques ressemblant à la schizophrénie, qui sont rarement observés dans les cas d'encéphalite des récepteurs GABAA. Les mécanismes qui sous-tendent les symptômes neuropsychiatriques de l'encéphalite des récepteurs NMDA ne sont pas entièrement compris. On suppose que les auto-anticorps peuvent avoir des impacts distincts sur les populations d'interneurones inhibiteurs dans l'hippocampe, qui jouent un rôle crucial dans la régulation du comportement du circuit global. Ainsi, le dysfonctionnement des interneurones pourrait contribuer aux symptômes psychotiques observés dans l'encéphalite à récepteur NMDA. En outre, les interneurones de l'hippocampe dépendent davantage des récepteurs NMDA pour la transmission synaptique excitatrice que les cellules pyramidales. Cette dépendance plus élevée, ainsi que la présence de sous-unités spécifiques du récepteur NMDA, rendent les interneurones plus sensibles aux effets des auto-anticorps antirécepteurs NMDA. La perturbation de l'entraînement excitateur sur les réseaux inhibiteurs peut conduire à une désinhibition locale et à une activité épileptiforme généralisée. Globalement, si l'encéphalite des récepteurs NMDA et l'encéphalite des récepteurs GABAA ont des mécanismes sous-jacents différents, leurs manifestations cliniques se chevauchent considérablement. La compréhension des mécanismes communs et distincts de ces maladies est cruciale pour le développement d'interventions thérapeutiques ciblées.

L'objectif principal de cette thèse est de comprendre les mécanismes fonctionnels à l'origine de la pathophysiologie de l'hippocampe causée par les auto-anticorps des récepteurs NMDA et GABAA. L'étude vise à étudier les déficits synaptiques, cellulaires et de réseau induit par les actions des auto-anticorps dans les préparations de cellules hippocampiques. Le premier objectif consiste à examiner le phénotype synaptique en analysant l'activité synaptique excitatrice et inhibitrice à l'aide de l'électrophysiologie et de l'immunohistochimie. Le deuxième objectif se concentre sur le phénotype cellulaire en étudiant l'excitabilité intrinsèque des neurones hippocampiques CA1 et leur réponse aux auto-anticorps des récepteurs NMDA et GABAA. Le troisième objectif explore le phénotype du réseau en évaluant l'activité globale du réseau et la synchronisation des activations cellulaires dans les circuits hippocampiques exposés aux auto-anticorps.

L'objectif final est d'acquérir une compréhension globale de l'impact des auto-anticorps des récepteurs NMDA et GABAA sur la fonction hippocampique, afin de mettre en lumière les mécanismes sous-jacents à l'encéphalite auto-immune et aux symptômes qui lui sont associés.

Les premières études sur la pathogénicité moléculaire des auto-anticorps des patients étaient limitées par la disponibilité des échantillons, tels que le sérum et le liquide céphalo-rachidien (LCR). Les échantillons de patients contiennent une combinaison de différents auto-anticorps dont les cibles varient, ce qui rend difficile la détermination de la pathogénicité précise d'auto-anticorps spécifiques. Cependant, les progrès réalisés dans la production d'anticorps monoclonaux recombinants ont permis aux chercheurs de générer des auto-anticorps monoclonaux répliqués par les patients en quantités illimitées. L'utilisation de ces anticorps monoclonaux évite la présence de facteurs inconnus dans les échantillons de patients et permet un contrôle précis de la concentration des immunoglobulines pathogènes. Nous avons utilisé deux auto-anticorps pathogènes dans nos recherches : l'un ciblant le récepteur NMDA et l'autre ciblant le récepteur GABAA. Ces anticorps monoclonaux proviennent d'échantillons de patients et ont été largement caractérisés dans des études antérieures. L'anticorps monoclonal antirécepteur NMDA se lie à la sous-unité GluN1 du récepteur, ce qui affecte son regroupement dans les compartiments postsynaptiques et réduit les courants synaptiques médiés par le récepteur NMDA. L'anticorps monoclonal anti-récepteur GABAA se lie aux récepteurs GABAA contenant l' $\alpha 1$ et induit une réduction de la fonction du canal et un déplacement synaptique des récepteurs. L'utilisation de ces auto-anticorps monoclonaux dans la recherche permet de reproduire l'état pathologique et fournit un environnement contrôlé pour l'étude des mécanismes pathogènes de l'encéphalite auto-immune. Bien que les anticorps monoclonaux ne puissent pas reproduire entièrement la complexité des états pathologiques polyclonaux, ils offrent une plus grande reproductibilité et une caractérisation précise des effets des auto-anticorps sur des cibles réceptrices spécifiques. En fin de compte, les auto-anticorps monoclonaux sont des outils précieux pour l'étude de l'encéphalite auto-immune. Ils constituent un système contrôlé et reproductible pour étudier les effets pathogènes des auto-anticorps sur les protéines neuronales, ce qui permet de mettre en lumière les mécanismes de la maladie et les approches thérapeutiques potentielles.

Dans le chapitre II, nous avons cherché à évaluer la qualité et l'adéquation de l'auto-anticorps monoclonal antirécepteur NMDA d'origine humaine, #003-102, pour étudier la pathologie médiée par les anticorps dans l'encéphalite auto-immune. Nous avons effectué des analyses immunocytochimiques et histochimiques pour déterminer sa réactivité à la surface neuronale, sa spécificité pour la cible et son impact sur les astrocytes et la microglie dans des cultures organotypiques d'hippocampe. Les résultats ont montré que l'anticorps se liait aux neurones de l'hippocampe, qu'il était en corrélation avec l'expression des récepteurs NMDA et qu'il répondait aux critères préétablis de maintien de la liaison et de la spécificité. En outre, nous avons constaté

qu'il ne provoquait pas d'activation immunitaire des cellules astrocytaires et microgliales dans le système de culture organotypique. Cependant, nous reconnaissons que la possibilité d'une libération de cytokines par les auto-anticorps sans modification de la morphologie cellulaire et de l'expression des marqueurs immunitaires ne peut être exclue, ce qui suggère la nécessité d'une étude plus approfondie. Sur la base de ces résultats, nous avons déterminé qu'une concentration de 0,5 µg/ml et une durée d'exposition de 24 heures de l'anticorps étaient appropriées pour le travail expérimental prévu dans les préparations hippocampiques in vitro.

Compte tenu de l'ensemble des systèmes homéostatiques en place, chacun dépendant d'une diaphonie efficace entre la signalisation synaptique excitatrice et inhibitrice, les fondements mécaniques de l'épileptogènes auto-immune pourraient résider dans la corégulation pathologique de la neurotransmission excitatrice et inhibitrice. On pourrait s'attendre à ce que les anticorps anti-NMDAR et anti-GABAAR provoquent des perturbations en aval dans les deux systèmes de neurotransmission, inhibiteur et excitateur. Cependant, la caractérisation de cette perturbation intersynaptique n'a pas encore fait l'objet d'une étude approfondie. Dans le chapitre III, nous émettons l'hypothèse que l'interférence de l'organisation et de la fonction des récepteurs NMDA ou GABAA à l'aide d'anticorps se métastase en un dérèglement global de la signalisation synaptique ionotropique, ayant un impact sur la neurotransmission glutamatergique et GABAergique. L'étude de la corégulation des systèmes glutamatergiques et GABAergiques, dans le contexte de l'encéphalite auto-immune, peut fournir de nouvelles pistes pour le développement thérapeutique et l'intervention dans ces états pathologiques. En outre, l'identification de la corégulation de l'IE dans ces maladies pourrait élargir nos connaissances sur l'interaction entre les systèmes excitateurs et inhibiteurs dans le maintien de l'homéostasie de l'hippocampe à un niveau plus fondamental. Dans ce chapitre, nous avons cherché à étudier les effets synaptiques des auto-anticorps des récepteurs NMDA et GABAA sur la neurotransmission excitatrice et inhibitrice. Nous avons démontré que les deux types d'auto-anticorps entraînaient une réduction des courants médiés par les récepteurs AMPA, ce qui indique une diminution de la transmission synaptique excitatrice. Nous avons également observé une diminution modérée de la localisation des récepteurs AMPA dans les sites postsynaptiques. Il est intéressant de noter que la réduction de l'amplitude du courant était plus importante que la déplétion des récepteurs AMPA synaptiques, ce qui suggère des altérations possibles de l'organisation de ces récepteurs à l'échelle nanométrique. En outre, les deux types d'auto-anticorps ont provoqué une réduction substantielle des courants médiés par les récepteurs GABAA, ce qui indique une diminution de la transmission synaptique inhibitrice. Cette réduction était attendue dans le cas des auto-anticorps anti-récepteurs GABAA, mais elle a également été observée avec les auto-anticorps anti-récepteurs NMDA. Ces résultats suggèrent que les auto-anticorps ciblant des antigènes neuronaux

uniques peuvent avoir un impact sur la fonction synaptique par le biais de mécanismes multiples, affectant à la fois l'activité synaptique excitatrice et l'activité synaptique inhibitrice.

En outre, nous avons étudié l'équilibre entre les entrées synaptiques spontanées excitatrices et inhibitrices, et nous avons constaté que l'équilibre était déplacé vers l'hyperexcitation malgré la réduction globale de l'excitation et de l'inhibition. En outre, nous avons exposé les cellules principales CA1 à des auto-anticorps et enregistré des courants synaptiques excitateurs et inhibiteurs en l'absence d'activité induite par un potentiel d'action. Au cours de ces expériences, nous avons constaté que les auto-anticorps des récepteurs NMDA réduisaient les entrées excitatrices médiées par les récepteurs AMPA, tandis que les auto-anticorps des récepteurs GABAA ne modifiaient pas de manière substantielle les amplitudes des courants postsynaptiques excitateurs miniatures (mEPSC). Cependant, nous avons observé une réduction de la fréquence des événements de courant postsynaptique inhibiteur miniature (mIPSC), après exposition à l'un ou l'autre des échantillons d'auto-anticorps pathogènes. Ces données ont été utilisées pour modéliser la multiplicité synaptique, en tant que mesure du degré de connectivité entre les cellules principales et les interneurons vers les cellules principales. Ces données suggèrent qu'aucun type d'auto-anticorps ne modifie la densité des synapses excitatrices, mais qu'ils provoquent tous deux une régulation à la baisse des synapses inhibitrices sur les neurones pyramidaux CA1. Pour étudier les mécanismes moléculaires sous-jacents à la déstabilisation synaptique, nous avons ensuite examiné le rôle des protéines d'échafaudage synaptique excitatrices et inhibitrices, l'homer-1c et la géphyrine, respectivement. Nous n'avons pas trouvé d'impact significatif sur les échafaudages synaptiques excitateurs ; cependant, l'incubation avec des auto-anticorps en l'absence d'astrocytes a empêché la réduction des récepteurs AMPA localisés au niveau synaptique, ce qui suggère que les astrocytes peuvent jouer un rôle dans les altérations pathogènes induites par les auto-anticorps. En outre, nous avons également observé une diminution de la densité de la protéine d'échafaudage synaptique inhibitrice, la géphyrine, qui était médiée par l'hyperphosphorylation d'un résidu sérine spécifique, S270. Il est intéressant de noter que des niveaux similaires de phosphorylation et de dégradation des échafaudages synaptiques inhibiteurs ont été observés avec les auto-anticorps des récepteurs GABAA et NMDA, ce qui indique un substrat commun pour la pathogénicité dans l'encéphalite auto-immune, indépendamment des antigènes cibles spécifiques.

Alors que nous avons observé des perturbations significatives des phénotypes synaptiques excitateurs et inhibiteurs, après incubation avec des auto-anticorps des récepteurs NMDA et GABAA, nous avons émis l'hypothèse que ces auto-anticorps pourraient avoir un impact sur les propriétés fonctionnelles des canaux ioniques extrasynaptiques impliqués dans l'excitabilité neuronale. Alors que l'on pourrait s'attendre à des réductions de l'inhibition tonique GABAergique dans des conditions de réduction de l'activité synaptique

inhibitrice, les données suggèrent que la perte d'inhibition synaptique pendant l'état de mal épileptique n'altère pas - ou au contraire peut augmenter - la conductance tonique GABAergique. En outre, de nombreuses publications ont démontré que les récepteurs GABAA situés au niveau du segment initial de l'axone peuvent influencer la fonction neuronale intrinsèque et la génération du potentiel d'action. En ce qui concerne les auto-anticorps antirécepteurs NMDA, ils peuvent réduire les courants toniques excitateurs, en particulier dans les populations neuronales inhibitrices. La présence de récepteurs NMDA insensibles au magnésium et contenant le GluN2D sur les interneurons inhibiteurs contribue à leur dépendance à l'égard des canaux toniques actifs. Ce mécanisme de désinhibition a été impliqué dans les effets psychotomimétiques et les effets antidépresseurs rapides. Le texte émet l'hypothèse que les auto-anticorps des récepteurs NMDA peuvent diminuer les courants excitateurs toniques, ce qui pourrait entraîner des phénotypes de crises et de psychoses. L'ensemble fournit une base solide pour la possibilité que l'action des auto-anticorps des récepteurs NMDA et GABAA, ciblant des populations de récepteurs non synaptiques, puisse moduler fortement l'excitabilité intrinsèque des neurones de l'hippocampe. Pour étudier ces idées, au chapitre IV, nous avons effectué des enregistrements par pince à courant à cellule entière et par pince à tension à cellule attachée sur des neurones pyramidaux CA1 et des interneurons inhibiteurs pour mesurer la sortie du potentiel d'action, l'excitabilité intrinsèque et l'activité spontanée sans manipulation intracellulaire. Nos résultats ont montré que les auto-anticorps des récepteurs GABAA augmentaient l'excitabilité des neurones pyramidaux, entraînant une augmentation du potentiel d'action, une dépolarisation du potentiel de membrane au repos, une diminution de la rhéobase et de la latence des pointes, ainsi qu'une augmentation de la résistance à l'entrée. En revanche, les auto-anticorps des récepteurs NMDA n'ont pas eu d'effet significatif sur ces paramètres. La perte de l'inhibition tonique GABAergique sur les cellules principales a été observée, ce qui peut favoriser l'hyperactivité cellulaire et la dépolarisation du potentiel de repos. En outre, il a été constaté que les auto-anticorps dirigés contre les récepteurs GABAA modifiaient les formes d'onde du potentiel d'action, en réduisant l'amplitude et en prolongeant la durée, peut-être en affectant la fonction des canaux sodiques et potassiques voltage-dépendants. Inversement, les auto-anticorps anti-récepteurs NMDA ont sélectivement réduit l'excitabilité neuronale GABAergique, entraînant un décalage négatif des courbes entrée-sortie, une hyperpolarisation du potentiel de repos et une augmentation de la rhéobase. Le mécanisme par lequel ces auto-anticorps exercent leurs effets n'est pas entièrement compris, mais il suggère fortement la modulation des récepteurs GABAA et NMDA extrasynaptiques. Des expériences de voltage-clamp sur cellules ont en outre démontré que les sorties cellulaires inhibitrices étaient réduites au silence après exposition aux auto-anticorps antirécepteurs NMDA, tandis que l'activité excitatrice des neurones augmentait après exposition à l'un ou l'autre des auto-anticorps. L'ensemble de ces résultats suggère que les effets combinés des altérations des entrées synaptiques et des changements dans l'excitabilité cellulaire contribuent à l'hyperexcitation des cellules pyramidales dans les circuits hippocampiques.

Compte tenu de nos résultats antérieurs et du fait que les phénotypes épileptiques sont très répandus dans les encéphalites des récepteurs NMDA et GABAA, nous avons émis l'hypothèse que les actions des auto-anticorps convergeraient pour induire une hyperactivité dans les réseaux hippocampiques. Pour répondre à cette hypothèse dans le chapitre V, nous avons mené des expériences d'imagerie calcique sur des tranches d'hippocampe en culture exposées à des auto-anticorps antirécepteurs NMDA et GABAA, afin d'examiner leur impact sur l'activité des réseaux. Étant donné le rôle important du sous-champ hippocampique CA1 dans le traitement de la mémoire et le dysfonctionnement cognitif, ainsi que son association avec l'épilepsie, nous nous sommes principalement concentrés sur le dysfonctionnement de ce sous-champ. Nous notons également que l'utilisation de techniques d'enregistrement par réseaux multi-électrodes (MEA) pour approfondir l'étude de l'activité du réseau a entraîné une certaine disparité dans les rapports précédents, notamment en ce qui concerne l'impact des auto-anticorps anti-récepteurs NMDA sur l'activité du réseau dissocié. Nous suggérons que les deux auto-anticorps sont susceptibles d'induire une hyperexcitabilité au niveau du réseau, les auto-anticorps des récepteurs NMDA pouvant affecter la synchronisation par leur impact spécifique sur les interneurons GABAergiques. Nos expériences utilisant l'imagerie calcique ont révélé que les deux auto-anticorps entraînent une hyperactivité dans les neurones pyramidaux CA1, ce qui indique un état hyperactif du réseau. Ainsi, nous pensons que cette hyperactivation médiée par les anticorps est probablement due à des entrées synaptiques modifiées favorisant l'hyperexcitation et des impacts sélectifs sur l'excitabilité cellulaire. Nous proposons en outre que la déstabilisation et l'hyperactivation des réseaux hippocampiques soient un facteur clé dans la survenue des crises chez les patients atteints d'encéphalite auto-immune. L'hippocampe est largement associé à diverses fonctions cognitives, et l'hyperactivation de l'hippocampe peut entraîner des déficits dans le traitement cognitif. C'est pourquoi nous avons étudié l'importance d'une coordination fine de l'activité des cellules de sortie de CA1 pour des processus cognitifs sains. Nous avons découvert une altération sélective par les auto-anticorps des récepteurs NMDA, conduisant à un état hypersynchrone du réseau. Avec ces données, il est tentant de spéculer que le dysfonctionnement des interneurons inhibiteurs et la réduction de la rétroaction et de l'inhibition latérale pourraient être liés au développement de décharges hippocampiques hypersynchrones. Nous suggérons en outre que les travaux futurs visent à caractériser l'impact des auto-anticorps sur des sous-populations spécifiques d'interneurones GABAergiques. En outre, nous avons également effectué des enregistrements de réseaux multi-électrodes (MEA) sur des cultures d'hippocampes dissociées et avons constaté que les auto-anticorps des récepteurs NMDA augmentaient l'activité du réseau, tandis que les auto-anticorps des récepteurs GABAA n'avaient pas d'effet perceptible. Le manque de cohérence entre les résultats de l'imagerie calcique et les enregistrements MEA peut être attribué au degré élevé de variabilité - inhérent aux ensembles de données MEA - ou peut refléter les impacts des auto-anticorps dépendant de l'architecture du circuit hippocampique. Dans l'ensemble, nous soulignons le rôle des auto-anticorps des récepteurs NMDA et GABAA dans l'altération de

l'activité du réseau hippocampique et mettons en évidence les implications potentielles pour les crises, les dysfonctionnements cognitifs et la plasticité synaptique.

I

INTRODUCTION

Excitatory-Inhibitory Balance and Homeostasis in Healthy and Diseased Brain Networks

Throughout the central nervous system, homeostatic activity levels across diverse neuronal populations are maintained within strict physiological control via a fine-tuned balance between excitatory drive and an opposing inhibitory control. The precise maintenance of this excitation-inhibition balance is crucial for healthy brain function, and its dysregulation has been implicated in a wide array of neurological and neuropsychiatric disorders, including epilepsy and schizophrenia (Fritschy, 2008; Gao and Penzes, 2015; Dehghani *et al.*, 2016; Y. Liu *et al.*, 2021). The developmental trajectory of excitation-inhibition balance is varied throughout the lifespan: where early life is associated with a high degree of local synchronicity and reduced long-range neuro-communication; however, transition into the mature state through adolescence and adulthood is associated with a reversal of these parameters, whereby local bursts of excitation become desynchronised in favour of long-range and high-precision connections in complex whole brain architecture (Bonifazi *et al.*, 2009; Kirkby *et al.*, 2013; Griguoli and Cherubini, 2017; Caballero, Orozco and Tseng, 2021). This intricate co-modulation of excitation and inhibition, both throughout neurodevelopment and homeostatic plasticity, is indicative of the variety of endogenous neurological mechanisms that are interconnected with this physiological process. A greater understanding of the multiple components that comprise excitation-inhibition balance – including synaptic or *phasic* neurotransmission, and extra synaptic or *tonic* modulations – is necessary before we can adequately apply models of its dysfunction to neurological disease states. Ultimately, excitation-inhibition balance is a highly complex phenomenon, which is developmentally and homeostatically regulated across a range of synaptic and cellular functions.

Interneuron Development and Maintenance of Excitation-Inhibition Balance in the Healthy Brain State

Although representing a minority of neuronal cells, conservatively estimated between 10-20%, GABAergic interneurons exert potent inhibitory control over whole brain activity (Gentet, Stuart and Clements, 2000; Kim *et al.*, 2017; Keller, Erö and Markram, 2018; Lecumberri *et al.*, 2018). This function of local inhibitory modulation is particularly crucial for controlling self-recurrent circuitry, such as those found in microcircuits of the trisynaptic pathway between hippocampus and entorhinal cortex (Maccaferri, 2011; Hu, Gan and Jonas, 2014; Paz and Huguenard, 2015). It has been demonstrated that pharmacologically relieving hippocampal

circuits of local GABAergic control elicits spontaneous seizure generation, largely driven by recurrent and reciprocal principal cell connections in the CA3 subfield (Miles and Wong, 1983) (Figure 1A-C). Conversely however, benzodiazepine-enhanced receptivity to endogenous GABA has been shown to disrupt the formation of hippocampal-dependent memory (McNaughton and Morris, 1987). Evidently, bidirectional perturbation of inhibitory potency over hippocampal network function is pathogenic in nature. Hence, the integrity of hippocampal interneuron function is required to maintain a healthy brain state, within a narrow margin of physiological activity. An additional layer of complexity is provided by the great diversity in interneuron subtypes, which must act in concert with one another to correctly orchestrate hippocampal function (Booker and Vida, 2018).

The stratification of interneuron subpopulations is based on the expression of molecular markers, premigrational origin, morphology and axonal projection targets (Danglot, Triller and Marty, 2006) (Figure 1D). The fine targeting of inhibitory axonal projections to principal cells, at subcellular precision, is exemplified by the *basket* and *chandelier* interneuron subtypes. The former selectively form synapses located on cell bodies and perisomatic dendrites, while the latter provides inhibitory innervation of the axon initial segment (Kosaka, 1983; Somogyi *et al.*, 1983; Cajal *et al.*, 1995). This intricate feature of interneuron axonal morphology generates distinct cellular populations that hold varying forms of inhibitory control over the excitatory cell network. For example, a high density of somatic inhibitory synapses provides potent inhibitory effects to intercede with somatic depolarisation, whereas inhibitory innervation of the axon initial segment is well placed to disrupt action potential propagation after somatic depolarisation has occurred (Boivin and Nedivi, 2018). Moreover, distally located inhibitory synapses, such as those formed by somatostatin-positive interneurons, are suited to modulate local calcium spiking activity in neighbouring excitatory synapses (Miles *et al.*, 1996).

The inhibitory control exerted over principal cell networks is also tightly coupled to the intrinsic firing properties of interneuron subgroups. Many GABAergic basket cells express the calcium-binding protein parvalbumin, and display high-frequency action potential spike trains (Pelkey *et al.*, 2017). This high-frequency firing property, coupled with the somatically targeted inhibition pattern, allows precise time-dependent inhibition of principal cell action potential generation, and is known to underpin the development of synchronous network oscillatory activity (Dasgupta and Sikdar, 2019; Antonoudiou *et al.*, 2020). However, other parvalbumin-negative basket cells have been identified with different firing properties, which express the neuropeptide cholecystokinin (CCK) (Kawaguchi and Kondo, 2002). Some calretinin-expressing interneuron subtypes have been identified, which selectively form inhibitory connections with other inhibitory cells (Chung and Moore, 2009). It has further been demonstrated that parvalbumin-positive basket cells also innervate other inhibitory basket cells (Deleuze, Pazienti and Bacci, 2014). Consideration of interneuron-to-interneuron connectivity yields a more complex relationship between excitation and inhibition, since global inhibitory reductions may differentially impact principal cells and other inhibitory networks alike. As such, the

physiological mechanisms that maintain a homeostatic balance of inhibitory-excitatory network activity must effectively co-regulate across these complex circuit dynamics (Lourenço, Koukoulis and Bacci, 2020).

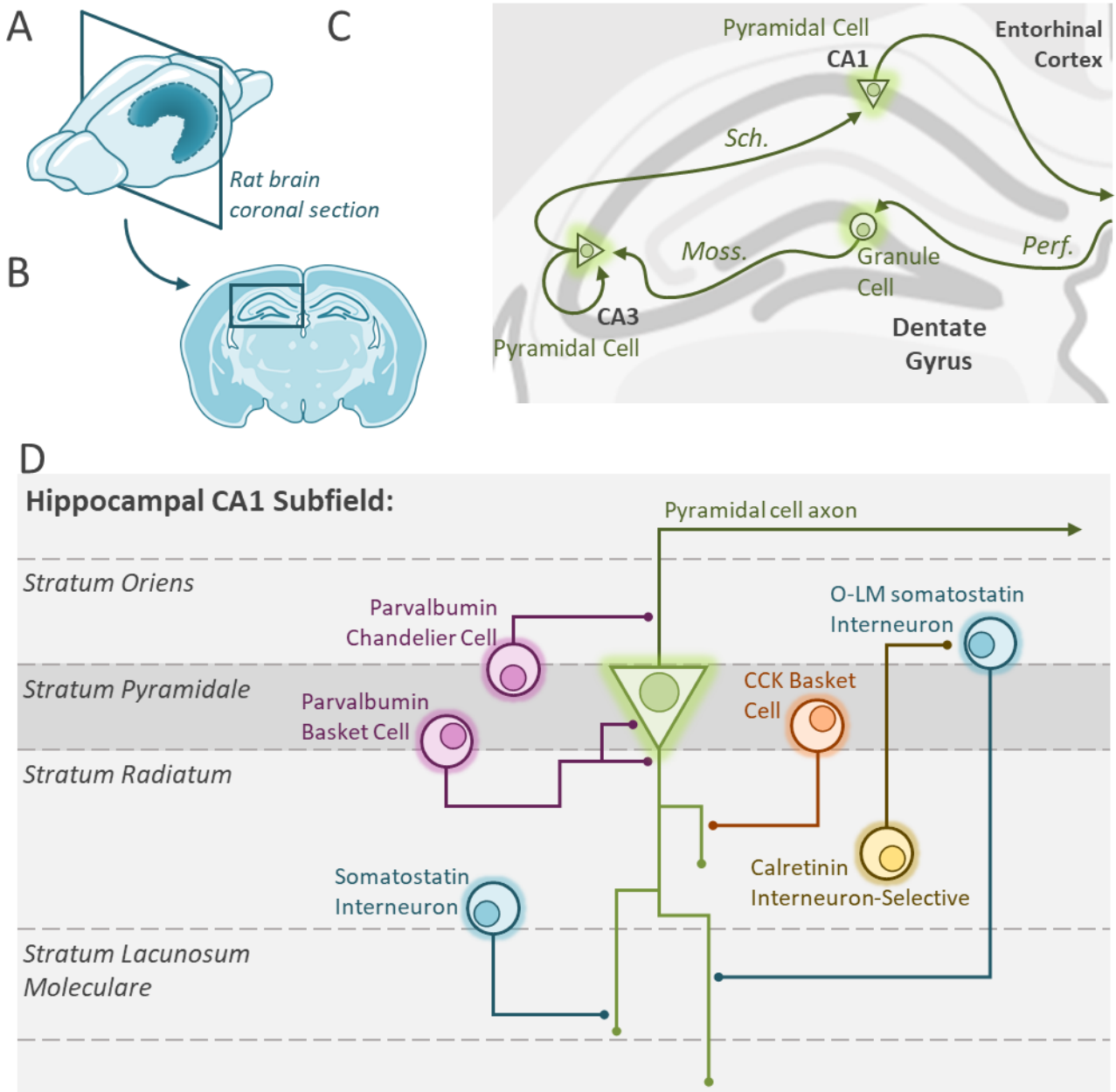


Figure 1: Diversity of Inhibitory Hippocampal Networks.

(A) Schematic of rodent hippocampal formation and (B) coronal section. (C) Schematic representation of the hippocampal trisynaptic pathway: The perforant pathway (*Perf.*) is formed of axons originating from the entorhinal cortex and projecting to the dentate gyrus. Mossy fibres (*Moss.*) from the dentate gyrus project to the CA3 region, and synapse with neurons of the Schaffer collaterals (*Sch.*) projecting onto the CA1. Pyramidal neurons in the CA1 complete the circuit, projecting back to the entorhinal cortex. (D) Schematic representation of interneuron diversity of the CA1 hippocampal subfield illustrating typical interneuronal connectivity with a pyramidal neuron.

The canonical view of homeostatic control over network activity is that it serves to modulate neuronal firing rates within a set physiological range (Turrigiano, 2012). Regulation of net neuronal firing rates within distinct brain regions permits global information processing throughout the brain (Chen *et al.*, 2022). This is achieved at the synaptic and cellular scales through modulations of excitatory and inhibitory synaptic potency, and also

dynamic fluctuations in intrinsic cellular excitability (Turrigiano, 2012; Cea-Del Rio *et al.*, 2020; Niemeyer, Schleimer and Schreiber, 2021; Trojanowski, Bottorff and Turrigiano, 2021; Rathour and Kaphzan, 2023). Effective excitation-inhibition balance is therefore achieved by a synergistic relationship between synaptic and cellular processes, working in parallel to one another. The governance of synaptic inputs is achieved through multiple co-regulatory mechanisms of inhibitory and excitatory synapses, including synaptogenesis, plasticity and pruning (Cline, 2005; Kirischuk, 2022). Whereas cellular outputs are modulated through shifts in intrinsic excitability, which adjust the computational response of individual neurons to altering synaptic inputs (Rutecki, 1992; Ohtsuki, 2020; Gonzalez, Losonczy and Negrean, 2022; Chancey *et al.*, 2023). The proper functioning of these two regulatory mechanisms is fundamental to conserving healthy brain function and effective information processing (London and Häusser, 2005; Carasatorre, Ramírez-Amaya and Díaz Cintra, 2016; Dunn and Kaczorowski, 2019; Price and Duman, 2020; Daou and Margoliash, 2021).

Homeostatic regulation of neuronal firing has been well established for many years, it has been shown that bidirectionally augmenting the activity of cultured hippocampal networks will result in the network autonomously returning to a baseline state within a matter of hours (Turrigiano *et al.*, 1998; Burrone, O'Byrne and Murthy, 2002). This can be achieved through a global modulation of excitatory synapses throughout the network to counterbalance the generalised shift in network activity, a process termed *synaptic scaling* (Turrigiano, 2012). Electrophysiological investigations have revealed that during synaptic scaling, neurons uniformly adjust the expression level of both NMDA- and AMPA receptors at excitatory postsynaptic compartments, while maintaining the relative differences in transmission strength between synapses (Desai *et al.*, 2002; Pérez-Otaño and Ehlers, 2005; Gainey *et al.*, 2009). This type of globalised synaptic plasticity has also been observed in organotypic hippocampal slices and also in *in vivo* networks, suggesting that it is likely an important physiological mechanism contributing to network homeostasis in living mammalian organisms (Kim and Tsien, 2008; Knogler, Liao and Drapeau, 2010).

Notably however, homeostatic regulation of neuronal activity is not a purely glutamatergic system of maintenance, but also incorporates inhibitory network modulation. In fact, synaptic scaling has more recently been shown to occur also at inhibitory synapses (Joseph and Turrigiano, 2017). It has also been demonstrated that directed pharmacological downregulation of GABAergic signalling, disinhibits neuronal networks leading to an increase in overall firing at the acute timescales (Miles and Wong, 1983), and firing rates under similar disinhibitory conditions also return to baseline after hours of chronic exposure (Corner and Ramakers, 1992). However, this type of generalised down-scaling of GABAergic inhibition is ineffective at compensating for large fluctuations in network activity (Eichler and Meier, 2008). For example, in rodent models of alcoholism, chronic alcohol administration induced ethanol-mediated potentiation of GABAergic transmission; subsequent withdrawal from alcohol consumption reduced the seizure threshold in experimental animals (Kokka *et al.*, 1993; Cagetti, Baicy and Olsen, 2004). Suggesting that compensatory downregulation of the GABAergic

system, in response to a transient perturbation of the network, puts the system at high risk of rebound excitation after removal of the modifying stimulus (Eichler and Meier, 2008). Interestingly however, seizure is a major health risk associated with alcohol withdrawal in humans which can be treated with the use of benzodiazepines (Koh *et al.*, 2021), supportive of the notion that ethanol-mediated GABAergic synaptic scaling may also play a role in this, and potentially other, human neuropathologies.

Implications of Excitation-Inhibition Imbalance in Epilepsy and Seizure States

Expectantly, seizures are an intuitive hallmark symptom associated with an underlying imbalance of excitatory and inhibitory systems (Bonansco and Fuenzalida, 2016). Although the specific underlying mechanisms of complex seizure phenotypes are known to be vastly heterogeneous in nature, neurological disorders associated with prominent and repetitive seizure phenotypes are typically associated together under the umbrella term of *epilepsy* (Pitkänen *et al.*, 2015; Liu *et al.*, 2018). As such, diverse human epileptic disorders have been extensively studied to elucidate the various potential routes towards network imbalance, including avenues to pathologically enhanced glutamatergic signalling, reduced GABAergic potency and altered intrinsic excitability of either excitatory or inhibitory cell populations (Chapman, 1998; Armijo *et al.*, 2005, 2005; de Curtis and Avoli, 2016; Khazipov, 2016; Williamson, Singh and Kapur, 2019; Wang *et al.*, 2021; Whitebirch *et al.*, 2022).

Epileptogenic roots of cellular hyperexcitation can be underpinned by tonic shifts in excitatory or inhibitory membrane conductance or phasic changes in synaptic transmission (Tasker and Dudek, 1991; Casillas-Espinosa, Powell and O'Brien, 2012; Owen, Bichler and Benveniste, 2021; Goisis *et al.*, 2022). In recent decades, the advances made in genetic sequencing technologies have permitted significant insights regarding genetic causes of various epileptic diseases (Symonds, Zuberi and Johnson, 2017; Perucca and Perucca, 2019). This is illustrated by the rapidly growing number of identified monogenic causes of human epilepsy, for instance, the GRIN Portal database collated by the Broad Institute currently contains 239 seizure causing mutations in NMDA receptor subunit-coding genes alone. Of those with functional characterisations performed, many appear to be gain-of-function mutations (Strehlow *et al.*, 2019; X. Chen *et al.*, 2020). Taking the example of the *GRIN2A* missense mutation, c.2434C>A, resulting in a methionine substitution in place of a highly conserved leucine residue in the M4 region of the GluN2A subunit (GluN2A-L812M), this mutation results in a profound gain-of-function phenotype with prolonged current decay kinetics in the resulting NMDA receptor ion channel (Pierson *et al.*, 2014). As one may expect, this mutation is associated with a severe epileptic encephalopathy, with comorbid developmental delay (Pierson *et al.*, 2014). This clinical phenotype is due to the substantial increase in synaptic excitatory drive, which then overwhelms the capacity of the inhibitory network, leading to a shift towards synaptically driven hyperexcitation. Similarly, epilepsy-causing

loss-of-function mutations have been identified in GABA_A receptor subunit-coding genes, such as the missense mutation K289M of the $\gamma 2$ subunit, which results in a rapid decay of inhibitory postsynaptic currents (Baulac *et al.*, 2001; Krivoshein and Hess, 2006). Such a reduction in GABAergic neurotransmission also yields an overall shift in excitation-inhibition balance, towards synaptic hyperexcitation.

The manifestation of seizures can also be elicited through an alteration of the intrinsic excitability of neurons and increasing the action potential output relative to typical synaptic inputs (Koch *et al.*, 2005; Meisel *et al.*, 2015; Nadella, Ghosh and Chu, 2022). Such a mechanism has recently been observed in the CA2 subfield of the hippocampus, in a widely used model of temporal lobe epilepsy, pilocarpine-induced *status epilepticus* (Whitebirch *et al.*, 2022). It has been shown that pilocarpine administration increases the intrinsic excitability of CA2 pyramidal neurons, albeit through an – as yet – unidentified mechanism (Whitebirch *et al.*, 2022). However, an increase in input resistance of these cells was also observed which may provide a possible route towards heightened cellular outputs in response to synaptic depolarisations (Whitebirch *et al.*, 2022). Intriguingly, while shifts in tonic glutamatergic and GABAergic conductance are known to induce alterations of intrinsic neuronal excitability, there is a wealth of experimental evidence suggesting that tonic inhibitory GABA_A receptor-mediated signalling is maintained or enhanced across many experimental models of epileptic disorders (Walker and Kullmann, 2012; Pavlov and Walker, 2013; Riebe *et al.*, 2016; Kammel *et al.*, 2018; Debanne and Russier, 2019; Pandit *et al.*, 2020). While it remains unclear why reductions in tonic GABA signalling are not observed in across common disease models, in fact often demonstrated to be increased – opposing the shift towards cellular hyperexcitation – it may be the case that tonic GABAergic conductance through extrasynaptic GABA_A receptors represents a major homeostatic regulatory tool in epileptogenic disease states (Walker and Kullmann, 2012). For this reason, the tonic GABAergic system has been a primary focus for the development of anti-epileptic drugs, with the aim of enhancing tonic inhibition as a means to restore healthy brain function (Davies, 1995; Deidda, Crunelli and Di Giovanni, 2021).

However, while the preceding sections have focussed on cell-autonomous pathways towards excitation-inhibition imbalance, it is essential to note that seizures are inherently network-based phenomena. Over recent years, multiple counter-intuitive routes towards seizure generation have been identified. For instance, in Dravet syndrome – a severe treatment-resistant epileptic disorder – is commonly underpinned by a loss-of-function mutation in the voltage-gated sodium channel, Nav1.1 (Catterall, Kalume and Oakley, 2010). Such a mutation would be predicted to cause a brain-wide reduction in action potential generation, through a reduced propensity for membrane depolarisation, essentially shifting excitatory-inhibitory balance in favour of *hypo*-excitability and away from a seizure-promoting network state. However, it has been demonstrated that this Nav1.1-induced hypoexcitability is predominantly observed in GABAergic cell populations, due to the substantially enriched expression of the voltage-gated channel in inhibitory neurons (Catterall, Kalume and Oakley, 2010; Yamakawa, 2011; Qiao *et al.*, 2013). Therefore, when placed in the context of physiological brain

networks, this modification of intrinsic cellular excitability results in a selective silencing of inhibitory networks and reduced inhibitory control of excitatory neuron populations, a mechanism termed *disinhibition*.

Implications of Excitation-Inhibition Imbalance in Neuropsychiatric Disorders

Beyond epilepsy, cortical and hippocampal disinhibition has also been strongly implicated in the development of neuropsychiatric disturbances, including schizophrenia (Heckers and Konradi, 2010; Gilani *et al.*, 2014; McGarrity *et al.*, 2017; Williams *et al.*, 2022). Post-mortem investigations into human brain tissue from schizophrenic patients has revealed a selective deficit of parvalbumin-expressing inhibitory interneurons in both cortical and hippocampal brain regions (Benes and Berretta, 2001; Pietersen *et al.*, 2014; Dienel and Lewis, 2019). Further, lower levels of GABA have been observed in the cerebrospinal fluid of patients displaying a range of psychiatric disturbance, including schizophrenia, bipolar disorder, major depressive disorder and anorexia nervosa (Lichtshtein *et al.*, 1978; Gerner and Hare, 1981; Gerner *et al.*, 1984; Orhan *et al.*, 2018; Mousten *et al.*, 2022). Remarkably, the magnitude of GABA reduction in the CSF was found to be predictive of disease severity in a cohort of patients enduring first episode psychosis (Orhan *et al.*, 2018). This would suggest that excitation-inhibition misbalance, underpinned by alterations in GABAergic inhibitory neurotransmission, represents a central mechanism of pathogenesis across a diverse array of human psychiatric conditions. Although there is a consistent body of evidence for reduced GABAergic transmission in schizophrenia, the impacts on glutamatergic function are substantially more elusive (Hoshino, 2013; Xu and Wong, 2018; Jun *et al.*, 2022; Wiera *et al.*, 2022). While early investigations suggested that levels of ambient glutamate were also diminished in schizophrenia (Kim *et al.*, 1980; Tsai *et al.*, 1995), a recent meta-analysis – collating reports of approximately 300 schizophrenic patients and matched healthy controls – has revealed a significant increase in peripheral glutamate concentrations (Song *et al.*, 2014). It is possible that this heterogeneity in reporting is due to a complex interaction between excitatory and inhibitory systems that is variable over the time course of psychiatric illness, and variably modulated by therapeutic intervention. It is therefore imperative to develop a greater understanding of the intricate relationship between excitatory and inhibitory systems throughout the brain, and to characterise the complex interplay between glutamatergic and GABAergic signalling across the synaptic, cellular and network levels of neuronal function in both the healthy and the disease state.

In line with a glutamatergic deficit being an underlying pathogenic avenue to the development of psychosis, dysfunction of the glutamatergic NMDA receptor has also been specifically implicated in psychiatric disorders (Wang *et al.*, 2004; Kristiansen *et al.*, 2007; Guo *et al.*, 2009). Specifically, reduced expression NMDA receptor subunit, GluN1, and its associated mRNA levels has been demonstrated in post-mortem samples from schizophrenic patients (Catts *et al.*, 2016); However it should be noted that this finding does remain somewhat controversial (Hu *et al.*, 2015). Yet, induction of membrane expression deficits in rodent models, for instance

by genetic manipulation of *SynGAP* – an NMDA receptor-associated synaptic-protein – successfully recapitulates some behavioural elements of psychiatric disturbance observed in humans (Guo *et al.*, 2009). In fact, a surprisingly effective and commonly used rodent model of schizophrenia involves the simple administration of NMDA receptor antagonists, such as MK-801 and ketamine (Eyjolfsson *et al.*, 2006; Frohlich and Van Horn, 2014; Abram *et al.*, 2022). Antagonism of the NMDA receptor alone is sufficient to induce typical clinical characteristics of schizophrenia, spanning across the positive, negative and cognitive symptom domains, and this has been demonstrated in both rodent and human experimental designs (Krystal *et al.*, 2005; Gunduz-Bruce, 2009; Neill *et al.*, 2010; Cadinu *et al.*, 2018; Lee *et al.*, 2020). Recent explorations into the precise role of NMDA receptor dysfunction in antagonist-based models of psychosis indicate that the NMDA receptor population on GABAergic interneurons is the key locus of pathogenesis, whereby abolition of a tonic NMDA receptor-mediated current on inhibitory interneurons drives a disinhibition of pyramidal cells which display a reduced sensitivity to pharmacological antagonism (Homayoun and Moghaddam, 2007; Jones, Corbin and Huntsman, 2014; Cohen *et al.*, 2015; Nakazawa and Sapkota, 2020). In fact, it has also been observed that chronic administration of lead (Pb^{2+}), a potent NMDA receptor antagonist, also recapitulates the selective loss of parvalbumin-expressing inhibitory interneurons in the brain of adolescent rats, a feature commonly observed in human tissue samples from schizophrenic patients (Stansfield *et al.*, 2015).

Expanding on this perspective of glutamatergic involvement in the development of schizophrenia, AMPA receptor expression has also been found to be significantly reduced in hippocampi obtained from post-mortem brain samples (Meador-Woodruff and Healy, 2000; Yonezawa *et al.*, 2022). Intriguingly, while reports regarding changes in AMPA receptor expression outside of the hippocampus have been greatly heterogeneous, reductions in hippocampal expression appear a remarkably consistent by contrast (Freed, Dillon-Carter and Kleinman, 1993; Noga *et al.*, 1997; Healy *et al.*, 1998). Taken together with findings regarding NMDA receptor perturbations, it is clear from these findings that aberrant glutamatergic neurotransmission in the hippocampus, in the form of both NMDA and AMPA receptor dysfunction, plays a pivotal role in the manifestation of psychosis across neuropsychiatric conditions.

While there is consensus regarding the causative role of hippocampal disinhibition on memory and learning deficits commonly observed in schizophrenic patients, less is understood regarding the possible hippocampal contributions to other clinical elements of the disease (Bast, Zhang and Feldon, 2001; Bast, Pezze and McGarrity, 2017; McGarrity *et al.*, 2017; Modinos *et al.*, 2018; Beck *et al.*, 2022; Williams *et al.*, 2022). However, data from rodent models have suggested that the disinhibited hippocampal outputs can elicit pathological brain activity in a plethora of downstream projection sites, causing a greater variety of behavioural and cognitive alterations (McGarrity *et al.*, 2017; Leung and Ma, 2022). This view implicates the hippocampus as a central hub for pathogenesis in neuropsychiatric conditions, both for typically hippocampus-

dependant processes such as memory and learning; but also in further non-hippocampal behavioural functions including prefrontal-dependant cognition, attention and psychosis (Leung and Ma, 2018).

Subsection Summary

The maintenance of a healthy brain state is critically dependant on the fine-tuned dynamics of excitatory and inhibitory networks. The complex development of excitatory and inhibitory cell populations is initiated during early neurodevelopmental stages, in which the GABAergic synaptogenesis dominates over the lagged developmental trajectory of the glutamatergic networks (Tyzio *et al.*, 1999; Akerman and Cline, 2006). During these early stages however, GABAergic signalling is excitatory rather than inhibitory, owed to high intracellular chloride concentrations relative to the extracellular environment (Cherubini *et al.*, 2011; Deidda *et al.*, 2015; Salmon *et al.*, 2020). Following the *postnatal GABA switch*, in which the release of GABA from presynaptic terminals system begins to elicit *hyper*-polarisation – resulting in the true emergence of an effective inhibitory network – excitatory glutamatergic synaptogenesis begins to mature in parallel, and typical patterns of local burst excitation are observed in hippocampal networks (Akerman and Cline, 2007). As excitatory-inhibitory connections are refined, this local hypersynchronous burst activity is reduced, and sporadic asynchronous firing patterns begin to arise (Menendez de la Prida, Bolea and Sanchez-Andres, 1996; Valeeva *et al.*, 2019; Graf *et al.*, 2022). These multifaceted phases of development are all made possible by the intricate interplay between a balanced glutamatergic and GABAergic systems; and perturbation of either network is substantially pathogenic, causing marked shifts in excitation-inhibition balance yields a variety of disease states from cognitive deficits, intellectual disability, epilepsy and neuropsychiatric disease.

The past decades of research into both epileptic pathologies and diverse neuropsychiatric diseases have converged on a dysfunction of excitatory-inhibitory balance, with the hippocampus as a particularly striking node of pathogenesis. There are many identified routes to epileptogenic hyperexcitability, including increased synaptic excitatory drive by genetic mutations in glutamatergic ion channels (Strehlow *et al.*, 2019; Sun *et al.*, 2021; Vieira, Jeong and Roche, 2021; X.-R. Liu *et al.*, 2021; Coombs *et al.*, 2022; Elmasri, Hunter, *et al.*, 2022), to changes in basic intrinsic cellular properties leading to hyperexcitable microcircuits (Niday *et al.*, 2017; Arnold *et al.*, 2019; Sun *et al.*, 2019; Whitebirch *et al.*, 2022). Characterisation of excitatory-inhibitory dysfunction in psychiatric conditions, however, has been frequently associated with a specific reduction of the excitatory drive onto inhibitory cell populations, resulting in disinhibition of excitatory networks (Fuchs *et al.*, 2017; Bygrave *et al.*, 2019; Howes and Shatalina, 2022). It is not surprising that, given the importance of well-governed network functioning, alterations at any scale of excitatory and inhibitory neurotransmission can yield a wide variety of disease phenotypes. It is also for this reason that it is crucial to precisely characterise the locus of pathogenesis in disorders of network misbalance, whether there be a loss of GABAergic signalling or

heightened cellular excitability, since it is not possible to develop targeted therapeutic interventions for a range of human diseases without this knowledge. Beyond this, identification of the variable routes towards network instability can provide valuable insights regarding the variety of mechanisms that exist in the healthy brain to maintain proper neurological functioning.

Role of Glutamatergic and GABAergic Receptors at Synaptic, Cellular and Network Levels

The most fundamental principal of neuronal function is electrochemical cellular excitability, since it is this distinct feature of neurons that permits inter-neuronal communication and translation of synaptic inputs to action potential outputs. This basic property is the foundational building block, conferring the immense computational capacity of the brain. Cellular excitability is allowable by the electrochemical gradient – formed by the differing ion concentrations in the intracellular environment relative to the extracellular space. Opposing ionic concentrations in these compartments are formed and maintained by the presence of ion-selective transporters and cotransporters – which actively or passively passage ions across the cellular membrane (Neverisky and Abbott, 2015; R. Liu *et al.*, 2019; Côme *et al.*, 2020; Zhao and Cao, 2022; Pressey *et al.*, 2023). Establishment of such gradients permits current flux through the opening of synaptic ionotropic receptors, allowing the passive movement of ions to follow their chemical gradient. For instance, the control of intracellular chloride concentrations by membrane transporters determines the polarity and magnitude of GABAergic transmission upon activation of GABA_A receptors (Cupello, 2003). The setup of electrochemical gradients also forms the basis of the action potential during the voltage-dependant permeability of sodium and potassium conductance (Hodgkin and Huxley, 1952, 1952; Hodgkin and Keynes, 1955). While the action potential waveform is a relatively invariant phenomenon, of set amplitude and propagation speed (relative to myelination rate and axonal width), currents flowing through synaptic compartments are comparatively highly dynamic and subject to constant alteration by a vast array of plasticity processes. As previously discussed, excitatory neurotransmission is predominantly conferred by the release of glutamate into the synaptic cleft, where it induces sodium and calcium influx at the postsynaptic site through activation of AMPA and NMDA receptors, respectively (Johnson and Koerner, 1988; Parsons, Danysz and Zieglgänsberger, 2005; Espinosa and Kavalali, 2009; Greger, Watson and Cull-Candy, 2017). Similarly, inhibitory neurotransmission in the brain is principally elicited by the release of GABA acting on GABA_A receptors (Tremblay, Lee and Rudy, 2016; Lee, Lee and Lee, 2019; Tang, Jaenisch and Sur, 2021). This section will focus on the functional capabilities of these three major ionotropic receptors, and their various roles at synaptic and, where relevant, at extrasynaptic compartments.

Structure and Function of the Glutamatergic AMPA Receptor

Fast excitatory neurotransmission is predominated by the synaptic function of the glutamatergic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (Traynelis *et al.*, 2010; Kamalova and Nakagawa, 2021). Changes to the synaptic density of AMPA receptors is a key mechanism of synaptic plasticity, which can be elicited by alterations in the surface dynamics and synaptic localisation of the receptor or variation in the surface expression through modulation of endo- and exocytosis (van der Sluijs and Hoogenraad, 2011;

Constals *et al.*, 2015; Watson, Ho and Greger, 2017; Choquet, 2018; Getz *et al.*, 2022). Synaptic enrichment of particular receptor subunits – which confer variable functional properties to the ion channel – and changes in the phosphorylation state of the receptor can also modulate the functional profile of the synapse (Carvalho, Duarte and Carvalho, 2000; Håkansson *et al.*, 2006; Emond *et al.*, 2010; Wang *et al.*, 2014; Diering and Hugarir, 2018). At the most basic level, the AMPA receptor structure follows the same overall form of all eukaryotic glutamatergic ion channels, being comprised of subunits, each with a layered pattern of protein domains: a large N-terminal domain (NTD) at the most extracellular portion of the amino acid sequence; an intermediary ligand binding domain (LBD) which operates with a clamshell motif; a transmembrane domain (TMD) constituting four segments of amino acid sequences which either cross or form re-entrant loops within the phospholipid bilayer of the neuronal membrane; and finally a cytoplasmic C-terminal domain (CTD) containing multiple sites for the binding of intracellular partners (Hollmann, Maron and Heinemann, 1994; Kim and Sheng, 2004; Rossmann *et al.*, 2011; Hugarir and Nicoll, 2013; Kamalova and Nakagawa, 2021) (Figure 2A).

The AMPA receptor is comprised of four of these structured subunits, which can assemble into functional homo- or heterotetramers (Midgett and Madden, 2008; Herguedas *et al.*, 2016; Coombs *et al.*, 2019). The inclusion of specific subunits into the receptor complex yields a wide variety of functional diversity in the resulting ion channel. There are four distinct AMPA receptor subunits, GluA1-4, each encoded by individual genes, *GRIA1-4*, respectively (Collingridge *et al.*, 2009; Traynelis *et al.*, 2010). Adding a further layer of complexity, each of these subunits undergo alternative splicing resulting in one of two possible isoforms, termed *flip* or *flop*, with differing amino acid sequences in the M4-proximal region of the extracellular loop (Pei *et al.*, 2009; Penn and Greger, 2009; Perozzo, Brown and Bowie, 2023). Moreover, the majority of AMPA receptors are calcium impermeable, a property controlled by the inclusion of calcium-blocking GluA2 subunits (Tempia *et al.*, 1996; Greger, Watson and Cull-Candy, 2017). This GluA2-conferred functional block is controlled by the substitution of a glutamine residue with an arginine within the pore-lining transmembrane segment (M2) (Sommer *et al.*, 1991; Bass, 2002). While the presence of glutamine at this site will permit the flux of calcium, arginine substitution prevents the passage of calcium through the channel and also prevent receptor blockade by endogenous polyamines (Dingledine *et al.*, 1999; Panchenko *et al.*, 1999). Although the majority of expressed AMPA receptors are GluA2-containing and calcium-impermeable, functional GluA2-lacking calcium-permeable channels do form a minority subset of expressed AMPA receptors in neurons (Man, 2011; Rozov, Sprengel and Seeburg, 2012; Yuan and Bellone, 2013). This combination of variable functional properties conferred by the presence of particular receptor subunits, which themselves can express variable functional states depending on a variety of post-translational modifications, results in a highly heterogeneous set of AMPA receptor populations which in turn yield a wide array of excitatory synaptic phenotypes (Isaac, Ashby and McBain, 2007; Emond *et al.*, 2010; Han, Lin and Niu, 2017; Diering and Hugarir, 2018). Beyond this, the AMPA receptor forms part of a larger protein complex with many possible auxiliary proteins which also

alter the functional properties of the receptor (Sumioka, 2013; Abdollahi Nejat *et al.*, 2021; Jacobi and von Engelhardt, 2021; Yu *et al.*, 2021).

Characterisation of the CTD, and its contribution to receptor functions, has been of considerable interest over recent years. This domain is highly heterogeneous between subunits, each containing distinct regions for intracellular binding partners and phosphorylation sites (Greger, Watson and Cull-Candy, 2017). While it is well understood that C-terminal sequences are crucial for the induction of long term potentiation, which specific regions of this domain that contribute to plasticity changes remain to be elucidated (Boehm *et al.*, 2006; Granger *et al.*, 2013; Hosokawa *et al.*, 2015). Further, the phosphorylation status of C-terminal regions are known to regulate the surface trafficking of the AMPA receptor, alter the binding to intracellular proteins and auxiliary subunits, and can also exert direct influence on receptor function through modulation of open probability and ionic conductance (Derkach, Barria and Soderling, 1999; Kristensen *et al.*, 2011; Lu and Roche, 2012; Corti and Duarte, 2023). It is reasonable to conclude that this major segment of the intracellular portion of the receptor is involved in its regulation by key intracellular mechanisms of homeostatic plasticity and therefore contribute to precise balancing of synaptic excitation relative to overall cellular activity and inhibitory drive (Coleman *et al.*, 2003; Wang, Gilbert and Man, 2012; Yong *et al.*, 2020).

A notable difference between ionotropic glutamate receptors (iGluRs), including the AMPA receptor, and other large families of ionotropic channel such as P2X and Cys-loop receptors is that iGluR subunits have dedicated ligand binding domains that are not formed by the interface of receptor subunits (Stern-Bach *et al.*, 1994; Armstrong and Gouaux, 2000; Saul *et al.*, 2013). These ligand binding domains form a clamshell-like structure which closes around a bound glutamate molecule, causing mechanical pressure and opening of the ion channel (Quiocho and Ledvina, 1996; Mayer, 2006; Zhao *et al.*, 2016). Pairs of ligand binding domains form dimers at the back of the clamshell-like structure, so the fully assembled four-subunit receptor is essentially a dimer of dimers (Yonkunas *et al.*, 2017). Desensitisation of the channel occurs when this closure of the clamshell structure causes a separation of dimerised subunits pairs (Klykov *et al.*, 2021; Aittoniemi *et al.*, 2023). Notably, stabilization of this dimer structure reduces receptor desensitisation which can be achieved by genetic manipulation of the subunit coding genes, through pharmacological agents including positive allosteric modulators (Stern-Bach *et al.*, 1998; Sun *et al.*, 2002; Jin *et al.*, 2005; Partin, 2015). Interestingly, the *flip* and *flop* splice variants of the GluA2 subunit display different desensitisation kinetics, through modulating the dimerization stability of subunit pairs, the *flip* variant desensitises slower than its counterpart (Sommer *et al.*, 1990; Mosbacher *et al.*, 1994; Koike *et al.*, 2000; Grosskreutz *et al.*, 2003). Altered trafficking of splice isoforms has also been demonstrated, whereby the *flip* isoform undergoes complex glycosylation events before release from the endoplasmic reticulum, and is trafficked to the cell membrane at significantly higher rates than the *flop* variant (Coleman *et al.*, 2006; Penn, Williams and Greger, 2008; Zheng, Sabirzhanov and Keifer, 2012; La Via *et al.*, 2013; Kandel *et al.*, 2018; Morise *et al.*, 2020). Moreover, chronic activity blockade in neuronal

networks has been shown to induce changes in splice isoform expression in the CA1 subfield of the hippocampus (Penn *et al.*, 2012). As such, isoform enrichment is a possible mechanism of homeostatic regulation, which may alter the abundance of AMPA receptor expression in the cellular membrane, and the functional profile of synaptically expressed receptor population (Penn *et al.*, 2012).

Advances in microscopy technologies have revealed a super-resolved sub-synaptic organisation of AMPA receptors, forming dense clusters within synaptic compartments, termed *nanodomains* (Barrera-Ocampo and Chater, 2013; Nair *et al.*, 2013; Tang *et al.*, 2016). The localisation of these AMPA receptor nanodomains is closely associated to the excitatory postsynaptic scaffolding protein, PSD-95, and the presynaptic protein, *RIM1* (Nair *et al.*, 2013; Sun *et al.*, 2022). *RIM1* itself is associated with *Munc13* and *syntaxin* to form presynaptic machinery necessary for glutamate release at the active zones of the axon terminal (Betz *et al.*, 2001; Magdziarek *et al.*, 2020; Wang *et al.*, 2020; Rizo, 2022). While the transsynaptic component of this pre- and postsynaptic alignment of AMPA receptors with glutamate release sites is not fully understood, the transsynaptic cell adhesion proteins, *neurexin* and *neuroligin*, are promising candidates (Peixoto *et al.*, 2012; Trotter *et al.*, 2019; Choquet and Hosy, 2020). Presynaptically expressed *neurexin* binds *neuroligin* at postsynaptic compartments, and the intracellular component of *neuroligin* is able to recruit PSD-95 and in turn anchor AMPA receptor clusters (Craig and Kang, 2007; Mondin *et al.*, 2011; Südhof, 2017; Jeong *et al.*, 2019). Interestingly, abolition of the *neuroligin* and PSD-95 interaction has been shown to disrupt the alignment of AMPA receptors with presynaptic release sites (Mondin *et al.*, 2011). As such, it is likely that all of these proteins form complex interactions, bridging across the pre- and postsynaptic compartments, to maintain high-density AMPA receptor nanodomains directly opposite to sites of glutamate release. These transsynaptic structures are termed a *nanocolumn* (Figure 2B). Disruption of this nanocolumn organisation has been shown to reduce the efficacy of AMPA receptor signalling, causing reduction in the amplitude of synaptic currents without significant changes to the number of AMPA receptors in the synapse (Haas *et al.*, 2018). Evidently, preservation of the nanodomain and nanocolumn architecture of synaptic AMPA receptors is vital to maintain healthy synaptic function.

A final consideration of AMPA receptor physiology is their lateral mobilisation during synaptic plasticity. A relevant example can be demonstrated by the alteration of AMPA receptor anchoring to PSD-95 during NMDA receptor-dependent *long term depression* (LTD) (Lüscher and Malenka, 2012; Compans *et al.*, 2021). Low-level activation of the NMDA receptor, has been demonstrated to induce long lasting depression of the excitatory synaptic efficacy through a reduction in AMPA receptor expression at postsynaptic sites (Thiels *et al.*, 1996; Toyoda, Zhao and Zhuo, 2006; Kang, Noh and Chung, 2020; Chen, Li and Zhuo, 2021; Compans *et al.*, 2021). Interestingly, this reduced expression of AMPA receptors at synapses is not necessarily accompanied by a major reorganisation in the nanoscale topography of the synaptic AMPA receptor population. Instead, lower densities of AMPA receptors are found in subsynaptic nanodomains without, without dramatic changes in

overall nanodomain number or dimension (Savtchenko and Rusakov, 2014; Sinnen *et al.*, 2017; Haas *et al.*, 2018). This likely reflects a robustness of the synapse to maintain the nanocolumn structure, even in the context of fluctuations in AMPA receptor expression. Further, it has been demonstrated that the surface dynamics of AMPA receptors are increased during NMDA receptor-dependent LTD – a feature that is relatively unique to NMDA receptor-mediated plasticity, as this upregulation of AMPA receptor motility is not found in other forms of synaptic depression, such as P2X receptor-dependent LTD (Compans *et al.*, 2021). It has been elucidated that this shift in AMPA receptor dynamics is the result of NMDA receptor-dependent phosphorylation of PSD-95 by glycogen synthase kinase 3 beta (GSK3 β), specifically at a tyrosine residue in position 19 (Compans *et al.*, 2021). The phosphorylation of PSD-95 at this site causes degradation of the synaptic scaffold and therefore an un-anchoring of AMPA receptors to postsynaptic compartments (Nelson *et al.*, 2013; Delgado, Nall and Selvin, 2020; Compans *et al.*, 2021). These findings demonstrate that the ionotropic function and lateral mobility of synaptic receptors, both NMDA and AMPA receptors, are intricately linked. In fact, they typically co-regulate one-and-other in order to maintain a healthy ratio of NMDA-to-AMPA receptors at excitatory synapses (Watt *et al.*, 2000; Myme *et al.*, 2003; Lafourcade *et al.*, 2022). While the example discussed here was NMDA receptor-dependent LTD, there are – of course – many other routes towards synaptic plasticity (Johnston *et al.*, 1992; Kemp and Bashir, 1997; Kessey and Mogul, 1997; Wang, Rowan and Anwyl, 1997; Anwyl, 2006; Connor and Wang, 2016; Alkadhi, 2021; Sanderson *et al.*, 2022). And similarly other NMDA receptor dependent processes that can alternatively drive a strengthening of synaptic connections, as in long-term potentiation (Kullmann and Siegelbaum, 1995; Bliss and Collingridge, 2013; Tokay *et al.*, 2014; Wiera *et al.*, 2017; Yang *et al.*, 2017; Giese, 2021). Evidently, synaptic ion-channel co-regulation is highly complex, and perturbations to these mechanisms – particularly to a central plasticity regulating channel like the NMDA receptor – is expected to cause severe deficits in the maintenance of homeostatic activity and synaptic plasticity.

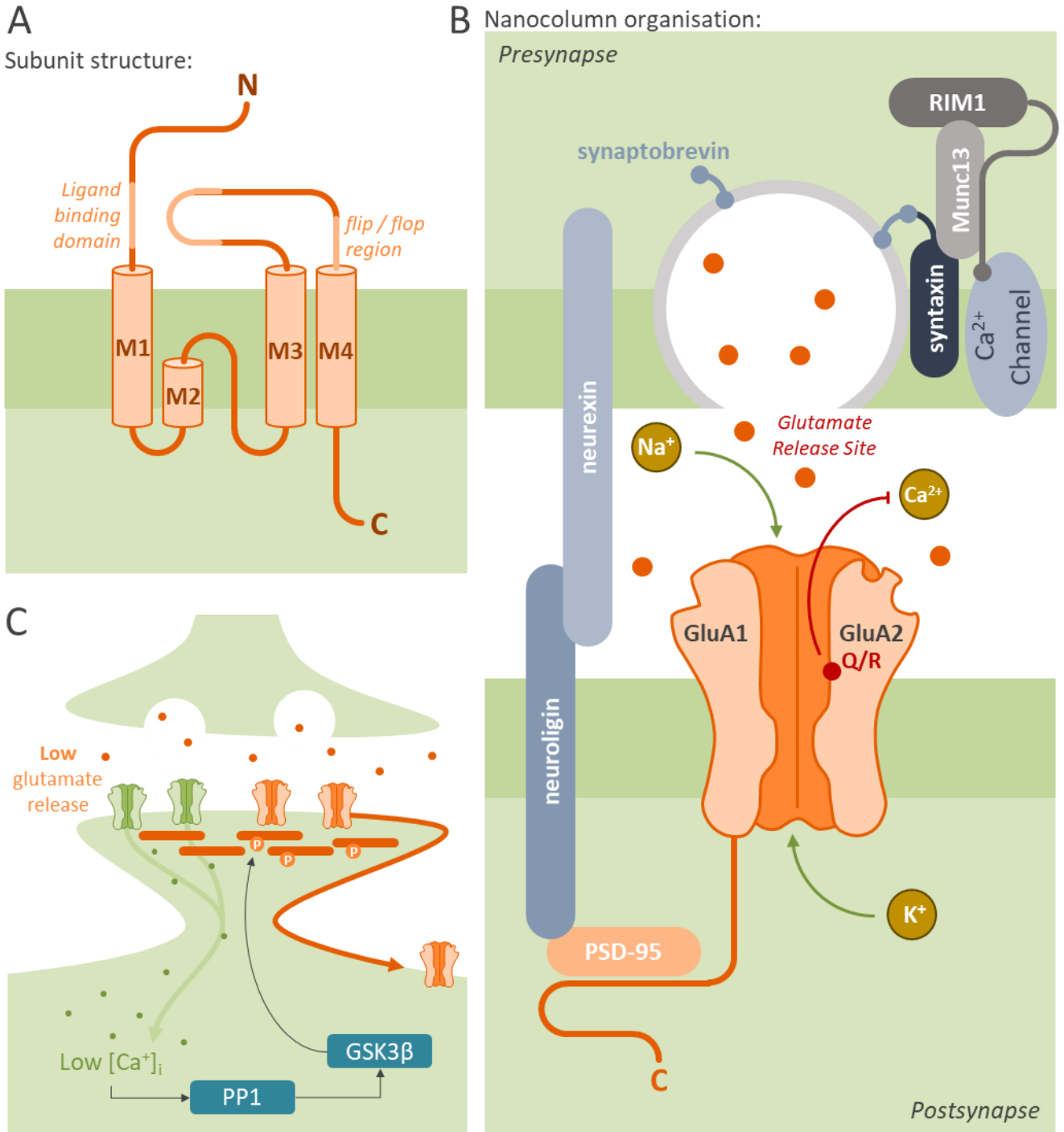


Figure 2: AMPA Receptor Structure and Function.

(A) Schematic of typical AMPA receptor subunit structure, including 4 membrane domains (M1-4), a ligand binding domain comprised of N-terminal and M3-M4 loop sequences, and a site of flip/flop splice variation extracellularly proximal to M4. (B) Schematic representation of AMPA receptor nanocolumn alignment architecture, involving the neuroligin/neurexin/PSD-95 alignment of postsynaptic AMPA receptors with glutamate release sites; and the presynaptic release machinery involving vesicle-associated synaptobrevin, its interaction with presynaptic membrane-bound syntaxin. Additionally, Munc13 and RIM1 associate glutamate containing vesicles with presynaptic calcium channels. Ion flux through the activated AMPA receptor is shown, and the GluN2A subunit-dependent block of calcium permeability. (C) Schematic representation of NMDA receptor dependent long term depression, where low influx of calcium through NMDA receptors (green), preferentially activated PP1 and, in turn, GSK3 β . Phosphorylation of PSD-95 by this pathway, at T19, leads to the destabilisation of AMPA receptors from the postsynaptic density, and their eventual depletion from the synaptic compartment.

Structure and Function of the Glutamatergic NMDA Receptor

Although AMPA receptor currents dominate fast excitatory neurotransmission, the presence of slower NMDA receptor mediated currents at postsynaptic compartments are crucial for shaping the later phases of excitatory synaptic phenotypes, and controlling many synaptic plasticity processes (Vargas-Caballero and Robinson, 2004; Traynelis *et al.*, 2010; Reiner and Levitz, 2018). As previously highlighted, the vast majority of synaptic AMPA receptor populations are calcium impermeable; NMDA receptors however are major contributors to calcium influx which drive a variety of downstream intracellular pathways, vital for correct and healthy brain function (Regehr and Tank, 1990; Burnashev *et al.*, 1992; Xin *et al.*, 2005; Kapitein *et al.*, 2011; Bliss and Collingridge, 2013; Glebov, 2020; Puri, 2020). This prime feature of NMDA receptors – serving as an initiator to a diverse range of cellular processes – implicates NMDA receptor dysfunction in a variety of neurological disease mechanisms, including epilepsy, intellectual disability neurodevelopmental disorders and neuropsychiatric conditions (Hynd, Scott and Dodd, 2004; Fromer *et al.*, 2014; Burnashev and Szepetowski, 2015; Strehlow *et al.*, 2019; Vieira, Jeong and Roche, 2021; Elmasri, Hunter, *et al.*, 2022; Elmasri, Lotti, *et al.*, 2022; Mangano *et al.*, 2022). The general architecture of the NMDA receptor follows that of other iGluRs: being composed of four subunits, each of which has a typical structure of large extracellular N-terminal and ligand-binding domains, followed by a transmembrane domain with three transmembrane helices and one re-entrant intracellular loop, and finally a large intracellular C-terminal domain (Dingledine *et al.*, 1999; Traynelis *et al.*, 2010; Lee *et al.*, 2014). While there is remarkable similarity between the NMDA and AMPA receptor structures, there are many key differences in the function and gating properties of NMDA receptors that confers them distinct physiological capabilities (Reiner and Levitz, 2018).

In addition to the ability to flux calcium, NMDA receptors also display other properties that distinguish their function from that of the AMPA receptor population. Predominantly, in the physiological environment, NMDA receptor channels are blocked by an ambient concentration of extracellular magnesium (Mori *et al.*, 1992; Clarke and Johnson, 2006). This magnesium-blockade is alleviated by AMPA receptor-dependant depolarisation of the synaptic compartment, and action potential backpropagation, causing expulsion of the magnesium ion from the central channel (Vargas-Caballero and Robinson, 2004; Fuenzalida *et al.*, 2010). Additionally, while being glutamatergic receptors, NMDA receptors also require the binding of endogenous co-agonists, glycine or D-serine, for the opening of the ion pore (Dingledine, Kleckner and McBain, 1990; Thomson, 1990; Le Bail *et al.*, 2015). As such, opening of synaptically expressed NMDA receptors requires both glutamate release from the presynaptic axon terminal and successful postsynaptic depolarisation to expel the magnesium blockade before calcium can be permitted through the channel. This ligand-gating coupled to voltage-dependency of channel function allows NMDA receptors to act as *coincidence detectors*, permitting influx of calcium to the dendritic spine selectively during the co-occurrence of both pre- and postsynaptic activation (Seeburg *et al.*, 1995).

All identified NMDA receptors require the inclusion of exactly two co-agonist binding GluN1 subunits (Scatton, 1993; Stephenson, 2001; Henson *et al.*, 2010). The obligatory GluN1 protein is encoded by a single gene, *GRIN1*, but exists in eight distinct isoforms as a result of three alternatively spliced exons (Durand, Bennett and Zukin, 1993; Laurie and Seeburg, 1994; Bai and Hoffman, 2009). One of these exons, exon 5, encodes a 21 amino acid sequence in the N-terminal domain of the receptor; whereas exons 21 and 22, both encode C-terminal domain sequences of 37 or 38 amino acids, respectively (Hansen *et al.*, 2018). These isoforms of the GluN1 subunit display regional and cell-specific developmental expression profiles, and also confer unique functional properties to the assembled receptor (Laurie and Seeburg, 1994; Zhong *et al.*, 1995; Paupard, Friedman and Zukin, 1997). For instance, the inclusion of the exon 5-encoded sequence in the N-terminal domain reduces the subunits potency for glycine, resulting in an accelerated decay of the post synaptic current (Traynelis, Hartley and Heinemann, 1995; Traynelis *et al.*, 1998; Rumbaugh *et al.*, 2000; Vance, Hansen and Traynelis, 2012; Yi *et al.*, 2018). Alternatively, splice isoform variation of the intracellular C-terminal domain impacts the subcellular localisation of receptors (Scott *et al.*, 2001; Mu *et al.*, 2003; Wenthold *et al.*, 2003). The intracellular domain is also rich in phosphorylation sites which act as targets for a large variety of kinase and phosphatase activity (Tingley *et al.*, 1993, 1997; Iacobucci and Popescu, 2017). However, it is important to note that intracellular anchoring to PSD-95 is not an exclusive mechanism for synaptic localisation. Recent evidence has demonstrated that a positive surface charge on the extracellular N-terminal domain of the NMDA receptor is crucial for its association to the negatively charged phospho-tyrosine residue of EphB2 (Washburn *et al.*, 2020). In fact, this extracellular association with EphB2 was shown to be necessary for the maintenance of NMDA receptors in the dendritic spines, and abolition of EphB2-GluN1 interaction increases the lateral mobility of NMDA receptors in the cellular membrane (Washburn *et al.*, 2020). Essentially, while the obligatory GluN1 subunit of the NMDA receptor contains many intracellular binding sites, the surface diffusion of the receptor is a complex and multifactorial phenomenon, controlled by numerous intracellular and extracellular processes.

Although there are also some subtle functional properties conferred by the GluN1 splice isoform present in the assembled receptor, it is the inclusion of two glutamate-binding GluN2 subunits that dominates in governing the overall NMDA receptor kinetic profile (Monyer *et al.*, 1992; Punnakkal, Jendritza and Köhr, 2012; Wyllie, Livesey and Hardingham, 2013; Hansen *et al.*, 2014; Glasgow, Siegler Retchless and Johnson, 2015; Sun, Hansen and Jahr, 2017). There are four GluN2 subunits, named GluN2A-D, each encoded by distinct genes: *GRIN2A* through to *GRIN2D*, respectively (Traynelis *et al.*, 2010). While GluN2 subunits do not undergo alternative splicing, they share the same basic structure as GluN1 subunits with largely conserved amino acid sequences (Monyer *et al.*, 1992). Despite overall similarity on their native architecture, control over the functional profiles of different GluN2 subunit-containing receptors is mediated by changes across a range of receptor gating and kinetic properties (Tu and Kuo, 2015; McDaniel *et al.*, 2020). A clear example can be shown

by considering the potency for glutamate, the glutamate EC₅₀ of GluN2D-containing NMDA receptors is more than 6-fold lower than that conferred by GluN2A subunit inclusion (Erreger *et al.*, 2007). This dramatically increased potency for glutamate by GluN2D subunits may reflect a differential functional role for this receptor subtype, being enriched in extrasynaptic sites on dopaminergic and GABAergic interneuron populations, where local glutamate concentrations are substantially reduced relative to synaptic compartments (Riebe *et al.*, 2016; Morris, Mishina and Jones, 2018; Yao *et al.*, 2022). Intriguingly, GluN2 subunit inclusion also alters the potency for co-agonists at the glycine binding domain on GluN1 subunits (Sheinin, Shavit and Benveniste, 2001; David *et al.*, 2010; Jessen *et al.*, 2017; Maolanon *et al.*, 2017). This modulation of GluN1 agonist potency is strikingly effective, with GluN2D subunits inducing a ten-fold increase in glycine potency relative to GluN2A-containing receptors, similarly in line with the reduced co-agonist availability outside synapses (Chen *et al.*, 2008; Harsing and Matyus, 2013). This overriding impact of GluN2 subunits on GluN1-ligand interactions is conferred by a conformational modulation of GluN1 ligand-binding domains by their association with GluN2 subunit sequences (Chen *et al.*, 2008). Evidently, the structural assembly of NMDA receptors with specific GluN2 subunits substantially modulates synaptic phenotypes, via a functionality-defining dominance that is either conferred directly by the GluN2 subunit or exerted over GluN1 subunit actions by GluN1-GluN2 subunit interactions.

Further, additionally to modulating agonist – and co-agonist – potency, the GluN2 subunit also determines receptor deactivation after unbinding of glutamate. It is this kinetic property that controls the decay phase of the NMDA receptor-mediated excitatory postsynaptic currents (Lester *et al.*, 1990; Vicini *et al.*, 1998; Wyllie, Béhé and Colquhoun, 1998; Yuan *et al.*, 2009). Arguably, this feature is the most apparent and functionally significant parameter controlled by the GluN2 subunit, with decay time constants, termed τ_{decay} , which can vary by orders of magnitude between receptor subtypes (Vicini *et al.*, 1998; Hansen *et al.*, 2018). GluN2A containing NMDA receptors display the most rapid deactivation, with a τ_{decay} typically estimated around 50 milliseconds. This is in stark contrast to the extensive decay time of GluN2D containing channels, with a τ_{decay} of approximately 4000 milliseconds (Vicini *et al.*, 1998). Modulation of biophysical properties also modulate the kinetics of NMDA receptor-mediated postsynaptic currents. For instance, GluN2A and GluN2B subunit inclusions confer higher channel conductance and calcium permeability, relative to GluN2C and GluN2D containing receptors (Erreger *et al.*, 2004; Qian, Buller and Johnson, 2005; Siegler Retchless, Gao and Johnson, 2012; Paoletti, Bellone and Zhou, 2013; Wyllie, Livesey and Hardingham, 2013). This conference of ion permeability and conductance modulates the rise time constant, τ_{rise} , and amplitude of synaptic NMDA receptor currents (Maki and Popescu, 2014). A further GluN2 subunit-conferred functional property of the NMDA receptor channel is sensitivity to magnesium blockade. Specifically, it has been demonstrated that heightened sensitivity to magnesium block is imparted to the ion channel by the presence of a serine residue in the M3 transmembrane segment of the GluN2A and GluN2B subunits, proximal to the intracellular loop

(Siegler Retchless, Gao and Johnson, 2012). However, in both GluN2C and GluN2D subunits – which display a markedly reduced block by extracellular magnesium – this serine residue is substituted for a leucine (Siegler Retchless, Gao and Johnson, 2012). Experimental investigations revealed that genetic manipulation of GluN2A subunits, to contain a leucine at this site, relieves magnesium block from the resulting ion channel (Siegler Retchless, Gao and Johnson, 2012). Notably, reduced sensitivity to magnesium block again confers an increased suitability of GluN2D-containing NMDA receptors to extrasynaptic sites, as AMPA receptor-mediated local membrane depolarisation is normally a prerequisite to magnesium unblocking of synaptic NMDA receptors (Riebe *et al.*, 2016; Morris, Mishina and Jones, 2018; Yao *et al.*, 2022).

Beyond the detailed characterisation of GluN2 subunit-conferred functional properties, the developmental and regional expression profiles for each GluN2 subunit has been extensively characterised (Watanabe *et al.*, 1992; Akazawa *et al.*, 1994; Monyer *et al.*, 1994; Suzuki *et al.*, 2023). These expression profiles are of considerable importance, considering that temporal and regional control of GluN2 subunits offers an avenue towards significant reshaping synaptic phenotypes across time, brain regions and cell types. For instance, GluN2C subunit expression is confined to cerebellar structures, whereas in excitatory neurons of the hippocampus GluN2B is the dominant mediator of NMDA receptor currents at neonatal stages, which is rapidly dwarfed by the upregulation of GluN2A subunit expression during early postnatal development (Watanabe *et al.*, 1992; Akazawa *et al.*, 1994; Monyer *et al.*, 1994; Zhong *et al.*, 1995; Sanz-Clemente, Nicoll and Roche, 2013). This shift in cell surface enrichment from GluN2B to GluN2A subunits is commonly referred to as *the developmental switch*. Interestingly, initiation of the developmental switch is dependent on network activity (Williams *et al.*, 1993; Ramoa and Prusky, 1997; Lee *et al.*, 2008). This has been demonstrated in *in vitro* neuronal networks, where pharmacological manipulations to inhibit network activity prevented GluN2A subunit upregulation; and *in vivo* where ocular deprivation obstructs developmental switching in the visual cortex of ferrets (Ramoa and Prusky, 1997; Lee *et al.*, 2008). Further, hippocampal inhibitory cells show an enriched expression of GluN2D-containing NMDA receptors, relative to the local excitatory cell population (Perszyk *et al.*, 2016; Hanson *et al.*, 2019; Garst-Orozco *et al.*, 2020; Gawande *et al.*, 2023). Given the vast divergence in functional profiles of distinct NMDA receptor subtypes, the maintenance of proper NMDA receptor signalling and plasticity throughout development, and across brain regions and cell types, is likely underpinned by finely controlled expression and localisation of GluN2 subunit-containing receptors.

Beyond the ionotropic function of the NMDA receptor, an emerging area of research has begun to explore the *metabotropic*-like behaviour of intracellular receptor interactions within the C-terminal domain (Gray, Zito and Hell, 2016; Dore *et al.*, 2017; Stein *et al.*, 2020, 2021; Park, Stein and Zito, 2022). For instance, it has been known for two decades that the C-terminal domain of the GluN2B subunit contains a high affinity binding site for calcium/calmodulin-dependent protein kinase ii alpha, CaMKII α , which is not present on the C-terminal domains of other GluN2 subunits (Strack, McNeill and Colbran, 2000). In fact, it has been further demonstrated

that the C-terminal domains from GluN2A or GluN2B subunits recruit substantially different intracellular complexes of membrane-associated guanylate kinase (MAGUK) scaffolding proteins (Frank *et al.*, 2016, 2017). Evidently, the interactome of different NMDA receptor subunits not only permit variation in subcellular localisation, but also positions them with unique capabilities in terms of initiating intracellular signalling cascades (Hardingham, 2019).

In addition to the bidirectional regulation of AMPA receptor localisation and expression in LTP and LTD, NMDA receptor activity has also been demonstrated to modulate GABA_A receptor distribution in the neuronal membrane. Specifically, it has been demonstrated that overexpression of GluN2B-containing NMDA receptors at the surface of hippocampal neurons promotes the internalisation of α 5-containing GABA_A receptors (Wu *et al.*, 2021). Conversely, conditional knockout of *Grin2a* or *Grin2b* reduced or increased tonic inhibitory GABAergic currents, respectively (Wu *et al.*, 2021). Further, studies have reported a bidirectional effect of glutamate signalling on GABA_A receptor clustering. Specifically, high levels of synaptic glutamate – inducing large NMDA receptor-dependent calcium influx – has been shown to induce dispersion of GABA_A receptors at neighbouring inhibitory synapses, through a calcineurin-dephosphorylation-dependant pathway (Muir *et al.*, 2010). Ultimately, glutamatergic signalling – and NMDA receptor function specifically – has been numerously demonstrated to co-regulate GABA_A receptors in the neuronal membrane (Matsuyama, Nei and Tanaka, 1997; Gault and Siegel, 1998; Marsden *et al.*, 2007; Muir *et al.*, 2010; P. Liu *et al.*, 2019; Wu *et al.*, 2021). Given the role of the GABA_A receptor in transducing the vast majority of inhibitory neurotransmission, it is clear that correct NMDA receptor functioning is vital in maintaining physiological balance between excitation and inhibition.

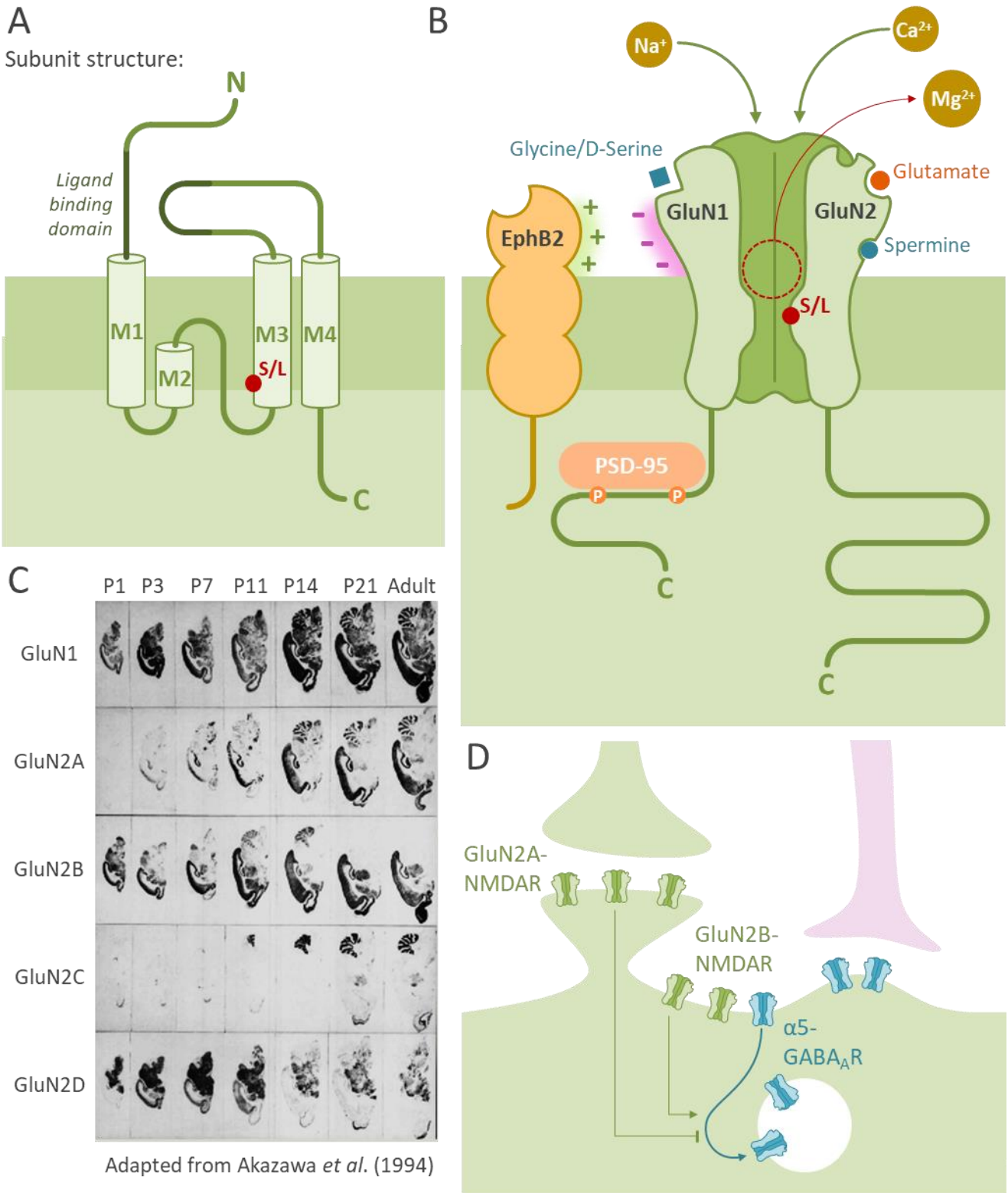


Figure 2: NMDA Receptor Structure and Function.

(A) Schematic of typical NMDA receptor subunit structure, including 4 membrane domains (M1-4), a ligand binding domain comprised of N-terminal and M3-M4 loop sequences, and a site of magnesium sensitivity-conferring serine residue in M3. (B) Schematic representation of NMDA receptor architecture, including the presence of agonist and co-agonist binding sites, extracellular interaction with charged domains of the EphrinB2 receptor and intracellular association of GluN1 with PSD-95. (C) Panel taken from Akazawa *et al.* (1994), showing the regional and temporal expression profiles of five principal NMDA receptor subunits. (D) schematic showing bidirectional impacts of NMDA receptor signalling on extrasynaptic GABA_A receptor internalisation, by GluN2A- and GluN2B-containing channels.

Structure and Function of the GABAergic GABA_A Receptor

As discussed in the prior section, the vast majority of inhibitory neurotransmission is mediated by the GABA_A receptor (Roberts and Frankel, 1950; Miles, 1999). In the hippocampus, GABA_A receptors are primarily localised to dendrites, somata and axon initial segment of excitatory and inhibitory neurons (Mohler *et al.*, 1990; Trigo, Marty and Stell, 2008; Kasugai *et al.*, 2010; Muir and Kittler, 2014). The assembled GABA_A receptor is a heteropentamer with a central chloride-permeable ion channel, belonging to the Cys-loop ligand-gated ion channel superfamily (Schwartz, 1988; Zhu *et al.*, 2018; Kim and Hibbs, 2021). The receptor typically contains two ligand binding sites for GABA and alternative binding sites for endogenous functional-modulators (Sigel and Ernst, 2018; Masiulis *et al.*, 2019; Sente *et al.*, 2022). Although ligand binding to a single active site is sufficient to induce opening of the channel, chloride conductance is dramatically increased upon binding of two ligands to the active sites (Baumann, Baur and Sigel, 2003). Upon binding of GABA released from inhibitory presynaptic compartments, influx of chloride ions induces a hyperpolarisation of the membrane potential, thereby reducing the propensity for action potential generation (Miles, 1999). As such, the overarching effect of GABA release acts via GABA_A receptors to reduce activity of neuronal networks, providing a counterbalance to the converse excitation of hippocampal networks by glutamate. In comparison to the iGluR family, GABA_A receptor subunit composition is considerably more complex, with a total of 19 subunit-coding genes identified to date. These genes uniquely encode individual subunits, namely: α 1-6 (encoded by *GABRA1-6*), β 1-3 (*GABRB1-3*), γ 1-3 (*GABRG1-3*), ρ 1-3 (*GABRR1-3*), δ (*GABRD*), ϵ (*GABRE*), π (*GABRP*) and θ (*GABRQ*) (Backus *et al.*, 1993; Steiger and Russek, 2004; Darlison, Pahal and Thode, 2005; Daniel and Ohman, 2009). The relative expression of these genes, and their associated subunits, is developmentally and regionally regulated, resulting in a highly heterogeneous set of GABA_A receptor isoforms, with differing functional properties (Sigel *et al.*, 1990; Araki, Kiyama and Tohyama, 1992; Gutiérrez *et al.*, 1997; Minier and Sigel, 2004; Olsen and Sieghart, 2008). For instance, the δ subunit-containing receptors exhibit high affinity for GABA and GABA-site-antagonist, muscimol, compared to the δ subunit-lacking channels (Saxena and Macdonald, 1994; Quirk *et al.*, 1995; Benkherouf *et al.*, 2019). However, the predominant isoform in adult hippocampal neurons is thought to be composed of two α 1, two β 2, and one γ 2 subunits (Baur, Minier and Sigel, 2006). Further, mutations in GABA_A receptor subunit-coding genes have been identified in various neuropsychiatric disorders, including epilepsy, autism spectrum disorder, bipolar disorder and schizophrenia (Coon *et al.*, 1994; Papadimitriou *et al.*, 2001; Korpi and Sinkkonen, 2006; Gallagher *et al.*, 2007; Chiu *et al.*, 2008; Kang and Barnes, 2013; Maillard *et al.*, 2022). Evidently, normal physiological functioning of the GABA_A receptor is vital to maintain brain health, and perturbation to receptor activity will result in altered neurological function, and ultimately disease.

Despite the relatively large number of subunit-coding genes, the structure of individual subunits is well conserved, with each subunit consisting of approximately 450 amino acid residues (Ghit *et al.*, 2021). Each subunit is comprised of a large extracellular N-terminal domain and four transmembrane domain sequences

(TMD1-4, Figure 3A) (Zhu *et al.*, 2018). The second transmembrane domain (TMD2) is believed to form the centralised chloride-permeable ion pore, and a large intracellular loop connecting TMD3 and TMD4 contains many amino acid sequences permitting intracellular protein interactions, such as those with inhibitory synaptic scaffolding protein gephyrin (Nyman-Andersen, Sawyer and Olsen, 2002; Trudell and Bertaccini, 2004; Chen and Olsen, 2007; Saiepour *et al.*, 2010; Thompson, Lester and Lummis, 2010; Zhu *et al.*, 2018, Figure 3B-C)). Moreover, posttranslational modifications can further alter the functional profile of resulting GABAergic currents (Chen *et al.*, 1990; Krishek *et al.*, 1994; Sigel, 1995; Lo *et al.*, 2010; Sallard, Letourneur and Legendre, 2021). The binding site for GABA is located at the interface between the N-terminal domains of α and β subunits (Michałowski, Kraszewski and Mozrzymas, 2017; Gottschald Chiodi *et al.*, 2019). The interface of N-terminal domains between α and γ subunits have been demonstrated to harbour binding sites for psychotropic drugs such as benzodiazepines, which induce a positive allosteric modulation of the channel (Rudolph and Antkowiak, 2004; Sigel and Lüscher, 2011, Figure 3C)). Endogenous neurosteroids can also positively modulate channel function through binding at transmembrane sequences of the α and β subunits (Brussaard and Koksma, 2002; Wang, 2011; Adams, Thomas and Smart, 2015; Johansson *et al.*, 2016, Figure 3C).

Moreover, the inclusion and arrangement of particular GABA_A receptor subunits greatly influences the gating and functional phenotype of the resulting ion channel (Pritchett *et al.*, 1988; Angelotti and Macdonald, 1993; Gingrich, Roberts and Kass, 1995; Sigel *et al.*, 2006; Sente *et al.*, 2022). Throughout the central nervous system, expression of GABA_A receptor subunits is highly regulated (Montpied *et al.*, 1988; Poulter *et al.*, 1992; O'Hara *et al.*, 1995; Pávai *et al.*, 2010). For instance, $\alpha 6$ subunit is uniquely expressed in the cerebellum and the ρ subunit is predominantly expressed in the retina (Sieghart and Sperk, 2002). Beyond such regional segregation, particular subunits are preferentially localised to specific subcellular compartments. Postsynaptic clusters of GABA_A receptors typically contain the $\alpha 1-3$, $\beta 1-3$, and $\gamma 2$ subunits, which display a lower affinity for GABA (Ghit *et al.*, 2021). In these postsynaptic compartments, presynaptic release of GABA from inhibitory axon terminals leads to a high concentration of GABA in the synaptic cleft, leading to a short chloride conductance (Ghit *et al.*, 2021). Conversely, extrasynaptic GABA_A receptors are typically composed of $\alpha 4-6$, $\beta 2-3$ and δ subunits, which display a substantially increased affinity for GABA (Kasugai *et al.*, 2010; Luscher, Fuchs and Kilpatrick, 2011). This increased affinity results in activation of the channels by physiological ambient levels of GABA outside of synaptic compartments, which contribute to longer tonic inhibitory conductance (Scimemi *et al.*, 2005; Wlodarczyk *et al.*, 2013; Kasaragod *et al.*, 2022). Intriguingly, this tonic inhibition is known to modulate stress-induced memory impairment, hippocampal synaptic plasticity and neurogenesis (Platel, Lacar and Bordey, 2007; Smith, 2013; Feng *et al.*, 2014; Lee *et al.*, 2016; Catavero, Bao and Song, 2018; Wyroślak, Lebida and Mozrzymas, 2021). The role of tonic inhibition on hippocampal plasticity has been shown to involve conductance arising from $\alpha 5$ -containing GABA_A receptors on CA1 pyramidal neurons (Wyroślak, Lebida and

Mozrzymas, 2021). Genetic deletion or pharmacological antagonism of these specific channels has been shown to increase neuronal depolarisation during 10 Hz stimulation (Martin *et al.*, 2010). Further, alterations in subunit-coding gene expression, believed to underpin changes to the functional profile of surface expressed GABA_A receptors, have been identified in post-mortem neural tissue of depressed patients and victims of suicide (Merali *et al.*, 2004; Poulter *et al.*, 2008, 2010). As such, the functional heterogeneity of expressed GABA_A receptor subtypes demonstrate that the importance GABAergic signalling extends beyond simple maintenance of overall network activity. In fact, the physiological functioning of the GABAergic system supports and upholds many complex neurological functions, from learning and memory to resistance against development of neuropsychiatric disease.

The intracellular trafficking of GABA_A receptors is a highly complex process, regulated by a vast range of protein-protein interactions (Figure 3D). Briefly, ubiquitin-related protein, PLIC1, interacts with GABA_A receptors in the endoplasmic reticulum, serving as a quality control regulator to stabilise correctly folded and assembled receptors in this subcellular domain (Saliba, Pangalos and Moss, 2008). Interaction with the Golgi-specific DHC zinc finger protein (GODZ) has been shown to palmitoylate the cytoplasmic loop of $\gamma 2$ subunits – a process which is necessary to induce clustering of the $\gamma 2$ subunit-containing GABA_A receptors at postsynaptic sites (Keller *et al.*, 2004; Rathenberg, Kittler and Moss, 2004; Fang *et al.*, 2006). Further, reduced expression of GODZ by shRNA-silencing results in reduced synaptic clustering and corresponding decreases in GABA_A receptor-mediated inhibitory current amplitudes and frequency (Fang *et al.*, 2006). Further, interaction of β subunits with brefeldin A-inhibited GDP/GTP exchange factor 2 (BIG2) permits the vesicular budding of GABA_A receptors through the trans-Golgi network towards the neuronal membrane (Charych *et al.*, 2004). This forward trafficking of GABA_A receptors to the neuronal surface is counterbalanced by clathrin-mediated endocytosis. Both β and γ subunits have been shown to interact with the clathrin adaptor protein, AP2, in a dephosphorylation-dependent manner (Kittler *et al.*, 2000, 2008; Herring *et al.*, 2003). Further, inhibition of this internalisation process has been demonstrated to increase GABA_A receptor clustering at synapses and increase the amplitude of inhibitory synaptic currents (Kittler *et al.*, 2005). Since palmitoylation and phosphorylation are reversible and dynamic processes, it is easy to appreciate the rich variety of intracellular processes which can continuously alter the expression, localisation and function of GABA_A receptors at the neuronal membrane (Figure 3D).

As for glutamate receptors, the trafficking of GABA_A receptors to-and-from the neuronal membrane occurs at extrasynaptic compartments. And the surface diffusion of receptors is dynamically regulated to maintain an equilibrium of ion channels at both synaptic and extrasynaptic sites (Bruneau and Akaaboune, 2006; Vithlani, Terunuma and Moss, 2011). Synaptic localisation of GABA_A receptors is underpinned by their association with the inhibitory synaptic scaffolding protein, gephyrin (Tretter *et al.*, 2008; Wu *et al.*, 2012; Kowalczyk *et al.*, 2013). While gephyrin scaffolds form the substrate of both GABAergic and glycinergic synapses, the latter are

principally expressed in the spinal cord; whereas GABAergic synaptic transmission dominates brain-based inhibition (Legendre, 2001; Alvarez, 2017). Gephyrin is a multi-domain protein, where the N-terminal domain forms a trimeric structure and the C-terminal domain forms a dimer (Kasaragod and Schindelin, 2018; Pizzarelli *et al.*, 2020). This combination of tri- and dimerization results in oligomerisation of the scaffolds, to form a postsynaptic hexagonal protein lattice, serving to stabilise the GABA_A receptor (Fritschy, Harvey and Schwarz, 2008). It has been well-known that gephyrin contains many sites for phosphorylation, which dynamically modulate the integrity of the scaffold (Zacchi, Antonelli and Cherubini, 2014). Among other kinases now recognised to phosphorylate gephyrin, glycogen synthase kinase 3 β (GSK3 β) has been shown to exert control over degradation of gephyrin scaffolds, specifically through phosphorylation of a serine residue at position 270 (Tyagarajan *et al.*, 2011). Tyagarajan *et al.* (2011) identified that this S270 residue is constitutively phosphorylated in basal states, and transfection with a phosphor-null mutant (S270A) increased gephyrin cluster density, GABAergic current amplitudes and frequency in hippocampal neurons. Specifically, phosphorylation of the scaffold by GSK3 β promotes the recruitment of the Ca²⁺-dependent cysteine protease, calpain (Tyagarajan *et al.*, 2011). Upon recruitment, calpain then cleaves and degrades the scaffold; which can in turn lead to the dispersion of GABA_A receptors from the synaptic compartment (Kawasaki *et al.*, 1997; Petrini and Barberis, 2014; Battaglia *et al.*, 2018).

In addition to alterations of synaptic scaffolding proteins and surface expression, the functionality of GABA_A receptors is also modulated by their surface diffusion in the neuronal membrane (Lombardi, Kinzler and Jacob, 2020; Merlaud *et al.*, 2022). Experimental data investigating the surface dynamics of GABA_A receptors, by quantum-dot single particle tracking (QD-SPT), revealed a significantly reduced mobility at synaptic compartments, compared to extrasynaptic sites (Bannai *et al.*, 2009; de Luca *et al.*, 2017; Hannan *et al.*, 2020). This indicated that association of GABA_A receptors with gephyrin reduces the mobility of the ion channels, enabling their retention and stabilisation within postsynaptic densities (Renner, Specht and Triller, 2008). Further studies have demonstrated that the synaptic population of GABA_A receptors is constitutively and rapidly replenished by receptor exchange between synaptic and extrasynaptic compartments, indicating the importance of well-regulated transport of GABA_A receptors in the plasma membrane (Maynard and Triller, 2019). Intriguingly, increasing neuronal excitability with potassium channel blocker, 4-aminopyridine, increased the diffusion of both synaptic and extrasynaptic GABA_A receptors (Niwa *et al.*, 2012). This increase in surface dynamics was shown to result in reduced clustering of both GABA_A receptors and gephyrin at inhibitory synapses and reduce GABAergic synaptic transmission (Niwa *et al.*, 2012). Such impacts were demonstrated to rely on NMDA receptor-mediated calcium influx, triggering the activation of calcineurin (Niwa *et al.*, 2012). A similar pathway has been described, in which excitatory LTP-inducing stimulation of CA1 Schaffer collaterals in the hippocampus results in LTD of inhibitory synapses (Wang *et al.*, 2003). In this cascade, inhibitory LTD is dependent on NMDA receptor-mediated activation of calcineurin preceding its

recruitment to GABA_A receptor complexes (Wang *et al.*, 2003). Calcineurin dephosphorylates a serine residue on the $\gamma 2$ subunit (S327), resulting in increased mobility and reduced residency within postsynaptic compartments (Wang *et al.*, 2003; Muir *et al.*, 2010). Yet, the precise molecular mechanisms linking $\gamma 2$ subunit-phosphorylation and alterations to lateral mobility remain to be fully elucidated.

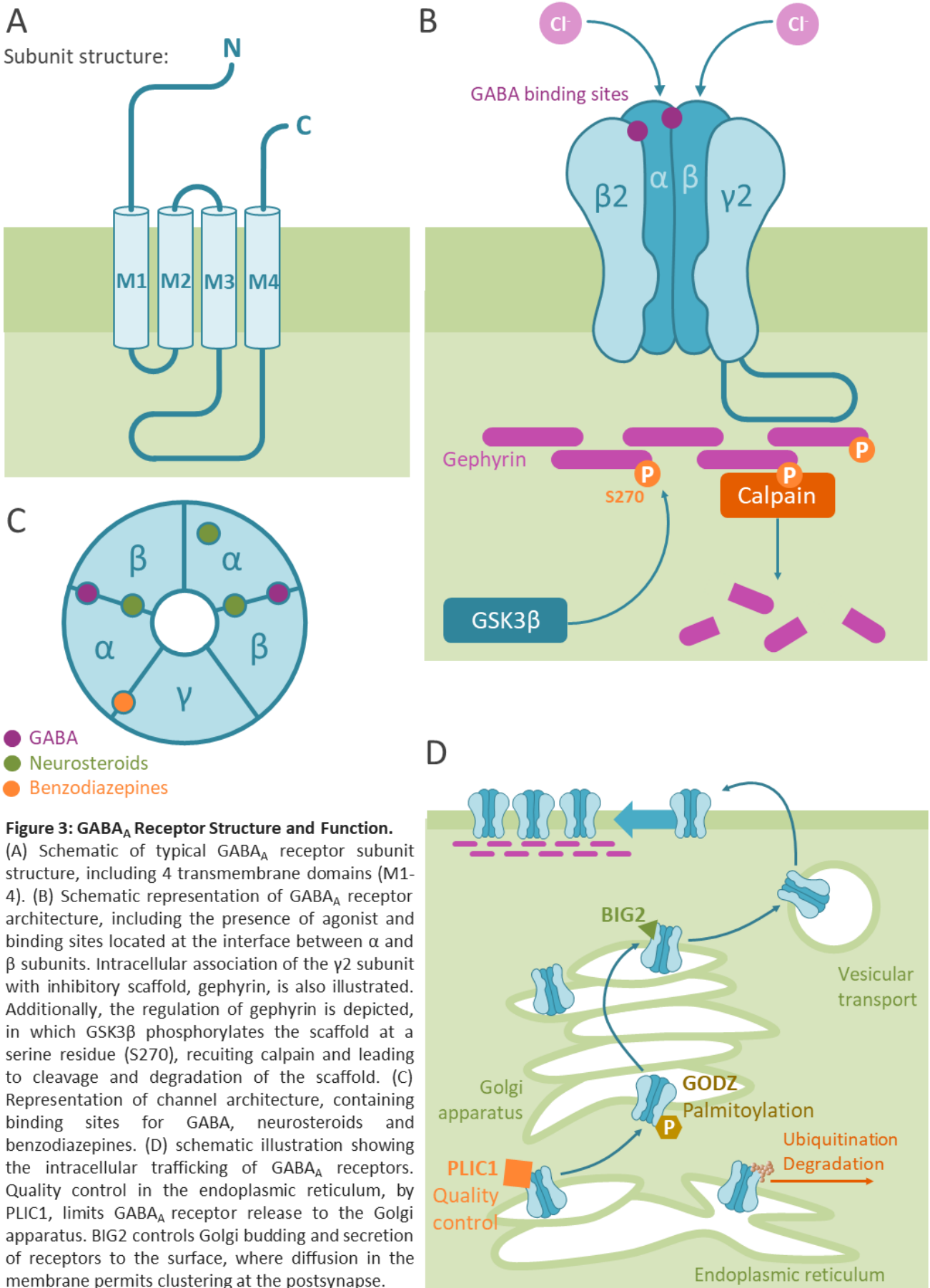


Figure 3: GABA_A Receptor Structure and Function. (A) Schematic of typical GABA_A receptor subunit structure, including 4 transmembrane domains (M1-4). (B) Schematic representation of GABA_A receptor architecture, including the presence of agonist and binding sites located at the interface between α and β subunits. Intracellular association of the $\gamma 2$ subunit with inhibitory scaffold, gephyrin, is also illustrated. Additionally, the regulation of gephyrin is depicted, in which GSK3 β phosphorylates the scaffold at a serine residue (S270), recruiting calpain and leading to cleavage and degradation of the scaffold. (C) Representation of channel architecture, containing binding sites for GABA, neurosteroids and benzodiazepines. (D) schematic illustration showing the intracellular trafficking of GABA_A receptors. Quality control in the endoplasmic reticulum, by PLIC1, limits GABA_A receptor release to the Golgi apparatus. BIG2 controls Golgi budding and secretion of receptors to the surface, where diffusion in the membrane permits clustering at the postsynapse.

Subsection Summary

Fast excitatory neurotransmission relies on the glutamatergic AMPA receptor, which undergoes changes in synaptic density to facilitate synaptic plasticity (Diering and Huganir, 2018). This plasticity can be achieved through alterations in receptor surface dynamics, synaptic localization, and modulation of endo- and exocytotic processes (Choquet and Hossy, 2020). Advances in microscopy have revealed the sub-synaptic organization of AMPA receptors in nanodomains closely associated with scaffolding proteins such as PSD-95 and presynaptic proteins like RIM1 (Heine and Holcman, 2020; Fukata *et al.*, 2021; Sun *et al.*, 2022). Neurexin and neuroligin are potential transsynaptic proteins involved in aligning AMPA receptors with presynaptic release sites, forming nanocolumns (Choquet and Hossy, 2020; Fukata *et al.*, 2021). Disruption of this architecture can impair AMPA receptor signalling and synaptic function (Tang *et al.*, 2016; Heine and Holcman, 2020). During synaptic plasticity, AMPA receptors can undergo lateral mobilization, as observed in NMDA receptor-dependent LTD (Compans, Choquet and Hossy, 2016; Choquet, 2018). NMDA receptor activation induces a reduction in AMPA receptor expression without major changes in nanoscale topography, but with increased surface dynamics. Phosphorylation of PSD-95 mediates this effect by causing degradation of the synaptic scaffold, unanchoring AMPA receptors from postsynaptic compartments (Nelson *et al.*, 2013; Compans *et al.*, 2021). The interplay between NMDA and AMPA receptors is critical for maintaining a healthy ratio at excitatory synapses and regulating synaptic activity. Perturbations in these mechanisms can disrupt synaptic plasticity and homeostatic function.

NMDA receptors are distinct from AMPA receptors and play a crucial role in calcium influx, which is necessary for various cellular processes in the brain. NMDA receptors require both glutamate release and postsynaptic depolarization to allow calcium entry, making them coincidence detectors (Seeburg *et al.*, 1995). The NMDA receptor is composed of four subunits, including two obligatory GluN1 subunits and two GluN2 subunits (Vyklícky *et al.*, 2014). The GluN1 subunit isoforms and GluN2 subunits confer unique functional properties to the receptor, affecting agonist potency, kinetics, and sensitivity to magnesium blockade (Paoletti and Neyton, 2007; Flores-Soto *et al.*, 2012). The GluN2 subunit also determines receptor deactivation and impacts synaptic currents (Tu and Kuo, 2015; Y.-S. Chen *et al.*, 2020). The expression profiles of GluN2 subunits are developmentally and regionally regulated, shaping synaptic phenotypes over time and in different brain regions and cell types (Bai and Hoffman, 2009; Herrera-Zamora *et al.*, 2019; Haddow, Kind and Hardingham, 2022; Ramírez *et al.*, 2023). Additionally, NMDA receptor interactions within the C-terminal domain and their activity have been found to modulate other receptor distributions, such as GABAA receptors, further influencing the balance between excitation and inhibition in the brain (Gault and Siegel, 1998; Marsden *et al.*, 2007; Wu *et al.*, 2021).

GABA_A receptors are responsible for the majority of inhibitory neurotransmission in the brain. GABA_A receptors contain binding sites for the neurotransmitter GABA, as well as alternative binding sites for variety of endogenous neuromodulators (Sallard, Letourneur and Legendre, 2021; Sente *et al.*, 2022). Activation of GABA_A receptors by GABA leads to the influx of chloride ions, resulting in hyperpolarization of the membrane and a reduction in neuronal activity. GABA_A receptors are trafficked to and from the neuronal membrane through intricate protein-protein interactions (Jacob, Moss and Jurd, 2008; Luscher, Fuchs and Kilpatrick, 2011). Palmitoylation and phosphorylation play important roles in receptor clustering at postsynaptic compartments, membrane trafficking, and synaptic function (Kittler and Moss, 2003; Keller *et al.*, 2004; Lüscher and Keller, 2004; Comenencia-Ortiz, Moss and Davies, 2014). Gephyrin, the major inhibitory synaptic scaffolding protein, stabilizes GABA_A receptors at synapses (Groeneweg *et al.*, 2018). Phosphorylation of gephyrin can modulate its stability, and dysregulation of this process can also lead to the dispersion of GABA_A receptors from synaptic compartments (Kuhse *et al.*, 2012; Kalbouneh *et al.*, 2014; Zacchi, Antonelli and Cherubini, 2014; Flores *et al.*, 2015; Battaglia *et al.*, 2018).

The functionality of GABA_A receptors is also influenced by their surface diffusion in the neuronal membrane. Experimental studies have shown that the rate of diffusion of extrasynaptic receptors is higher than that of synaptic receptors (Lombardi, Kinzmaier and Jacob, 2020; Merlaud *et al.*, 2022). Increased neuronal excitability and calcium influx can enhance receptor diffusion and lead to reduced clustering of GABA_A receptors and gephyrin at inhibitory synapses, affecting GABAergic synaptic transmission (Bannai *et al.*, 2009; Battaglia *et al.*, 2018). Overall, GABA_A receptors play a vital role in maintaining brain health and are involved in various neurological functions. Dysregulation of receptor activity can contribute to plethora of neurological disorders (Jembrek and Vlainic, 2015; Walker, 2018; Maljevic *et al.*, 2019; Sakimoto *et al.*, 2021). Understanding the structure, trafficking, and functional modulation of GABA_A receptors, and their reciprocal coregulation with other major ion channels – including NMDA and AMPA receptors – is important for advancing our knowledge of excitation-inhibition balance, and its implications for brain function and disease.

Applications to the Clinical Challenges of Autoimmune Encephalitis

Recent years have seen the identification of an array of autoimmune neurological syndromes, whereby patients express antibodies directed against brain-endogenous antigens. Two prominent diseases in this category are anti-NMDA receptor autoimmune encephalitis and anti-GABA_A receptor autoimmune encephalitis, in which patients develop antibodies directed against major excitatory or inhibitory ionotropic channels, respectively (Dalmau *et al.*, 2007, 2008; Sansing *et al.*, 2007; Ohkawa *et al.*, 2014; Petit-Pedrol *et al.*, 2014). Molecular investigations have delineated an acute pathway of action of these autoantibodies, generally focussing on the isolated impacts on the target antigens. For instance, it has been demonstrated that anti-NMDA receptor autoantibodies bind to the extracellular N-terminal domain of the obligatory GluN1 subunit (Amrutkar *et al.*, 2012; Gleichman *et al.*, 2012). This antibody binding causes a synaptic destabilisation of the NMDA receptors through hindrance of the interaction between negatively-charged sequences on the GluN1 N-terminal domains with synaptically localised and positively-charged EphB2 receptors (Mikasova *et al.*, 2012; Ladépêche *et al.*, 2018; Washburn *et al.*, 2020). This destabilisation causes an increase in the surface dynamics of synaptic NMDA receptor populations at acute timescales, promoting displacement to extrasynaptic sites (Ladépêche *et al.*, 2018). Subsequent crosslinking of extrasynaptic NMDA receptor clusters, accompanied by a negative shift in surface mobility, promotes internalisation of receptors (Hughes *et al.*, 2010; Mikasova *et al.*, 2012). This depletion of surface expression results in an excitatory hypofunction on hippocampal neurons (Hughes *et al.*, 2010; Huang and Xiong, 2021). Conversely, anti-GABA_A receptor autoantibody binding elicits a direct antagonism of the inhibitory ion channel, with little focus placed on a possible alteration of surface organisation and diffusion (Noviello *et al.*, 2022). Direct antagonism of GABA_A receptor channels has been demonstrated to occur through competition by GABA_A receptor autoantibodies for orthosteric GABA binding sites at the interface between α and β subunits, and also through negative allosteric modulation of the channel (Noviello *et al.*, 2022). Such antagonism thus drives a hypofunction of the major inhibitory neurotransmission system. Essentially, NMDA- and GABA_A receptor autoantibodies have been shown to drive either excitatory or inhibitory hypofunction, respectively. Intriguingly, despite the considerable opposition in the function of these autoantibody targets, seizure phenotypes form a prominent facet of a largely shared symptomology in these two distinct autoimmune diseases (Petit-Pedrol *et al.*, 2014; Geis *et al.*, 2019; Kreye *et al.*, 2021).

Given the array of homeostatic systems in place, each dependent on effective cross-talk between excitatory and inhibitory signalling, the mechanistic underpinnings of autoimmune epileptogenesis may reside in pathological co-regulation of excitatory and inhibitory receptors (Hunter, Jamet and Groc, 2021; Andrzejak *et al.*, 2022). As such, we might expect NMDA- and GABA_A receptor antibodies to elicit downstream perturbations across both neurotransmission systems, inhibitory and excitatory. However, the characterisation of this cross-synaptic disruption remains to be fully investigated (Iizuka, Sakai and Mochizuki, 2010). We hypothesise that antibody-mediated interference of NMDA- or GABA_A receptor dynamics, organisation and function will

metastasise into global dysregulation of ionotropic signalling, impacting glutamatergic and GABAergic functions, expressed throughout the synaptic, cellular and network scales of neuronal activity. Investigations into the co-regulation of glutamatergic and GABAergic systems, in this context of autoimmune encephalitis, may provide novel avenues for therapeutic development and intervention for these disease states. Moreover, identification of excitatory-inhibitory co-regulation in these diseases may further expand our knowledge regarding the interaction between excitatory and inhibitory systems in the maintenance of hippocampal homeostasis on a more fundamental level.

Clinical Features of NMDA Receptor Encephalitis

In the last two decades, anti-NMDA receptor encephalitis – a single multistage disease – has been described. Initially, Dalmau *et al.* (2007) identified a patient cohort who presented initially with a viral-like prodromal phase followed sharply by psychotic symptoms bearing a striking resemblance to that of a first psychotic episode in schizophrenia, however often concurrent with subtle indicators of underlying neurologic pathology including dyskinesia (Dalmau *et al.*, 2008; Wandinger *et al.*, 2011). Later stages of the disease progression comprise a seizure phenotype and a worsening autonomic dysfunction leading to fatality if untreated (Wandinger *et al.*, 2011; Byun *et al.*, 2015; Veciana *et al.*, 2015; Mehr *et al.*, 2016). The Dalmau team and others identified that the root of this disorder was by the pathogenic action of patient-generated autoantibodies directed against the extracellular portion of the NMDA receptor (Dalmau *et al.*, 2008; Hughes *et al.*, 2010; Gleichman *et al.*, 2012). While the underlying cause of NMDA receptor encephalitis can vary from patient-to-patient, and in many cases may remain unknown, large cohorts of female patients present with underlying ovarian tumour (Dalmau *et al.*, 2007; Titulaer *et al.*, 2013; Lynch *et al.*, 2018). This is thought to trigger an immune response against NMDA receptors due to the high levels of expression of NMDA receptors within the teratoma itself (Dalmau *et al.*, 2007). Since this first description of anti-NMDA receptor encephalitis, there has been a considerable research effort to elucidate the mechanisms by which these patient autoantibodies destabilise neurological function, leading to a disease phenotype with such a heterogeneous selection of symptoms: progressing through the cognitive, behavioural and autonomic domains of neurological function.

A particularly striking feature of anti-NMDA receptor encephalitis is the reliable phases of disease progression (Wandinger *et al.*, 2011; Graus *et al.*, 2016; Dalmau *et al.*, 2019). It has been suggested that prior to encephalitic symptom onset, patients experience a viral-like prodromal phase with persistent intense headache, fever and rarely associated with emesis. In the majority of cases, this prodromal phase appears to self-resolve spontaneously, shortly before the presentation of encephalitic symptoms (Tominaga *et al.*, 2018; Gurrera, 2019; Ma *et al.*, 2019). In an early review of 100 patients, psychiatric symptoms were observed in all cases during the initial phase of proper encephalitic illness (Dalmau *et al.*, 2008). However, this initial

psychiatric phase is considerably heterogeneous between patients, experiencing symptoms of psychosis (auditory and visual hallucination), depression, mania, eating disorder, addiction (Kayser, Kohler and Dalmau, 2010; Schieveld *et al.*, 2019; Forrester *et al.*, 2020). Alarming, upon presentation, approximately 77% of patients are primarily examined by psychiatrists, and 40% of these patients were initially sectioned in psychiatric institutions as opposed to neurological wards, despite the majority showing concurrent neurologic symptoms during initial evaluation (Dalmau *et al.*, 2008; Lejuste *et al.*, 2016). Not only may misdiagnosis of anti-NMDA receptor encephalitis as psychiatric disorder impede effective and timely treatment, but may be directly damaging, as this encephalitis is known to associate with an intolerance to common psychiatric medication, including antipsychotics, as they can worsen motor dysfunction if present (Irani *et al.*, 2010; Mohammad *et al.*, 2014; Schieveld *et al.*, 2019; Warren *et al.*, 2021). Many have suggested that screening for all patients presenting with seemingly rapid onset psychiatric symptoms for possible prodromal symptoms or, in women, the concurrence of ovarian tumour, may aid the clinician in successfully identifying cases of anti-NMDA receptor encephalitis from generalised psychiatric disorders (Câmara-Pestana *et al.*, 2022).

Further, a secondary encephalitic feature commonly observed in this patient group is an epileptic phenotype, where seizure occurrence is intermittent with psychiatric features (Titulaer *et al.*, 2013; Dalmau *et al.*, 2019). A recent review of 86 paediatric cases of this encephalitis revealed that 72% developed some seizure phenotype during the course of disease, where most developed within the first two weeks after initial symptom onset, similar reports in adults estimate a seizure-phenotype prevalence of 73% (Qu *et al.*, 2020; Chen *et al.*, 2021). Similarly to the psychiatric phase, this seizure component of encephalitis is considerably heterogeneous, with patients developing focal, faciobrachial dystonic and tonic-clonic seizures, or status epilepticus (de Bruijn *et al.*, 2019). In a review of paediatric cases, over 40% of patients developed status epilepticus, with prolonged seizure activity and limited recovery in the interval period (Qu *et al.*, 2020). Moreover, in a large-scale review of electroencephalographic (EEG) data collected from over 400 anti-NMDA receptor encephalitis cases, abnormality in EEG activity was shown to correlate with case referral to intensive care units and latency to recovery from disease (Gillinder *et al.*, 2019). Specifically, abnormalities in the delta range were strong indicators of both of these factors (Gillinder *et al.*, 2019).

Continuing progression of anti-NMDA receptor encephalitis develops into autonomic instability in the vast majority of cases (Engen and Agartz, 2016; Lin and Lin, 2020). Hypoventilation, often requiring respirator support, is considered a particular hallmark symptom of this condition, however other autonomic manifestations can include hypo- and hypertension, hypo- and hyperthermia, bradycardia and tachycardia (Dalmau *et al.*, 2008, 2011; Florance *et al.*, 2009). Interestingly, it is suggested that these autonomic dysfunctions are irrespective of any epileptic phenotype, instead representing a separable mechanism of action for the pathogenic autoantibodies underlying each stage of encephalitic illness (Yan *et al.*, 2021). These late stages of severe autonomic distress which, if untreated will lead to fatality, is generally considered to be

the summit of disease progression (Dalmau *et al.*, 2011; Zhong *et al.*, 2022). Interestingly, anti-NMDA receptor autoantibody titres have been shown to correlate with both disease progression and eventual patient outcome, where high titres are associated with later stages of disease progression and poorer recovery outlook (Gresa-Arribas *et al.*, 2014; Ciano-Petersen *et al.*, 2021), although this has been disputed by some evidence demonstrating an unclear relationship between titre measurements and prognostic value (Broadley *et al.*, 2019). This may suggest that a dose-dependent effect of autoantibody exposure provokes discrete effects on network behaviour, giving rise to the variable disease phases.

Current treatments of NMDA receptor encephalitis should aim to address both the underlying cause and manage the clinical symptoms at presentation. Typically, first-line treatments are generalised immunomodulators, for instance the use of corticosteroids, intravenous immunoglobulins, or plasma exchange (Nissen *et al.*, 2020). If a teratoma is identified, it should be removed (Dalmau *et al.*, 2019). Early identification of disease, providing rapid therapeutic intervention, such as tumour resection in combination with immunomodulatory treatments, are effective in reducing antibody titers and improving prognosis (Titulaer *et al.*, 2013). Lack of responsiveness to first-line therapeutics, or delayed treatment onset can require subsequent second-line immunosuppression with drugs like rituximab or cyclophosphamide (Wang, 2016; Kimura, 2022). To address acute clinical symptoms, antipsychotics are often used in conjunction with other therapies to manage behavioural and psychotic disturbances, if present. However, antipsychotic intolerance has been associated with NMDA receptor encephalitis, providing a challenge to current clinical care of patients (Lejuste *et al.*, 2016; Giné Servén *et al.*, 2019; Pacheco-Barrios *et al.*, 2022; Sarmiento *et al.*, 2022). However, benzodiazepines and other medications have been used to address specific symptoms, including sleep disturbance and catatonia, with mixed reports of patient responses (Maat *et al.*, 2013; Kruse *et al.*, 2014; Kuppuswamy, Takala and Sola, 2014; Mohammad *et al.*, 2014). Ultimately, greater understanding of the functional and molecular mechanisms of this disease are urgently needed, in the hope to identify possible avenues to targeted therapeutic intervention. This is particularly evident considering the current lack of effective available therapies for symptom management, administered alongside generalised immunomodulation – in which patient responsiveness is typically delayed for weeks to months (Titulaer *et al.*, 2013; Dalmau *et al.*, 2019; Kimura, 2022). Since the identification of NMDA receptor encephalitis, there has been a concerted research effort to understand the mechanistic underpinnings of neuronal pathology, and a primary aim of this thesis is to contribute to that accumulation of understanding.

Molecular Mechanisms Implicated in the Hippocampal Pathology of NMDA Receptor Encephalitis

Since the identification of pathogenic autoantibodies directed against the NMDA receptor in encephalitis, there has been considerable interest in identification of the precise epitope – or epitopes – targeted by

immunoglobulins. Early experimental investigations suggested that the majority of NMDA receptor autoantibodies target the N-terminal domain of the GluN1 subunit, specifically in a region proximal to an asparagine glycosylation site at N368 (Gleichman *et al.*, 2012). However, it should be noted that this study analysed the binding of NMDA receptor autoantibodies to a library of GluN1 mutants expressed in human embryonic kidney (HEK) cells, and it has since been demonstrated elsewhere that GluN1-N368Q mutation impedes expression of NMDA receptors on the surface of this cell line, instead GluN1-N368Q is retained in the endoplasmic reticulum (Lichnerova *et al.*, 2015). Further studies have elucidated that NMDA receptor autoantibodies can arise from distinct B-cell lineages in single patients, and distinct monoclonal antibodies can effectively bind at non-overlapping epitopes, providing competing evidence that the reactivity of autoantibodies does not solely reside in this single N-terminal domain epitope (Sharma *et al.*, 2018; Taraschenko, Fox, Eldridge, *et al.*, 2021). Intriguingly, Sharma *et al.* (2018) also suggested that some autoantibodies display preference to either synaptic or extrasynaptic populations of surface NMDA receptors, and that such heterogeneity in immunoreactivity may be responsible for variable symptom presentation observed at the clinical-scale of disease. Autoantibodies directed against the GluN2A subunit of the NMDA receptor have also been identified more recently, in a patient with recurrent NMDA receptor encephalitis (Zhou *et al.*, 2022). Despite this current debate in the field, we would note that monoclonal autoantibody samples provide a methodological route to investigate antibody-mediated dysfunction – in the absence of confounding factors such as unknown modulatory factors in the cerebrospinal fluid and contamination with other uncharacterised anti-neuronal autoantibodies. Moreover, 2-week infusion of single monoclonal autoantibody samples into mice has been shown to induce seizures and behavioural deficits in memory, consistent with epilepsy and the cognitive dysfunction observed at the patient-scale (Malviya *et al.*, 2017; Taraschenko, Fox, Eldridge, *et al.*, 2021; Taraschenko, Fox, Zekeridou, *et al.*, 2021). These findings are also similar to those found in rodent models of the disease, produced by infusion with patient cerebrospinal fluid, suggesting that single monoclonal autoantibodies are – at least in some cases – sufficient to induce a substantive disease model (Li *et al.*, 2015; Planagumà *et al.*, 2015; Carceles-Cordon *et al.*, 2020; Taraschenko, Fox, Zekeridou, *et al.*, 2021). Evidently however, rigorous epitope mapping is further required to better understand the immunoreactivity of pathogenic autoantibodies in this disease state, and such work will enable us to more finely elucidate the possibility of clone-specific modulations on NMDA receptor function and disease presentation.

Elucidation of the precise pathogenic mechanisms of NMDA receptor autoantibodies has been heavily investigated in recent years. Considering the role of NMDA receptors to function as ionotropic channels, much focus has been directed towards modification in NMDA receptor-mediated currents after autoantibody exposure. Intriguingly, studies have demonstrated that exposure of hippocampal neuronal cultures to patient-derived cerebrospinal fluid resulted in reduced NMDA receptor-mediated currents – both synaptically-

mediated phasic currents and extrasynaptically-mediated tonic currents (Hughes *et al.*, 2010; Kreye *et al.*, 2016). However, characterisation of residual channel function revealed that NMDA receptor autoantibodies elicit no detectable alteration to individual channel function (Moscatto *et al.*, 2014). Taken together, these data suggest that autoantibodies drive a hypofunction of NMDA receptors through a depletion of their surface expression, not via a direct modification in the functional properties of the channel (Wollmuth, Chan and Groc, 2021, Figure 5). Although it should also be noted that some studies have suggested a preferential binding of some monoclonal NMDA receptor antibodies to receptors in the open conformation, and that autoantibody binding can promote the maintenance of an open state (Gleichman *et al.*, 2012). The depletion of NMDA receptors from synaptic compartments has been directly visualised in a range of literature, employing monoclonal and polyclonal antibody samples, and also patient cerebrospinal fluid (Hughes *et al.*, 2010; Moscatto *et al.*, 2014; Masdeu, Dalmau and Berman, 2016; Castillo-Gómez *et al.*, 2017; Ladépêche *et al.*, 2018; Amedonu *et al.*, 2019). These studies have demonstrated that a primary driver of disease is the reduction in synaptic localisation of NMDA receptors, which has been well replicated across various patient-derived samples.

Lateral mobility of NMDA receptors constitutes a key regulator of their function at the neuronal surface (Groc *et al.*, 2006; Newpher and Ehlers, 2008; Dupuis and Groc, 2020). Given that redistribution of receptors at the subcellular scale is implicated in the pathology of NMDA receptor encephalitis, a great deal of work has been carried out to finely characterise the impact of autoantibodies on the surface trafficking of NMDA receptors, after exposure to autoantibodies. These investigations have demonstrated that alterations to surface diffusion play a key role in aetiology of disease (Mikasova *et al.*, 2012; Gréa *et al.*, 2019). Principally, quantum-dot single particle tracking experiments have revealed that acute administration of polyclonal NMDA receptor autoantibodies increases the mobility of synaptic NMDA receptors, indicated by increase in both mean square displacement and diffusion coefficients of GluN2A-containing NMDA receptors (Mikasova *et al.*, 2012). It is believed that this increase in receptor mobility is – at least partially – induced by antibody-mediated hinderance of GluN1 interaction with EphB2 receptors in the postsynaptic compartment (Mikasova *et al.*, 2012). This hypothesis is supported by the finding that autoantibody application reduces the association of NMDA receptors with EphrinB2 receptors, and that activation of ephrinB2 receptors – which strengthens its interaction with GluN1 – hinders antibody-mediated synaptic displacement (Mikasova *et al.*, 2012). Interestingly however, autoantibody exposure promotes a converse immobilisation of extrasynaptically localised GluN2B-containing receptors, and this reduction on extrasynaptic mobility is consistent with other reports, demonstrating antibody-mediated crosslinking of NMDA receptors at extrasynaptic sites (Hughes *et al.*, 2010; Mikasova *et al.*, 2012; Ladépêche *et al.*, 2018; Ismail and Faustmann, 2020; Huang and Xiong, 2021; Rahman *et al.*, 2023). Together with prior results, this data supports a molecular model of disease, in which NMDA receptor autoantibodies disrupt synaptically anchoring protein-protein interactions resulting in a

displacement of receptors from postsynaptic compartments. Subsequent to mislocalisation, enrichment of NMDA receptors at extrasynaptic compartments leads to the generation of immobilised macro-clusters of crosslinked receptors, which are then internalised from the surface of the neuron (Figure 5). Ultimately this drives a functional reduction in NMDA receptor-mediated currents at synaptic compartments, despite a lack of direct antibody-mediated modification of channel function (Wright *et al.*, 2021). However, we would also note here that an alternative mode of action may explain observed synaptic deficits, whereby autoantibodies bind predominantly to extrasynaptic receptors, impeding the surface trafficking and entry to synaptic sites (Hunter, Jamet and Groc, 2021). In this case, autoantibodies may not directly bind to the synaptically localised channels, instead altering the equilibrium of lateral diffusion mediating entry and exit from postsynaptic compartments, reviewed in Hunter *et al.* (2021).

Given that NMDA receptor signalling is a complex regulator of synaptic plasticity, many studies have investigated the impact of patient autoantibodies on induction of long-term potentiation (Zhang *et al.*, 2012; Würdemann *et al.*, 2016; Blome *et al.*, 2018; Radosevic *et al.*, 2022). It has been widely demonstrated that exposure of neuronal cultures to NMDA receptor autoantibodies modulates plasticity, whereby a variety of potentiating stimuli fail to induce potentiated synaptic response, or in some cases instead promotes long-term depression (Zhang *et al.*, 2012; Dupuis *et al.*, 2014; Würdemann *et al.*, 2016; Blome *et al.*, 2018; Radosevic *et al.*, 2022). Evidently, autoantibody-induced deviation of physiological NMDA receptor function extends beyond isolated impacts on NMDA receptor-mediated current, and will have substantial long-term impacts on the function of the excitatory synapse and network homeostasis (Jurek *et al.*, 2019; Wright *et al.*, 2021; Andrzejak *et al.*, 2022). However, early investigations into autoantibody-mediated pathogenesis suggested that excitatory synaptic scaffolding protein, PSD-95, and the synaptic localisation of AMPA receptors were unaltered after acute exposure to patient antibody samples (Hughes *et al.*, 2010). More recent data from *in vivo* rodent models, however, has identified reductions in the amplitude of AMPA receptor-mediated synaptic currents after chronic infusion of NMDA receptor autoantibodies (Wright *et al.*, 2021). Given the pivotal regulatory role of NMDA receptor function on other membrane proteins – most notably synaptic AMPA receptors – further work is urgently required to unveil possible impacts of antibody-mediated NMDA receptor disorganisation on overall excitatory synaptic function (Ceanga *et al.*, 2022).

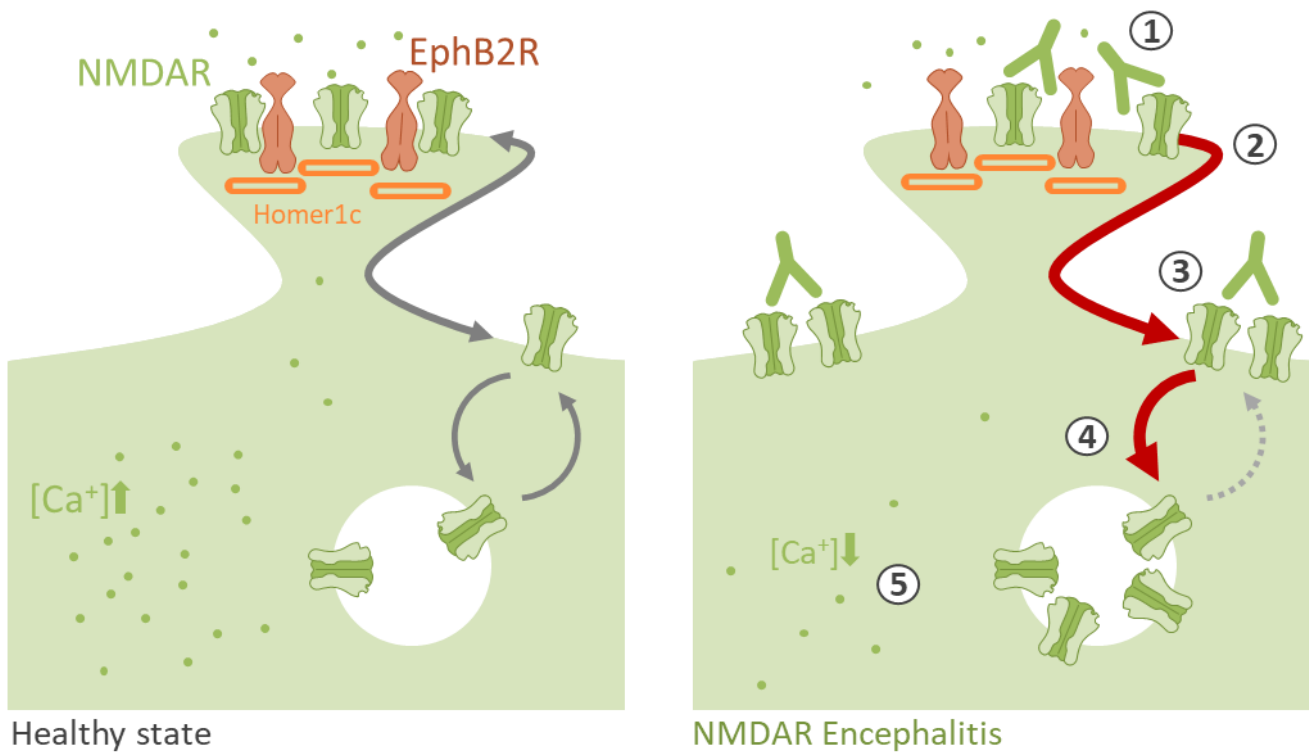


Figure 5: Molecular Mechanisms in NMDA Receptor Autoimmune Encephalitis.

Schematic representation of NMDA receptor autoantibody pathogenicity. Left panel denotes the healthy state in which receptors are trafficked to and from the membrane, freely diffuse, and anchor at synapses through interaction with Homer-1c and EphB2 receptors 1) Antibody binding to GluN1 subunits elicits 2) an increase in lateral mobility and synaptic displacement. 3) Crosslinking at extrasynaptic compartments promotes 4) receptor internalisation. 5) Loss of surface NMDA receptors drives a reduction in channel function and calcium influx.

Clinical Features of GABA_A Receptor Encephalitis

While NMDA receptor encephalitis constitutes the highest prevalence of encephalitis diagnoses, patients have also been identified with autoantibodies targeting the GABA_A receptor (Ohkawa *et al.*, 2014; Petit-Pedrol *et al.*, 2014). In 2014, Petit-Pedrol *et al.* were amongst the first to formally recognise the associated condition of anti-GABA_A receptor autoimmune encephalitis (herein referred to as GABA_A receptor encephalitis). The presence and action of GABA_A receptor autoantibodies in these patients leads to severe neurological dysfunction and a wide variety of symptoms. As for NMDA receptor encephalitis, the clinical presentation can vary between patients; however, a review of 26 patient case reports suggested there are a typical set of core-symptoms that are largely observed in affected individuals. These include seizures (88%), cognitive dysfunction (67%), behavioural alterations (46%), decreased level of consciousness (42%) and motor dysfunction (35%) (Spatola *et al.*, 2017).

Arguably, severe seizure phenotypes form the most prominent feature of GABA_A receptor encephalitis, present in approximately 90% of patients (Petit-Pedrol *et al.*, 2014; Spatola *et al.*, 2017; Guo, Gelfand and Geschwind, 2020). However, these seizure phenotypes can present in a variety of forms, including focal

seizures, generalized tonic-clonic seizures, and status epilepticus (Petit-Pedrol *et al.*, 2014). In many cases, patients display low responsivity to typical antiepileptic medications providing a clinical challenge to the currently available therapeutic interventions (Fukami *et al.*, 2017; Guo, Gelfand and Geschwind, 2020). In addition to seizure, cognitive dysfunction is also observed in a majority of GABA_A receptor encephalitis cases (Spatola *et al.*, 2017; Tappatà *et al.*, 2022). This typically takes the form of memory deficit, difficulties with maintaining attention and focus, impaired judgement, confusion and executive dysfunction (Petit-Pedrol *et al.*, 2014; Spatola *et al.*, 2017). Behavioural alterations in GABA_A receptor encephalitis are commonly reported, however the precise nature of such alteration can be difficult to determine from case report data. A recent review of 50 patients reported 26% (13/50) contained the terms “behavioural changes”, “personality changes”, “anxiety” or “depression” (Guo, Gelfand and Geschwind, 2020). This study suggested that dysautonomia has also been associated with GABA_A receptor encephalitis, identified in 8% (4/50) case reports in which patients may experience abnormalities in regulation of blood pressure, heart rate and body temperature (Guo, Gelfand and Geschwind, 2020). However, the authors also note here that one of these patients had NMDA receptor autoantibodies in serum samples. Given that NMDA receptor encephalitis is more commonly associated with autonomic dysfunction, this presentation may be more difficult to attribute directly to the presence of GABA_A receptor autoantibodies in this patient (Engen and Agartz, 2016; Guo, Gelfand and Geschwind, 2020; Lin and Lin, 2020).

Rates of motor dysfunction – including orofacial dyskinesia, dystonic and abnormal posture, generalized choreoathetosis and tremor – vary between reports, Spatola *et al.* (2017) identify movement disorder in 35% (9/26) of patients, whereas Guo *et al.* (2020) suggest a lower rate of 14% (7/50) noting that two of these seven cases also presented with NMDA receptor autoantibodies in serum, which may explain the apparent disordered motor function – otherwise misattributed to GABA_A receptor autoantibody-mediated pathology. Additionally, conflicting reports concerning psychiatric manifestations are apparent in GABA_A receptor encephalitis case literature. Some reports indicate the presence of hallucinations in this condition, at approximately 14% (7/50) of cases (Guo, Gelfand and Geschwind, 2020). However, in a subsequent review of the available cited cases, we identified 4 reports indicating the presence of hallucination; and in two of these cases, patients expressed concomitant autoantibodies against GABA_B receptors, acetylcholine receptors and voltage gated potassium channels (Ohkawa *et al.*, 2014; Petit-Pedrol *et al.*, 2014). In the other two cases, it is unclear if patients were also screened for the presence of other autoantibodies or the original nature of disease which lead to serum analysis (Pettingill *et al.*, 2015). As such, the association between GABA_A receptor autoantibodies presence and hallucination should be approached with some caution.

The underlying trigger for GABA_A receptor encephalitis appears to be somewhat heterogeneous, often comorbid with other autoimmune conditions and cancer (Simabukuro *et al.*, 2015; Graus *et al.*, 2016; Spatola *et al.*, 2017; Brändle *et al.*, 2021; Tappatà *et al.*, 2022). Spatola *et al.* (2017) identified concurrent illness in 42%

(11/26) cases, of which: 64% had an underlying tumour, 18% developed autoimmune encephalitis subsequent to viral (herpes simplex 1 and human herpesvirus 6) encephalitis, and 18% comorbid with myasthenia gravis. Intriguingly, both patients who developed autoimmune encephalitis subsequent to viral infection were positive for both GABA_A receptor and NMDA receptor autoantibody screening (Spatola *et al.*, 2017).

The standard diagnostic procedures involved in identification of GABA_A receptor encephalitis are similar to that of other autoimmune encephalitides, including an evaluation of clinical presentation, neuroimaging, electroencephalography, cerebrospinal fluid analysis and antibody screening (Graus *et al.*, 2016). Neuroimaging of patients with GABA_A receptor encephalitis revealed abnormalities in T2/fluid-attenuated inversion recovery (FLAIR) MRI (Petit-Pedrol *et al.*, 2014). Specifically, multifocal abnormalities of both grey and white matter across are regularly identified, impacting two or more of the following brain regions: temporal, frontal, parietal, occipital, basal ganglia, cerebellum and brainstem (Petit-Pedrol *et al.*, 2014; Vacchiano *et al.*, 2019; B. Deng *et al.*, 2022; Baqal, Vanood and Harahsheh, 2023). Further, FLAIR-MRI anomalies display asynchronicity, where impacted regions appear and resolve independently of one-and-other, typically within days to weeks (Petit-Pedrol *et al.*, 2014). Electroencephalography reveals epileptiform activity in the majority of cases, particularly in temporal lobe areas (Petit-Pedrol *et al.*, 2014). Analysis of cerebrospinal fluid also shows abnormalities in the majority of cases, particularly increased protein concentration and white blood cell count, and in a rare case in which brain biopsy was performed, patient neural tissue revealed lymphocytic infiltrates and microglial activation (Petit-Pedrol *et al.*, 2014; Fukami *et al.*, 2017). Given the association between GABA_A receptor encephalitis and cancer, tumour screening is also often performed in identified patients. Importantly, the positive identification of autoantibodies directed against the GABA_A receptor are a prerequisite for diagnosis, and this is typically screened for with cell-based assays, such as detection of immunofluorescent reactivity with human embryonic kidney cells expressing exogenous GABA_A receptor subunits (Planagumà *et al.*, 2015; Schuster *et al.*, 2019; Dalmau and Graus, 2023). However, many have criticised this approach for antibody detection, with evidence to suggest that commercial cell-based assays tend to result in a high proportion of false-negatives when tested with patient samples (Sinmaz *et al.*, 2015; Jézéquel, Rogemond, *et al.*, 2017; Giordano *et al.*, 2020; Ruiz-García *et al.*, 2021; Lenti *et al.*, 2022; Q. Deng *et al.*, 2022; Flanagan *et al.*, 2023). These concerns clearly highlight the urgent need for the development of novel antibody-detection strategies, in order to better aid in rapid treatment delivery to patients with suspected autoimmune encephalitis.

Comparably to NMDA receptor encephalitis, first-line treatments of GABA_A receptor encephalitis typically involve immunotherapy to suppress the autoimmune response (Graus *et al.*, 2016; Spatola *et al.*, 2017; Nakano *et al.*, 2022). This includes the use of corticosteroids, intravenous immunoglobulins and plasma exchange (Petit-Pedrol *et al.*, 2014). In cases of treatment-resistant disease or severe persistent symptoms, second-line immunosuppressive agents such as rituximab, azathioprine, cyclosporine, or cyclophosphamide

may be considered (Graus *et al.*, 2016; Figlerowicz *et al.*, 2018; Guo, Gelfand and Geschwind, 2020; Tappatà *et al.*, 2022). However, these more aggressive interventions carry considerable risk, and highlight the need for alternative therapeutics to manage clinical symptoms of this disease (Dou *et al.*, 2020; Labrosse *et al.*, 2021; Szeponowski *et al.*, 2021; Halliday *et al.*, 2022; Massa *et al.*, 2022). As for NMDA receptor encephalitis, the prognosis of GABA_A receptor encephalitis varies among individuals (Petit-Pedrol *et al.*, 2014; Venkatesan, 2015; Guo, Gelfand and Geschwind, 2020). However, early diagnosis and prompt initiation of immunotherapy (and tumour removal in appropriate cases) are associated with better healthcare outcomes. Despite the considerable severity of disease, the majority of patients show partial or complete recovery (44% and 36%, respectively), in response to immunotherapy; with very little reported incidence of relapse (Guo, Gelfand and Geschwind, 2020).

Molecular Mechanisms Implicated in the Hippocampal Pathology of GABA_A Receptor Encephalitis

The precise molecular mechanisms of action of autoantibodies directed against the GABA_A receptor is still under scrutiny. To date, several epitopes have been described across variable subunits of the channel, specifically of $\alpha 1$, $\beta 3$ and $\gamma 2$, although it has been noted that the majority of autoantibodies may be directed against the $\alpha 1$ subunit (Petit-Pedrol *et al.*, 2014; Ismail and Faustmann, 2020; Kreye *et al.*, 2021; Menke *et al.*, 2022). In a study of five patient-derived monoclonal autoantibodies, immuno-competition assays suggested that while some autoantibodies share similar reactive sites, others demonstrate no competition (Kreye *et al.*, 2021). Intriguingly, the binding of one of the monoclonals in this study enhanced the binding of an alternative monoclonal, indicating that the action of certain autoantibodies may induce a conformational change to the ion channel, to increase the binding efficacy of alternative monoclonals (Kreye *et al.*, 2021). Such a complimentary mechanism could suggest that some complex features of disease can only be elucidated in a polyclonal environment. However, it has also been revealed that cerebroventricular infusion of single monoclonal autoantibodies is sufficient to induce behavioural deficits consistent with disease progression in humans (Kreye *et al.*, 2021). Specifically, infusion of a single monoclonal against the $\alpha 1$ subunit induced a damaging neurological phenotype, in which experimental animals display seizures, catatonia and increased rates of mortality (Kreye *et al.*, 2021).

Many studies have investigated the action of GABA_A receptor autoantibodies on the surface distribution and organisation of the GABA_A receptor (Petit-Pedrol *et al.*, 2014; Kreye *et al.*, 2021; van Casteren *et al.*, 2022). These investigations have revealed that 24-hour exposure of $\alpha 1$ -targetting autoantibodies drives a reduction in the synaptic localisation of GABA_A receptors, which may be driven by an antibody-mediated alteration on lateral diffusion or induction of receptor internalisation (van Casteren *et al.*, 2022, Figure 6). However, similar exposure of a monoclonal autoantibody, selectively reactive against $\alpha 1\gamma 2$ -containing channels, did not alter

the synaptic localisation of these receptors (van Casteren *et al.*, 2022). Suggesting differential modes of action may exist between monoclonal antibodies.

Further, investigations have uncovered the impact of GABA_A receptor autoantibodies on GABAergic synaptic transmission. Interestingly, while 24-hour incubation with an anti- α 1 subunit monoclonal reduced the amplitude of evoked inhibitory postsynaptic currents in autaptic neuronal cultures, an autoantibody against α 1 γ 2-containing channels did not alter synaptic transmission (van Casteren *et al.*, 2022). Notably, reductions in GABAergic function have also been observed at much shorter timescales, for instance, 2-hour incubation of *ex Vivo* hippocampal slices with a monoclonal anti-GABA_A receptor autoantibody was sufficient to induce striking reductions in frequency and amplitude of spontaneous GABAergic currents in the CA3 subfield (Menke *et al.*, 2022). Further, Van Casteren *et al.* (2022) showed that 1-hour incubation of cortico-striatal cultures with the anti- α 1 subunit monoclonal was sufficient to elicit significant reductions in amplitude and frequency of miniature inhibitory postsynaptic currents. Theoretically, this rapid action of autoantibodies could be due to either a fast destabilisation of receptors from synaptic compartments leading to displacement towards extrasynaptic sites and internalisation, or alternatively through a direct antagonism of the channel function – for instance through competitive-binding for GABA active sites or negative allosteric modulation (Figure 6). Further experimental investigation revealed that incubation with Fab-fragments induced an immediate increase of network activity in corticostriatal cultures, reaching a peak of impact after only 4-minutes (van Casteren *et al.*, 2022). Given that Fab fragments are considered to have limited impacts on receptor redistribution and internalisation, and that the elicited alteration in network activity was considerably rapid, it is believed that these autoantibodies directly antagonise GABA_A receptors during the antibody-bound state (van Casteren *et al.*, 2022).

Notably, while the α 1 γ 2-reactive monoclonal used in the aforementioned studies did not elicit receptor redistribution or measurable alterations in inhibitory neurotransmission, infusion of this autoantibody into rodent brain provoked significant behavioural disturbance and increased epileptiform activity *in vivo* (van Casteren *et al.*, 2022). Additionally, this monoclonal has also been demonstrated to increase network activity *in vitro* (van Casteren *et al.*, 2022). It is compelling that, despite lack of clear evidence for any impact by this autoantibody on receptor organisation and function, it nevertheless elicits network and behavioural aberrations, through an as yet unidentified mechanism.

As for other ionotropic receptors, a finely-tuned equilibrium between exocytosis, surface trafficking, endocytosis and recycling controls the strength of GABAergic transmission (Jacob, Moss and Jurd, 2008; Luscher, Fuchs and Kilpatrick, 2011; Lorenz-Guertin and Jacob, 2018). While alterations in the surface mobility and expression of NMDA receptors have been clearly implicated in the pathophysiology of NMDA receptor encephalitis, relatively little attention has been paid to the possibility that GABA_A receptor autoantibodies

elicit similar impacts on their antigen channels. Lack of knowledge in this area is particularly striking considering that aberrant surface diffusion and expression of GABA_A receptors is linked to status epilepticus, a common clinical hallmark of GABA_A receptor encephalitis (Goodkin *et al.*, 2008; Eckel *et al.*, 2015; Cho *et al.*, 2017; Mele, Costa and Duarte, 2019; Naylor, 2023). Eckel *et al.* (2015) showed that exposure of primary hippocampal cultures to magnesium-free media, as an *in vitro* model of status epilepticus, reduces the surface stability of α 2-containing GABA_A receptors. This decrease in receptor stability appeared to be reliant on NMDA receptor-dependent activation of the phosphatase calcineurin (Eckel *et al.*, 2015). In consideration of such data, it is surprising that there are currently no investigations in the literature regarding the surface dynamics of GABA_A receptor in the context of autoimmune encephalitis. Given that seizure phenotypes are a prominent component of disease presentation, we might anticipate that alteration in the trafficking of the receptor – either through direct action of autoantibody-binding, or as a consequence of the induced epileptiform activity – and such alterations in trafficking and surface distribution may play a key role in the aetiology of disease progression.

Ultimately, a generalised consensus on the pathogenic mechanisms of autoantibodies against the GABA_A receptor centres around a model in which antibodies drive a functional reduction in channel activation, either through orthosteric and/or negative allosteric antagonism (Kreye *et al.*, 2016; van Casteren *et al.*, 2022). Conceptually, this reduction in GABAergic function can result in generalised network dysfunction, initiating the development of hyperactivity and epileptiform discharges in impacted brain regions. However, it should also be noted that autoantibody exposure also drives a reorganisation of GABA_A receptors, suggesting altered surface trafficking may also play a role in disease, as is considered to be the case in NMDA receptor encephalitis (Mikasova *et al.*, 2012; Petit-Pedrol *et al.*, 2014; Kreye *et al.*, 2021; van Casteren *et al.*, 2022). Additionally, this view is challenged by the identification of monoclonal autoantibodies that do not drive any detectable alteration of GABAergic channel function of inhibitory neurotransmission, even after 24-hours of exposure – yet still provoke epileptiform activity in *in vivo* models, as measured by electroencephalography (van Casteren *et al.*, 2022). Clearly, the precise molecular mechanisms implicated in GABA receptor encephalitis remain elusive and future work should aim to unveil the – likely multiple – pathogenic processes that can be evoked by autoimmune targeting of the GABAergic system. The generation of libraries of patient-derived monoclonal antibodies will expectantly provide useful tools for future investigations to shed light on this topic.

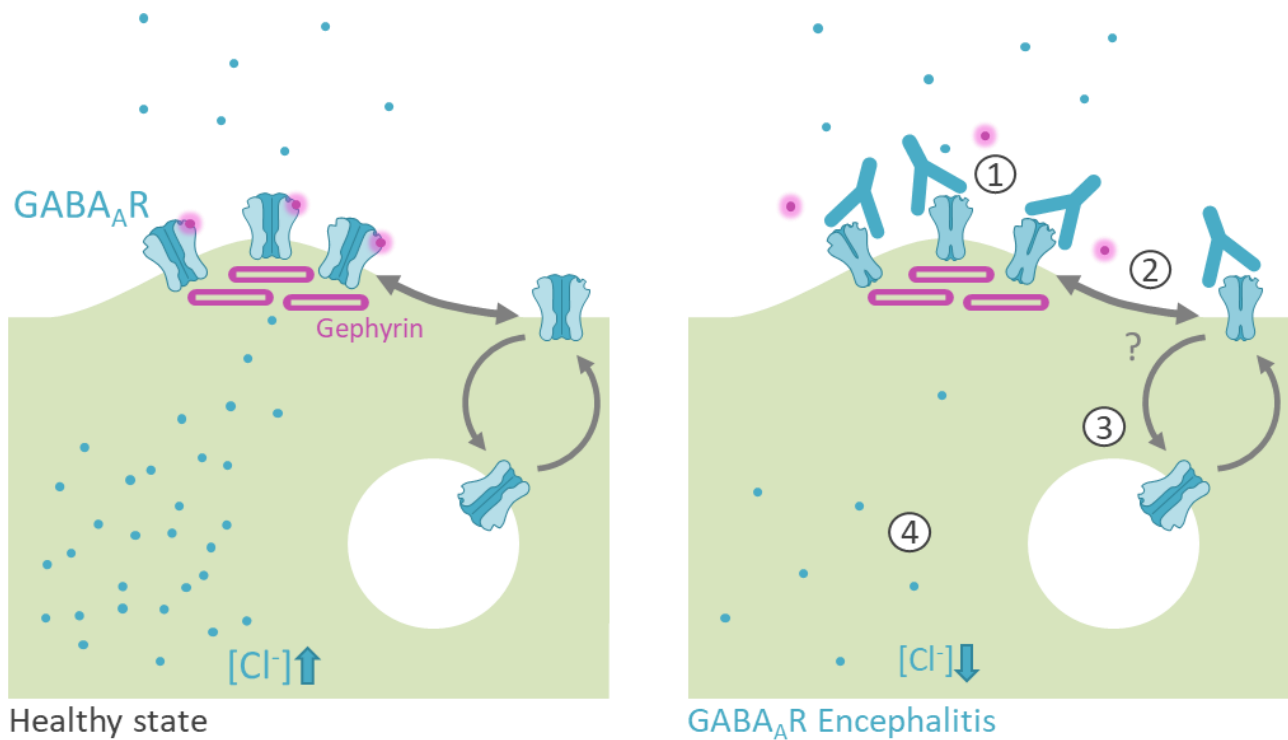


Figure 6: Molecular Mechanisms in GABA_A Receptor Autoimmune Encephalitis.

Schematic representation of GABA_A receptor autoantibody pathogenicity. Left panel denotes the healthy state in which receptors are trafficked to and from the membrane, where they freely diffuse, and anchor at inhibitory synapses through intracellular interaction with gephyrin. On the right, 1) autoantibody binding to GABA_A receptor subunits elicits an antagonistic effect on the channels, either through competitive binding at orthosteric sites, or negative allosteric modulation. 2) Autoantibodies might drive a synaptic displacement and 3) crosslinking-dependent receptor internalisation in some cases. 5) Antibody-mediated antagonism and possible synaptic depletion of GABA_A receptors drives a reduction in channel function and chloride influx.

Subsection Discussion and Outstanding Questions

Both NMDA- and GABA_A receptor encephalitis display a significant degree of clinical phenotypic convergence despite substantial heterogeneity of antigen targets and molecular pathogenesis (Table 1). As such, the principal objective of this thesis work was to elucidate the potential functional mechanisms leading to shared hippocampal pathology.

The canonical view of NMDA receptor autoantibody-mediated dysfunction is that a displacement of NMDA receptors from synaptic compartments, and promotion of internalisation, results in reduced cell surface expression and synaptic localisation of NMDA receptors (Hughes *et al.*, 2010; Lynch *et al.*, 2018). Given that calcium influx through these ion channels contributes to the late phase of excitatory postsynaptic currents, and initiates a variety of intracellular signalling cascades regulating synaptic plasticity, immune-targeting of these receptors in the disease state results in an excitatory hypofunction and altered network connectivity (Wenke *et al.*, 2019; Huang and Xiong, 2021; Andrzejak *et al.*, 2022). Conversely, the principal mechanisms implicated in GABA_A receptor encephalitis involve a direct antagonism of GABA_A receptors upon antibody binding, and potentially synaptic displacement (Petit-Pedrol *et al.*, 2014; Kreye *et al.*, 2021; van Casteren *et*

al., 2022). While it remains unclear whether this is the principal mechanism of action for the majority of pathogenic autoantibodies, or whether subsets of monoclonals elicit discrete impacts on receptor function and localisation; it is nevertheless anticipated that in the polyclonal human disease state, the presence of autoantibodies directed against the GABA_A receptor induce a substantial reduction in GABAergic neurotransmission, and ultimately an inhibitory hypofunction (Kreye *et al.*, 2021; Menke *et al.*, 2022; van Casteren *et al.*, 2022).

Given this substantial divergence of autoantibody actions, both at the level of their respective antigen functions and the functional modulations they elicit, we might anticipate similarly divergent clinical profiles of the two respective diseases. However, analysis of the prominent symptoms observed at presentation of NMDA- and GABA_A receptor encephalitis reveals considerable homogeneity. Most notably, seizure phenotypes, cognitive dysfunction and autonomic instability are common hallmarks of both autoimmune syndromes (Dalmau *et al.*, 2007; Wandinger *et al.*, 2011; Petit-Pedrol *et al.*, 2014; Spatola *et al.*, 2017). Markedly however, neuropsychiatric presentations resembling schizophrenia – including hallucinations, delusions paranoia etc. – are highly prevalent in patient cohorts diagnosed NMDA receptor encephalitis, while such symptomology is rarely identified in cases of GABA_A receptor encephalitis (Dalmau *et al.*, 2007; Wandinger *et al.*, 2011; Spatola *et al.*, 2017). While mechanisms evoking seizure and network hyperactivity in the context of GABA_A receptor encephalitis are relatively apparent – produced by a reduction in inhibitory neurotransmission – the mechanisms underpinning hyperactivity by excitatory hypofunction-inducing NMDA receptor autoantibodies remains somewhat less intuitive. Possible mechanisms by which NMDA receptor autoantibodies may induce seizure phenotypes and hippocampal hyperactivity are speculated upon in our review publication (Hunter *et al.*, 2021). Briefly, we hypothesise that NMDA receptor autoantibodies may elicit distinct impacts on inhibitory neuronal populations. To date, most research on the pathogenesis of antibodies in encephalitis has focused on pyramidal cell populations, largely neglecting the exploration of inhibitory interneuron networks. While the possibility of interneuron dysfunction contributing to NMDA receptor autoantibody pathogenesis has been suggested, there is a lack of direct experimental investigations in this area. Despite being a minority population in the hippocampus, interneurons play a crucial role in regulating the overall circuit behaviour of hippocampal networks and have been implicated in both psychotic disorders and epilepsy (Marín, 2012; Marx, Haas and Häussler, 2013; Heckers and Konradi, 2015; Pelkey *et al.*, 2017). These findings, coupled with the observation that NMDA receptor encephalitis presents with symptoms of psychosis and seizures, suggest that perturbations in hippocampal interneuron function may significantly contribute to the disease (Nakazawa and Sapkota, 2020). Recent studies on psychotic disorders propose that NMDA receptor hypofunction, specifically in interneuron populations, may be a key factor driving psychosis. Certain NMDA receptor antagonists have been known to induce poly-symptomatic psychotic disorders in humans, mimicking hallucinations, affect flattening, and paranoia (Javitt, 2004; Nakazawa, Jeevakumar and

Nakao, 2017). This behavioural similarity is accompanied by increased glutamate release in the medial prefrontal cortex and enhanced activity of cortical principal cells in animal models (Moghaddam and Javitt, 2012). The hypothesis is that the disinhibition of brain structures, including the frontal cortex and limbic system, mediated by excitatory cell action and glutamate release, is a primary route in the development of psychotic disorders. While the source of this disinhibition is still being investigated, local disinhibition has been observed in the CA1 subfield of the hippocampus, leading to effective disinhibition of the prefrontal cortex through downstream signalling disruption (Jodo *et al.*, 2005). Applying this line of reasoning to the study of NMDA receptor encephalitis, it is speculated that antibody-induced receptor hypofunction on interneurons could be a principal mechanism contributing to the psychotic symptoms observed in the early stages of the disorder. These findings align with the notion that autoantibody actions primarily target hippocampal and limbic regions in encephalitic patients, although the precise mechanism remains unidentified (Dalmau *et al.*, 2008).

Additionally, NMDA receptor autoantibodies may differentially modulate interneuron excitability, due to a differential role of NMDA receptors in controlling intrinsic properties of these cell populations, relative to hippocampal pyramidal neurons (Middleton *et al.*, 2008; Riebe *et al.*, 2016; Hanson *et al.*, 2019). Fast-spiking parvalbumin-positive (PV+) interneurons play a crucial role in locally controlling hippocampal excitation through their unique rapid and repetitive firing pattern (Stark *et al.*, 2014; Zaletel, Filipović and Puškaš, 2016; Antonoudiou *et al.*, 2020). These interneurons have garnered significant interest in the field of psychosis because their fast-spiking phenotype is known to regulate high-frequency gamma oscillations, which are disrupted in the electroencephalographs of psychotic patients (Marín, 2012). This suggests that PV+ interneurons are strong candidates for the disinhibition model of psychosis in NMDA receptor encephalitis. However, it remains unclear how patient autoantibodies, which presumably bind indiscriminately to NMDA receptors, can selectively affect interneurons without causing a widespread reduction in the activity of principal cells. One possibility is that the NMDA receptor serves different functional roles in interneurons compared to principal cells. Notably, interneuron populations exhibit more depolarized resting membrane potentials than nearby pyramidal cell populations, with PV+ interneurons being among the most depolarized, typically around -55 mV (Tricoire *et al.*, 2011). Despite this difference, all interneuron families and pyramidal cells have a similar threshold for generating action potentials, around -35 mV, except for PV+ interneurons, which are typically closer to the spike threshold (Tricoire *et al.*, 2011). PV+ interneurons have been found to have a higher expression of the magnesium-insensitive GluN2D-containing NMDA receptors, which contributes to the resting membrane potential during early developmental stages in the cortex (Hanson *et al.*, 2019). It is plausible that a tonic current mediated by NMDA receptors on PV+ interneurons could represent a unique pathological mechanism in encephalitic disease. Internalization of NMDA receptors on interneurons may drive the resting potential away from the spike threshold, leading to the loss of the fast-spiking phenotype

and inhibitory control. Although this hypothesis requires further investigation in the context of exposure to patient-derived NMDA receptor antibodies, studies have shown that genetically ablating NMDA receptor expression on PV+ fast-spiking interneurons disrupts their characteristic firing pattern (Carlén *et al.*, 2012).

Further, we speculated that excitatory synaptic transmission on hippocampal interneurons comprises a relatively increased reliance on NMDA receptor-mediated current, as compared the AMPA receptor dominance on the pyramidal cell population (Matta *et al.*, 2013; Pelkey *et al.*, 2017). In the CA1 area of the hippocampus, interneurons receiving Schaffer collateral inputs maintain a predominance of GluN2B-containing NMDA receptors at the synapse throughout development, whereas pyramidal cells upregulate the synaptic incorporation of GluN2A-containing channels after early postnatal development (Matta *et al.*, 2013). Notably, the relative contributions of NMDA and AMPA receptor populations at these synapses are roughly equal in interneurons, contrasting with pyramidal cells where the AMPA receptor number greatly exceeds the NMDA receptor population (Matta *et al.*, 2013; Pelkey *et al.*, 2017). Considering these observations, the presence of GluN2B-containing NMDA receptors, which have higher current transfer and a prolonged decay constant compared to their GluN2A-containing counterparts, along with a higher ratio of NMDA-to-AMPA expression at interneuron synapses, suggests that interneurons rely more heavily on NMDA receptor pools in the hippocampus (Hunter, Jamet and Groc, 2021). Consequently, in encephalitis, the uniform impact of NMDA receptor autoantibodies may disrupt synaptic function more profoundly in interneurons compared to principal cells. This, coupled with the increased vulnerability of interneurons due to the higher presence of tonic NMDA receptor-mediated currents, renders them significantly more susceptible to perturbation by immune-targeting (Povysheva and Johnson, 2012; Riebe *et al.*, 2016). Furthermore, it has been demonstrated that this loss of excitatory drive onto inhibitory networks not only leads to local disinhibition in the CA1 region but also induces wide-spread epileptiform activity (Kelsch *et al.*, 2014).

	NMDAR-Encephalitis	GABA _A R-Encephalitis
Seizures <i>Generalised seizures, focal seizures, status epilepticus</i>	73%	88%
Cognitive dysfunction <i>Attention, memory deficits, reduced executive functions</i>	88%	67%
Autonomic instability <i>Hypoventilation, cardiac dysrhythmias, coma, hypothermia</i>	47%	42%
Comorbid Pathology <i>Underlying tumour, other autoimmune disorder</i>	59%	42%
Psychiatric dysfunction <i>Bizarre behaviour, hallucination, anxiety, catatonia</i>	> 90%	< 14%
Motor dysfunction <i>Dyskinesia, rigidity, abnormal postures</i>	78%	< 35%

Table 1: Clinical Features of NMDA- and GABA_A Receptor Autoimmune Encephalitis.

Summary of principal clinical characteristic of NMDA- and GABA_A receptor autoimmune encephalitis. Data presented within the table are collected from Chen et al. (2021); Qu et al. (2020); Dalmau et al. (2008); Lejuste et al. (2016); Engen & Agartz (2016); Lin & Lin (2020); Spatola et al. (2017) and Guo et al. (2020).

Project Aims, Objectives & Hypotheses

The principal aim of this thesis work is to characterise the functional mechanisms underpinning hippocampal pathophysiology induced by exposure to NMDA- and GABA_A receptor autoantibodies. Full elucidation of the possible pathogenic avenues towards hippocampal dysfunction therefore comprises an understanding of the synaptic, cellular and network deficits driven by autoantibody actions in hippocampal cell preparations.

Aim I: Investigate the Synaptic Phenotype

A primary action of both NMDA and GABA_A receptors is their phasic signalling at excitatory and inhibitory synapses, respectively. Given the wide variety of coregulatory mechanisms at play – between AMPA-, NMDA- and GABA_A receptors – in the maintenance of neuronal activity and homeostasis, we anticipated that both autoantibodies may drive alterations in the excitatory and inhibitory synaptic activity of hippocampal cultures exposed to patient derived autoantibodies. To investigate this, we used whole-cell voltage-clamp electrophysiology, in organotypic hippocampal preparations, to uncover spontaneous excitatory (AMPA receptor-mediated) and inhibitory (GABA_A receptor-mediated) postsynaptic currents after 24-hour exposure to patient-derived monoclonal autoantibodies. We further investigated synaptic organisation and structuration with immunohistochemistry in dissociated hippocampal cultures. Given the association of seizure activity with both NMDA- and GABA_A receptor encephalitides, we anticipated to observe globalised disruption to synaptic transmission, with a shift in excitatory-inhibitory balance in favour of hyperexcitation of synaptic inputs to CA1 pyramidal neurons.

Aim II: Investigate the Cellular Phenotype

We further aimed to characterise the intrinsic excitability of CA1 hippocampal neurons. Since tonic conductance mediated by NMDA- and GABA_A receptors play prominent roles in the setting intrinsic firing of hippocampal interneurons and principal cells, respectively, we anticipated that autoantibodies may display divergence of their actions at this scale of neuronal function. Specifically, we expect that NMDA receptor autoantibodies may reduce the intrinsic excitability of hippocampal interneurons, which display a large reliance on NMDA receptor-mediated tonic excitatory current in controlling resting membrane potential. Conversely, we hypothesised that GABA_A receptor autoantibodies may ablate the tonic inhibitory GABAergic conductance and shunting inhibition, which is critical for maintaining intrinsic excitability of pyramidal neurons, thereby raising the intrinsic activity of this cell population. To investigate this, we used a whole-cell current-clamp approach to quantify various parameters related to intrinsic excitability, including input-output curves, resting membrane potential, rheobase, input resistance and action potential threshold. Moreover, we employed cell-attached voltage-clamp recordings to investigate the spontaneous activity of both excitatory and inhibitory interneurons in the CA1 subfield of organotypic hippocampal preparations.

Aim III: Investigate the Network Phenotype

Finally, we aimed to uncover the overall network phenotype of hippocampal circuits exposed to monoclonal autoantibodies. We expected that this would unveil a convergent overall dysfunction of hyperactivity, in terms of principal cell output, relating to the epileptogenesis and seizure commonly observed at the clinical scale. To investigate the network phenotypes, we used a calcium imaging approach, to quantify pyramidal cell activity in organotypic hippocampal cultures after 24-hour exposure to patent-derived monoclonal autoantibodies. With this dataset, we analysed overall network activity, in addition to synchronicity of cell activations in CA1 hippocampal networks. Additionally, we also performed multielectrode array recordings, with high-density dissociated hippocampal cultures to quantify generalised network deficits elicited by autoantibodies in disorganised neuronal networks.

Overall Objective

Taken together, our investigations allow us to map the functional pathways towards hippocampal dysfunction, mediated by both NMDA- and GABA_A receptor autoantibodies. This increased understanding of the consequences of immune-targeting of NMDA- and GABA_A receptors on hippocampal neurons, across synaptic, cellular and circuit levels of neuronal function will hopefully provide greater insights into the mechanisms by which autoantibodies – targeting opposing neurotransmission systems – can converge upon a similar overall dysfunction, that is hyperactivity resulting in seizure and cognitive deficits, in patients with discreet autoimmune encephalitic disease.

II

ANTIBODY VALIDATION

Monoclonal Autoantibodies Comprise a Valuable Research Tool for the Study of Autoimmune Encephalitis

The identification of autoantibodies directed against brain endogenous antigens has enabled the classification of a wide array of neurological syndromes, under the umbrella-term of autoimmune encephalitis. Currently, antibody screening technologies are permitting the identification of a range of functionally diverse autoantibody targets in the central nervous system (Prüss, 2021; Ramanathan *et al.*, 2021). Many of these identified targets are expressed on the neuronal surface, residing within variable neuronal domains including synaptic, extrasynaptic and axonal proteins (Prüss, 2021). Immune targeting of these antigens has been shown to induce alteration in neuronal function through a variety of mechanisms, such as direct antagonism of ion channels or disorganisation of synaptic receptors (Mikasova *et al.*, 2012; Kreye *et al.*, 2021; van Casteren *et al.*, 2022; Duong and Prüss, 2023). Expanding knowledge regarding the pathogenic mechanisms initiated by autoantibodies has aided in our understanding of the disease states in autoimmune encephalitides, but also bears the potential to shed light on fundamental aspects of neuronal protein function – specifically the processes by which dysfunction in a single surface protein can yield downstream perturbations across additional proteins and neuronal functions.

Early studies aiming to elucidate the molecular pathogenicity of patient autoantibodies were heavily restricted by the limited availability of samples, including serum and particularly cerebrospinal fluid (Duong and Prüss, 2023). Moreover, such patient derived samples invariably contain a polyclonal combination of autoantibodies, with variable epitopes against a single antigen, and variable antigen targets. While cerebrospinal fluid should contain a high concentration of pathogenic autoantibodies, serum samples are likely heavily diluted by the presence of systemic circulating immunoglobulins (Gresa-Arribas *et al.*, 2014). Additionally, cerebrospinal fluid from patients diagnosed with a specific autoimmune encephalitis, may contain concurrent autoantibodies against additional neuronal antigens. For example, investigation of autoantibody targets derived from the cerebrospinal fluid of a paediatric patient with GABA_A receptor encephalitis, showed that 7% (5/67) of unique IgG clones were reactive against the GABA_A receptor, while 27% of GABA_A receptor-negative clones (17/62) showed substantial staining of rat brain sections (Kreye *et al.*, 2021). Similar findings have also been gathered from analysis of the immune repertoire in cerebrospinal fluid from patients with NMDA receptor encephalitis (Kreye *et al.*, 2016). Such findings clearly indicate that patient biological samples contain a plethora of uncharacterised factors, including a significant volume of autoantibodies against unknown targets. The

concurrent presence of such unknowns in patient samples makes it difficult to determine the precise molecular pathogenicity of monospecific autoantibodies.

Advances in recombinant monoclonal antibody production has allowed for an expansion in research, despite the lack of availability of patient biological samples. These strategies permit an unlimited production of patient-replicated immunoglobulins, and also avoid contamination of experimental samples with unknown features present in serum and cerebrospinal fluid (Duong and Prüss, 2023). The use of recombinant patient-derived monoclonal autoantibodies has already produced valuable insights regarding the pathogenicity of autoimmune targeting of neuronal proteins, which well-replicate the disease state in *in vivo* models, and the elucidate some precise molecular pathways which may underpin disease in the clinical scale (Bennett *et al.*, 2009; Kreye *et al.*, 2016, 2021; Kornau *et al.*, 2020; Ramberger *et al.*, 2020; Andrzejak *et al.*, 2022; van Casteren *et al.*, 2022). Ultimately, the use of monoclonal autoantibody samples allows us to finely control the exact concentration of pathogenic immunoglobulins used and prevents confounding features resulting from possible confounding presence of uncharacterised immune mediators and off-target antigens. We are, however, mindful of the potential differences which can result from the use of a single monoclonal antibody, which may not fully replicate some features of the polyclonal disease state. Nevertheless, we would argue that employment of monoclonal autoantibodies in our investigations should permit greater replicability, and precise characterisation of the pathogenic mechanisms elicited by autoantibodies directed against the NMDA- and GABA_A receptor specifically, which we would have been unable to concretely conclude with the use of patient biological samples.

In our investigations, we have used a two high-affinity monoclonal autoantibodies, one reactive against the NMDA receptor (Kreye *et al.*, 2016) and the other reactive for the GABA_A receptor (Kreye *et al.*, 2021), developed, generated and generously provided by our collaborators. Briefly, the derivation of human monoclonal antibodies was performed by isolation of B-cells from cerebrospinal fluid of patients with autoimmune encephalitis, or the peripheral blood of a healthy donor. Antibody-coding genes, of both heavy and light chains of G-isotype immunoglobulins were purified from B-cell lysates and amplified by PCR, before insertion into bacterial plasmids (Kreye *et al.*, 2016, 2021). For the recombinant expression of monoclonal antibodies, paired heavy and light chain-coding plasmids were co-transfected into HEK293T cells and purified from cell culture supernatants, as previously described (Kreye *et al.*, 2016, 2021). Specifically, we have used the anti-NMDA receptor monoclonal autoantibody described in the study Kreye et al. (2016) as “#003-102”, which binds to the GluN1 subunit of the NMDA receptor. For our investigations of GABA_A receptor autoantibodies, we have used the patient derived monoclonal, reactive to α 1-containing GABA_A receptors, described as “#113-115” by Kreye et al. (2021). For our control conditions, we have employed an isotype-matched control antibody, derived from blood of a healthy donor, described as “mGo53” in an earlier publication (Wardemann *et al.*, 2003).

Characterisation of Recombinant Anti NMDA Receptor Monoclonal Autoantibody #003-102

The NMDA receptor autoantibody used in this thesis work has been well characterised by Kreye et al. (2016). These investigations identified nine immunoglobulins, from three patients, reactive for the GluN1 subunit of the NMDA receptor. GluN1-reactivity was determined by immunostaining of human embryonic kidney (HEK) cells, transfected with GluN1 subunit-coding DNA, following methanol fixation (Kreye *et al.*, 2016). Reactivity was subsequently confirmed using dissociated hippocampal cultures and rodent brain sections. In dissociated cultures, human monoclonal staining was visually inspected for colocalisation with a commercially available mouse anti-GluN1 and with excitatory presynaptic marker, VGLUT1 (Kreye *et al.*, 2016). In histochemical sections, staining distribution was visually inspected for characteristic patterning, comprised of strong labelling of hippocampal neuropil and cerebellar granule cells (Kreye *et al.*, 2016). Further, experiments with HEK cells transfected with either wildtype GluN1 or GluN1-N368Q, carrying a mutation in the N-terminal domain of the subunit, showed that the commercial anti-GluN1 bound to subunits on the surface of HEK cells irrespective of the N-terminal domain mutation; whereas the strong reactivity of the human monoclonal autoantibody was abrogated by the N368Q mutant (Kreye *et al.*, 2016). This finding would suggest that the epitope of the NMDA receptor monoclonal we have used in our investigations targets this region, or a proximal sequence, within the N-terminal domain of the GluN1 subunit. Subsequent investigations regarding the affinity of five identified monoclonal autoantibodies unveiled that #003-102 has a high affinity for the GluN1 subunit, demonstrating the highest level of maximal binding in GluN1-expressing HEK cells, and the lowest c_{50} (the concentration at which antibody binding was 50% of the maximal plateau) (Ly *et al.*, 2018). Moreover, in terms of the functional disturbance elicited by human monoclonals, Kreye et al. (2016) showed that 18-hour incubation with patient-derived monoclonal antibodies (at 0.5 $\mu\text{g}/\text{ml}$) was sufficient to reduce NMDA receptor clustering at postsynaptic compartments in dissociated hippocampal neurons, and also elicited reductions in NMDA receptor-mediated synaptic currents.

Accordingly with these investigations, we believe that the monoclonal autoantibody that we have used in our investigations binds with high affinity to the N-terminal domain of the GluN1 subunit, and 18-hour exposure of hippocampal neurons to 0.5 $\mu\text{g}/\text{ml}$ of this monoclonal is sufficient to drive a displacement of NMDA receptor from postsynaptic compartments and the resulting decrease in NMDA receptor-mediated currents, understood to be the primary drivers of disease, elucidated from investigations using both mono- and polyclonal samples (Hughes *et al.*, 2010; Mikasova *et al.*, 2012; Lynch *et al.*, 2018).

Characterisation of Recombinant Anti GABA_A Receptor Monoclonal Autoantibody #113-115

The anti-GABA_A receptor monoclonal autoantibody that we have used in our investigations has been previously isolated and characterised by previous studies (Kreye *et al.*, 2021; van Casteren *et al.*, 2022). Kreye *et al.* (2021) 67 distinct monoclonal autoantibodies from the cerebrospinal fluid of a paediatric case of severe GABA_A receptor encephalitis, 5 of which were reactive against $\alpha 1\beta 3$ -, and/or $\alpha 1\beta 3\gamma 2$ -containing GABA_A receptors in HEK cell-based assays. The monoclonal that we have used in our studies, #113-115, showed strong reactivity to hippocampal neuropil, cerebellar granule cells, putamen and olfactory bulb (Kreye *et al.*, 2021). Confirmation of GABA_A receptor-targeting was performed by analysis of colocalisation with a commercially available rabbit anti- $\alpha 1$ antibody, and inhibitory presynaptic marker, VGAT (Kreye *et al.*, 2021). Monoclonal antibody #113-115 bound to the surface of HEK cells expressing both $\alpha 1$ and $\beta 3$ subunits, but failed to show reactivity to cells expressing the $\beta 3$ subunit in isolation, additionally, inclusion of $\gamma 2$ did not alter the reactivity of #113-115, suggesting an epitope on the extracellular domain of the $\alpha 1$ subunit or formed at the interface between $\alpha 1$ and $\beta 3$ subunits (Kreye *et al.*, 2021). In terms of antigen affinity, #113-115 displayed the highest reactivity of the 5 isolated GABA_A receptor monoclonals, indicated by the lowest c_{50} , however – unlike anti-NMDA receptor monoclonals – GABA_A receptor autoantibodies displayed relatively heterogeneous rates of maximal binding (Kreye *et al.*, 2021). Intriguingly, immunocompetition assays of these 5 monoclonals unveiled that pre incubation with #113-115, in fact, enhanced the binding of another monoclonal, suggesting that #113-115 induced a conformational shift in the GABA_A receptor, eliciting increased availability of alternative epitopes for additional autoantibodies in a polyclonal setting (Kreye *et al.*, 2021). Functional investigations revealed that incubation of autaptic striatal neuronal cultures for 24-hours with 1 $\mu\text{g/ml}$ of monoclonal #113-115 was sufficient to induce reductions in GABA_A receptor-mediated evoked inhibitory postsynaptic currents. Kreye *et al.* (2021) identified no alteration in the total surface expression of GABA_A receptors, however, later investigation revealed that #113-115 does indeed elicit synaptic depletion of GABA_A receptor, irrespective of internalisation (van Casteren *et al.*, 2022). Further, *in vivo* pathogenicity of #113-115 was tested by intraventricular infusion (Kreye *et al.*, 2021). Mice exposed to high doses of #113-115 infusion by osmotic pump implantation developed symptoms associated with GABAergic dysfunction, including twitching, ataxia and circling behaviour, and 6/7 either died or developed status epilepticus resulting in humane sacrifice (Kreye *et al.*, 2021). Subsequent exposures to low-dosage of #113-115 in rats, also implanted with a wireless electroencephalographic recording device, showed spontaneous seizure generation and ictogenesis, and ex Vivo recording from brain slices of these animals uncovered increased epileptiform activity on CA1 and CA3 hippocampal subfields (Kreye *et al.*, 2021). Finally van Casteren *et al.* (2022) demonstrated that both intact IgG and Fab fragments of #113-115 are sufficient to drive network hyperexcitation at short timescales, reaching a peak of hyperactivity within 4-minutes. Suggesting therefore that this autoantibody acts as an allosteric or orthosteric antagonist of the GABA_A receptor (van Casteren *et al.*, 2022).

Ultimately, characterisation of anti GABA_A receptor monoclonal, #113-115, which we have employed in our investigations binds to the extracellular domain of α 1 β 3-containing GABA_A receptors, with high affinity. Exposure of hippocampal cultures to this monoclonal antibody drives a synaptic displacement of receptors, without induction of internalisation. Additionally, there is evidence to suggest that this monoclonal induces a conformational shift of the GABA_A receptor, and also that antibody-binding directly reduces channel function at rapid timescales. Such actions of GABA_A receptor autoantibodies are thought to be principal disease drivers in GABA_A receptor encephalitis, and as such we believe that this monoclonal represents an effective tool for the study of the pathogenic action of autoantibodies, more generally, in the disease state of GABA_A receptor encephalitis.

In-House Validation of NMDA Receptor Monoclonal #003-102

To ensure that transport of monoclonal autoantibody samples did not impact the integrity of samples, we analysed the reactivity of the NMDA receptor monoclonal, #003-102, with hippocampal neuronal cultures in our hands. Further, much of our planned experimental work was to be performed in organotypic hippocampal slice cultures, in which immune-reactive astrocytes and glial cells are present in the cultured tissue. Since autoimmune encephalitis is an immune-mediated syndrome that has been associated with inflammatory infiltrates and activation of microglia, as observed in human patient biopsy tissue, we aimed to understand if exposure to single monoclonal antibodies was able to elicit detectable microglial or astrocytic activation in organotypic hippocampal slices (Dalmau *et al.*, 2007; Petit-Pedrol *et al.*, 2014; Fukami *et al.*, 2017). This was particularly important since immune-activation of astrocytes and microglia can lead to secretion of cytokines, in turn altering neuronal function including excitability and promoting apoptosis (Liddelow *et al.*, 2017; Linnerbauer, Wheeler and Quintana, 2020; Vezzani *et al.*, 2022; Wang, Leak and Cao, 2022). To investigate these questions, we used the NMDA receptor monoclonal #003-102 in our *in vitro* model systems.

Methods

Primary hippocampal cell cultures

Hippocampal cultures were prepared from embryonic day 18 Sprague-Dawley rat pups. Briefly, hippocampi were dissected in ice-cold HBSS containing Penicillin-Streptomycin (PS) and HEPES. Hippocampi were incubated with trypsin-EDTA and dissociated by mechanical trituration. Cell suspension - containing neurons and glia - was diluted in 60mm sterile petri dishes containing pre-warmed Neurobasal culture medium supplemented with horse serum and poly-L-lysine coated 18mm coverslips, at a density of 250-275x10³ cells per ml. Dishes were maintained at 37°C in 5% CO₂ in a humidity-controlled incubator. For standard primary

cultures, at 3 days *in vitro* (DIV), a full media exchange with serum-free Neurobasal/B-27 culture media was performed. Full media exchanges continued twice weekly until use. Exogenous transgenes were introduced to neuronal cultures between DIV7-8 with a calcium phosphate transfection protocol. Briefly, a TE buffer solution containing purified bacterial plasmid DNA and 0.2M CaCl₂ was added dropwise to an equal volume HEPES-based phosphate buffer to form fine plasmid-containing calcium phosphate precipitates. The mass of DNA used for all transfection conditions did not exceed 2µg total DNA per coverslip. For these experiments *pRcCMVaa-SEP-rGrin1* was used to generate tagged NMDA receptor expression at the neuronal surface. Coverslips were transferred to 12-well culture plates, containing pre-warmed Neurobasal culture medium supplemented with kynurenic acid, 50µl of plasmid-precipitate suspension was added to each well and incubated for 90 minutes. Remaining precipitate suspension was then washed, and coverslips were returned to culture dishes until imaging at DIV 12-14.

Organotypic hippocampal slice culture

Hippocampal slice cultures were prepared from Sprague-Dawley rat pups at postnatal day 5. Hippocampi were dissected in ice-cold dissection solution composed of 0.5mM CaCl₂, 2.5mM KCl, 0.7mM KH₂PO₄, 2mM MgCl₂, 0.3mM MgSO₄, 50mM NaCl, 0.9mM Na₂HPO₄, 25mM glucose, 2.7mM NaHCO₃, 175mM sucrose and 2mM HEPES (pH 7.4, 320 mOsm). After isolation, 350µm thick transverse hippocampal slices were prepared using a McIlwain tissue chopper. Slices were rested in dissection solution at 4°C for 30 minutes, before plating onto PTFE membrane sections (FHLC01300, Millipore, UK) placed on Millicell cell culture inserts (PICM03050, Millipore, UK) and cultured in 6-well plates containing pre-warmed slice culture media. Slice culture media was composed of 50% BME, 25% HBSS, 25% horse serum and supplemented with 25mM glucose and GlutaMAX™ supplement. Slice cultures were maintained at 35 °C with 5% CO₂ in a humidity-controlled incubator. Full culture medium exchanges were performed the day after dissection, and then three times per week with pre-warmed slice culture medium, until fixation for immunostaining at DIV 14. slice cultures were treated with either NMDA receptor monoclonal antibody, #003-102, at 0.5 µg/ml or antibody-free culture media for 24-hours prior to fixation for histological analysis. This control condition was selected to identify whether the application of human IgG, regardless of reactivity with the tissue, was sufficient to induce immune-mediated activation of local microglial or astrocytic cells. Autoantibodies were applied to slice cultures by dilution into the slice culture media. A 20µl droplet of antibody-containing media was also applied directly on top the slice, above the membrane insert, to facilitate diffusion of autoantibodies into the cultured tissue.

Immunocytochemistry

Hippocampal cultures were immunostained in live with rabbit anti-GFP (Thermo Fisher A6455) at 1:1000, to uncover the surface expression of SEP-tagged GluN1 NMDA receptors, and either with or without human

monoclonal, #003-102, at 0.5 $\mu\text{g}/\text{ml}$, for 30 minutes, at 37°C. Cultures were then washed briefly with phosphate buffered saline (PBS), followed by fixation in a 4% paraformaldehyde 4% sucrose PBS solution, at room temperature for 15 minutes. Cultures were then washed three times with PBS, for 5 minutes with agitation. To quench aldehydic fluorescence, cultures were incubated with 50mM ammonium chloride in PBS for 15 minutes, and then washed again with PBS as before. Cultures were then blocked in 3% bovine serum albumin (BSA) PBS solution for 1 hour. Subsequently, cultures were incubated with goat anti-rabbit alexafluor-488 (Thermo Fisher A11008), at 1:500, overnight at 4°C under agitation. The next day, cultures were washed with three times with PBS and subsequently immunostained with goat anti-human alexafluor-568 at 1:500 (Thermo Fisher A21090), for 2 hours at room temperature. Coverslips were then washed again in PBS and ultrapure water prior to mounting in Vectashield anti-fade mounting medium and stored at 4°C until imaging.

Immunohistochemistry

Activation of astrocytes and microglial cells was investigated by immunostaining for GFAP or Iba1, respectively. Following incubation of organotypic hippocampal cultures with either NMDA receptor monoclonal antibodies, or antibody-free culture media, slices were briefly washed by submersion in PBS before fixation in 4% paraformaldehyde 4% sucrose PBS solution for 30 minutes at room temperature. Following fixation, slices were washed three times in PBS and aldehydic fluorescence was quenched with 50mM ammonium chloride in PBS for 30 minutes. Slices were then washed again in PBS as before and submerged in permeabilization solution (0.5% Triton X-100 in PBS) and maintained overnight at 4°C. Slices were washed in PBS three times, over 1 hour, in PBS before blocking with a 20% BSA PBS solution at 4°C until the following day. Slices were then immunostained by submersion in 5% BSA PBS solution containing either rabbit anti-GFAP (Abcam AB278054) or rabbit anti-Iba1 (WAKO 019-19741) at 1:1000 at 4°C until the following day. Slices were then washed in 5% BSA PBS solution 4 times, over 2 hours, at room temperature and under agitation. Secondary antibody (goat anti-rabbit alexafluor-488, Thermo Fisher A11008) was then added to 5% BSA PBS solution and slices were incubated at room temperature for 4 hours, with agitation. Finally, slices were washed 4 times in PBS, over 2 hours, before mounting in Vectashield anti-fade mounting medium, containing DAPI as a counterstain. A 200 μm spacer was added between the slide and cover glass to prevent compression and deformation of slices. Slices were kept at 4°C until imaging.

Image Acquisition and Analysis

Hippocampal cultures and organotypic slices were imaged using a spinning disk confocal microscope (Nikon Ni-E with spinning Yokogawa X1). For dissociated neuronal cultures, neuronal dendrites expressing immunostained GluN1-SEP were captured and maximum intensity projections were generated. Human monoclonal autoantibody binding to GluN1-positive puncta was determined using a custom-built script with FIJI image analysis software. Briefly, images were thresholded to produce a mask of GluN1-containing clusters

along the neuronal dendrite, based on commercial anti-GFP staining. This mask was then used to determine the fluorescence intensity of human monoclonal staining, or non-specific signal in control conditions, within these regions. Additionally, colocalisation analysis was performed by generating intensity line plots along raw fluorescence images of anti-GFP and anti-human channels, Spearman correlation coefficients generated by each image were tested against a hypothetical mean of 0 (signifying no correlation between signals). To quantify the level of GFAP and Iba1 expression and microglial and astrocytic cell morphology in organotypic slices, 20µm thick z-stacks were taken from the CA1 hippocampal subfield and maximum intensity projections were generated. A uniform threshold was applied across all projection images to identify the relative ratio of Iba1+ or GFAP+ pixels per stack projection, with the expectation that an increased activation of microglia or astrocytes will result in an increased expression of these intracellular proteins, and thus an increased positive-pixel ratio.

Results

Human NMDA receptor monoclonal antibody, #003-102, colocalises with GluN1

Quantification of the mean fluorescence intensity of commercial anti-GFP puncta along hippocampal dendrites revealed no significant difference between cultures exposed to antibody-free media and human anti-NMDA receptor monoclonals (Figure 7A-B, unpaired t test: $t(24) = 0.6085$, $p = 0.5486$). Indicating, as expected, that transfection rates of GluN1-SEP were stable across our two conditions, and that regions generated by thresholding these signals contain comparable levels of total surface GluN1. However, analysis of mean alexafluor-568 intensity, labelling for human IgG, within these GluN1-positive regions showed significant binding of #003-102, compared to control conditions (Figure 7C, unpaired t test: $t(24) = 6.877$, $p < 0.0001$). Indicating that integrity of human monoclonal antibody, #003-102, was maintained during transport, and continues to show binding to the surface of hippocampal neurons, in GluN1-positive puncta. Additionally, colocalisation of GluN1-SEP and human autoantibody signals was found to be statistically significant, with a mean Spearman correlation coefficient of 0.9228, example intensity plot shown in Figure 7D (one-sample t test: $t(12) = 34.35$, $p = 0.0001$, 95% confidence interval: 0.864 – 0.981). Suggesting that human monoclonal #003-102, binds selectively to regions of GluN1 expression.

Human monoclonal antibody exposure does not induce astrocytic or microglial activation

We found that antibody-free control and NMDA receptor monoclonal treated slices demonstrate equivalent levels of Iba1 and GFAP protein expression (Figure 7E-G, unpaired t test: $t(24) = 0.04$, $p = 0.966$ and $t(21) = 0.91$, $p = 0.375$, respectively), suggesting no detectable upregulation of micro-inflammatory processes, at least at the scale of microglial or astrocytic activation.

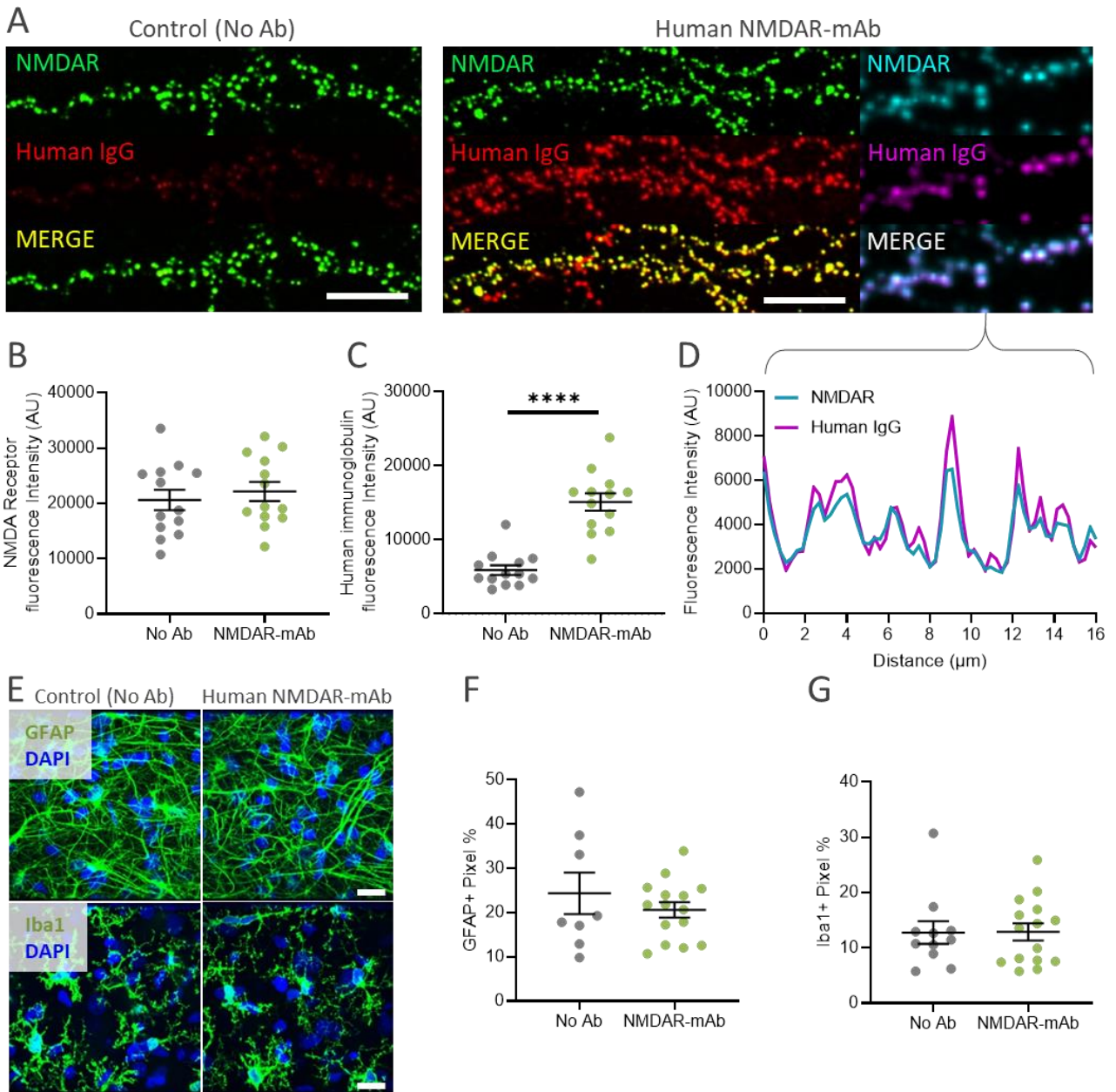


Figure 7: Monoclonal #003-102 is reactive for NMDA receptors and does not activate astrocytes or microglia.

(A) Representative images of DIV12 hippocampal neurons transfected with *pRcCMVaa-SEP-rGrin1* and immunostained for optically-tagged NMDA receptors (green or cyan), or human immunoglobulins (red or magenta). Left panel shows control condition, in which cells were not treated with any human monoclonal, and the right shown neurons exposed to anti-NMDA receptor monoclonal, #003-102. (B) Fluorescence intensity of immunostained optically-tagged NMDA receptors or (C) human immunoglobulins, after 30 minute exposure to antibody-free control media (N = 13 cells) or 0.5 $\mu\text{g/ml}$ of monoclonal #003-102 (N = 13 cells). (D) shows an example distance-fluorescence intensity plot for a 16 μm dendritic section (shown in panel A), used to calculate the Spearman correlation coefficient between signals. (E) Representative images of immunostainings of astrocytes (GFAP) and microglia (Iba1), from DIV14 organotypic hippocampal cultures exposed to antibody-free control media (GFAP: N = 8 slices; Iba1: N = 11 slices) or 0.5 $\mu\text{g/ml}$ of monoclonal #003-102 (GFAP: N = 15 slices; Iba1: N = 15 slices), for 24 hours.

Chapter Summary

Prior characterisation of human-derived monoclonal autoantibodies directed against the NMDA receptor, #003-102, and the GABA_A receptor, #113-115, indicated to us that these are valuable tools for the investigation of antibody-mediated pathology, relevant to the disease state of autoimmune encephalitis (Kreye *et al.*, 2016, 2021; van Casteren *et al.*, 2022). However, we wanted to ensure that these autoantibodies passed three quality control criteria in our hand: i) antibodies continue to show reactivity to neuronal surface antigens, ii) antibodies continue to show specificity for their relevant target and iii) antibody exposure does not elicit substantial immune activation of astrocytes and microglia in organotypic hippocampal preparations. To explore these criteria, we performed immunocytochemical and histochemical analysis of the human anti-NMDA receptor monoclonal, #003-102.

Analysis of autoantibody binding to the surface of dissociated hippocampal neurons demonstrated substantial binding of human monoclonal antibody, #003-102, which significantly correlated with NMDA receptor expression profiles. As such, we are confident that this autoantibody meets the first two criteria regarding maintained binding and specificity after transport. Additionally, exposure of organotypic hippocampal cultures elicited no alterations in the expression of Iba1 or GFAP, indicating a lack of immune activation of microglial and astrocytic cells, respectively. However, it should be noted that in a model of lipopolysaccharide-mediated immune-challenge in mice, elicited activation of both astrocytes and microglia only within the 24-48 hour time point (Norden *et al.*, 2016). Yet, upregulation of proinflammatory cytokine mRNA in microglia and astrocytes was detected at acute stages, within 2-4 hours of lipopolysaccharide insult, including Interleukin (IL)-6, IL-1 β , IL-10, TNF α and CCL2 (Norden *et al.*, 2016). As such, we cannot rule out the possibility that autoantibody exposure may promote the release of proinflammatory cytokines, without alteration in morphology and expression of GFAP and Iba1 in astrocytes and microglia in our model system. Future work may aim to perform a better characterisation of this possibility, perhaps with further timepoints of antibody exposure and detection of cytokine presence in hippocampal tissue in addition to morphological changes in non-neuronal immune-responsive cells.

Having fulfilled our defined criteria, we are confident that the use of human monoclonal autoantibodies, at a concentration of 0.5 μ g/ml and exposure time of 24 hours is appropriate for our planned experiments, aiming to investigate autoantibody-mediated dysfunction in *in vitro* hippocampal preparations. As such, this dose and exposure was used in all experiments described in subsequent chapters.

III

SYNAPTIC PHENOTYPES

Chapter Introduction

Recent years have seen the identification of an array of autoimmune neurological syndromes, whereby patients express antibodies directed against brain-endogenous antigens. Two prominent diseases in this category are anti-NMDA receptor encephalitis and anti-GABA_A receptor encephalitis, in which patients develop antibodies directed against major excitatory or inhibitory ionotropic channels, respectively (Dalmau *et al.*, 2007; Petit-Pedrol *et al.*, 2014). Molecular investigations have delineated an acute pathway of action of these autoantibodies, focussing on the isolated impacts on the target antigens. It has been demonstrated that anti-NMDA receptor autoantibodies bind to the extracellular N-terminal domain of the obligatory GluN1 subunit (Kreye *et al.*, 2016). This antibody binding causes a synaptic destabilisation of synaptic NMDA receptors through hindrance of the interaction between negatively-charged sequences on the GluN1 N-terminal domains with synaptically localised and positively-charged EphB2 receptors (Mikasova *et al.*, 2012; Ladépêche *et al.*, 2018; Washburn *et al.*, 2020). This destabilisation causes an increase in the surface dynamics of synaptic NMDA receptor populations at acute timescales, promoting displacement to extrasynaptic sites (Ladépêche *et al.*, 2018). Subsequent crosslinking of extrasynaptic NMDA receptor clusters, accompanied by a negative shift in surface mobility, promotes internalisation of (Hughes *et al.*, 2010; Mikasova *et al.*, 2012). This depletion of surface expression results in an excitatory hypofunction on hippocampal neurons (Hughes *et al.*, 2010; Kreye *et al.*, 2016; Huang and Xiong, 2021). Conversely, anti-GABA_A receptor autoantibody binding elicits a direct antagonism of the inhibitory ion channel, with relatively little focus placed on a possible alteration of surface organisation (Noviello *et al.*, 2022; van Casteren *et al.*, 2022). Direct antagonism of GABA_A receptor channels has been demonstrated to occur through competition by autoantibodies for GABA binding sites, located at the interface between α and β subunits, and also through negative allosteric modulation of the channel (Noviello *et al.*, 2022). Such antagonism thus drives a hypofunction of the major inhibitory neurotransmission system. Essentially, pre-clinical investigations regarding the action of NMDA- and GABA_A receptor autoantibodies have demonstrated either excitatory or inhibitory hypofunction, respectively.

Intriguingly, despite the considerable opposition in the function of these autoantibody targets, seizure phenotypes form a prominent facet of a largely shared symptomology in these two distinct autoimmune diseases (Petit-Pedrol *et al.*, 2014; Geis *et al.*, 2019; Dalmau and Graus, 2023). In the case of GABA_A receptor encephalitis, antagonism of inhibitory GABAergic transmission intuitively leads to epileptogenesis. Where loss

of inhibitory control results in uncontrolled excitation, particularly in brain regions that are dense in recurrent microcircuits such as the hippocampus. It is not surprising then that approximately 80% of patients in this cohort present with severe seizure phenotypes, elevating this to the predominant disease feature of GABA_A receptor encephalitis (Guo, Gelfand and Geschwind, 2020). However, the mechanisms by which NMDA receptor autoantibodies – which demonstrably drive excitatory hypofunction at the molecular scale – can result in a similar phenotype remains more elusive. Yet, seizures are observed in approximately 80% of cases, forming a considerable segment of clinical presentation, along with movement disorder (78%) and psychiatric dysfunction (90%) (Graus *et al.*, 2016; Dalmau *et al.*, 2019). It has been suggested that NMDA receptor autoantibodies may display a degree of selectivity in pathogenesis, whereby inhibitory interneuron populations are primarily affected (Huang and Xiong, 2021; Hunter, Jamet and Groc, 2021). Some experimental evidence has begun to emerge in support of this hypothesis, suggesting a disinhibitory action of NMDA receptor autoantibodies in cortical networks which may provide a route towards hyperexcitation in the context of encephalitis (Andrzejak *et al.*, 2022). These findings are particularly intriguing considering the proposed role of interneuron-specific hypofunction in other psychiatric conditions such as schizophrenia and bipolar disorder, which bear an acute and unique resemblance to early stages of NMDA receptor encephalitis (Kayser and Dalmau, 2016, p. 201; Nakazawa and Sapkota, 2020; Muñoz-Castrillo, Vogrig and Honnorat, 2022).

Ultimately, seizure phenotypes represent the clinical endpoint of significant perturbation to excitatory-inhibitory (EI) balance and network homeostasis. The maintenance of physiological EI balance in the healthy brain is supported by a complex interplay between many cell autonomous and network-level systems: including modulations of synaptic plasticity, scaling, synaptic density and intrinsic cellular excitability (Trasande and Ramirez, 2007; Murase, 2014; Jedlicka, Muellerleile and Schwarzacher, 2018; Nanou, Lee and Catterall, 2018; Jami *et al.*, 2021). It has been known for many decades that excitatory and inhibitory systems exert mutual influence over each other, in order to reach a physiological equilibrium. This can be achieved through intracellular signalling cascades, for example the bidirectional linking of NMDA receptor-mediated calcium influx to local GABA_A receptor mobility and reorganisation (Bannai *et al.*, 2015). Additionally, common interacting proteins such as type I and II dopamine receptors, which bind NMDA- and GABA_A receptors, respectively, can act as centralised orchestrators of excitatory and inhibitory signalling (Schoffemeer *et al.*, 2000; Ampe *et al.*, 2007; Maingret and Groc, 2021).

Given the array of homeostatic systems in place, each dependent on effective crosstalk between excitatory and inhibitory signalling, the mechanistic underpinnings of autoimmune epileptogenesis may reside in pathological co-regulation of excitatory and inhibitory receptors. We would expect NMDA- and GABA_A receptor autoantibodies to elicit downstream perturbations across both neurotransmission systems: inhibitory and excitatory. However, the characterisation of this cross-synaptic disruption remains to be fully investigated. We hypothesise that antibody-mediated interference of NMDA- or GABA_A receptor organisation

and function will metastasise into global dysregulation of ionotropic synaptic signalling, impacting glutamatergic and GABAergic neurotransmission. Investigations into the co-regulation of glutamatergic and GABAergic systems, in the context of autoimmune encephalitis, may provide novel avenues for therapeutic development and intervention for these disease states. Moreover, identification of EI co-regulation in these diseases may further expand our knowledge regarding the interaction between excitatory and inhibitory systems in the maintenance of hippocampal homeostasis on a more fundamental level.

Methods

Dissociated and organotypic hippocampal cultures

Primary cultures were generated and transfected as described in Chapter II. Exogenous transgenes used in these experiments were used: *pRK5CMV-rGabrg2-SEP*, *pRK5CMV-HA-SEP-rGria1*, *pcDNA3.1CMV-dsRed-rHomer1c* and *pEGFP-N1-CMV-mVenus-rGphn*. Autoantibodies and/or pharmacological reagents were added to neuronal cultures by dilution into the cell culture media, during a full media exchange the day prior to imaging. All autoantibody samples were diluted to a final concentration of 0.5 µg/ml of media. Where indicated, D-APV was applied to a final concentration of 1µM. For some experiments, astrocyte-free neuronal cultures were generated following the protocol of Kaech & Banker (2006). Briefly, coverslips were flipped onto astrocyte feeder layers, 3 hours after plating, and maintained in this inverted configuration. At DIV 3, a full media exchange with serum-free Neurobasal/B-27 culture media, containing 5µM cytosine arabinoside was performed to prevent astrocyte proliferation (Kaech and Banker, 2006). In these experiments, autoantibody incubations were performed in fresh culture media and in the absence of astrocyte feeder layers, for 24 hours, before fixation and immunostaining. Hippocampal slice cultures were prepared, and autoantibody samples applied as described in Chapter II.

Electrophysiology

Spontaneous excitatory and inhibitory postsynaptic currents (sEPSC and sIPSC, respectively) were recorded using a whole-cell patch clamp technique in voltage-clamp configuration. In summary, hippocampal slice cultures were transferred to the chamber of an upright microscope, containing 34°C carbogen-bubbled extracellular solution composed of 126mM NaCl, 3.5mM KCl, 2mM CaCl₂, 1.3 MgCl₂, 1.2mM NaH₂PO₄, 25mM NaHCO₃ and 12.1mM glucose (pH 7.4, 310 mOsm), perfused at approximately 2ml per minute. Neurons in the pyramidal cell layer of the CA1 hippocampal subfield were visualised using infrared differential interference contrast imaging. Patch pipettes for voltage-clamp recordings were filled with intracellular solution composed of 130mM CsMeS, 20mM HEPES, 0.2mM EGTA, 5mM QX-314·Cl, 2mM NaCl, 4mM Mg-ATP and 0.4mM Na-GTP (pH 7.3, 290 mOsm). Pipette resistance ranged from 4-6MΩ. After achieving a whole-cell configuration, AMPA receptor-mediated sEPSCs were recorded while clamping the membrane potential at -70mV. After 10

minutes of sEPSC recording, cells were then clamped at 0mV to record 10 minutes of GABA_A receptor-mediated sIPSCs. Miniature excitatory and inhibitory postsynaptic currents (mEPSC and mIPSC, respectively), were recorded with the same experimental setup, with the alteration that 2 μ M tetrodotoxin was added to extracellular solution during recordings. Traces were recorded using a Multiclamp 700B amplifier and a Digidata 1550B interface controlled by Clampex 10.7 (Molecular Devices). Series resistance was monitored throughout the experiment by a brief voltage step of -5 mV at 30 second intervals. Data was discarded if series resistance was found to vary by more than 20% during the recording session. Traces were analysed using ClampFit software, in which synaptic events were detected using an automated template search protocol. To permit correct characterisation of synaptic event amplitudes and kinetic parameters, extracted events were manually inspected for adherence to inclusion criteria. Specifically, events were discarded in instances where two synaptic events were found to be superimposed, where events occurred during a series resistance test or when peak amplitude failed to exceed 2 standard deviations of the baseline. Amplitudes of all detected events are displayed as cumulative frequency distributions, and for quantification, mean event amplitudes were calculated for each cell recorded. For quantification of event frequencies and burst parameters, automatically detected events in the template search protocol were counted, irrespective of inclusion criteria described for kinetic and amplitude analysis. Burst-like event distributions were evaluated by dividing the mean instantaneous frequency by the overall frequency of the recording, as such, a value of 1 would denote regular patterning of synaptic activity, and any increase above 1 denotes a clustering of events. Error bars represent the standard error of the mean.

Multiplicity modelling and analysis

To model multiplicity values from our miniature and spontaneous datasets, we calculated the mean and standard deviation of the frequency and amplitude distributions from experimental observation of spontaneous and miniature excitatory and inhibitory postsynaptic currents. This was necessary since spontaneous and miniature recordings were not performed in the same cell populations. Subsequently, we sampled values at random from these theoretical distributions and used these simulated datapoints to calculate the multiplicity with the following formula: $[(S_{\text{Freq}} \cdot S_{\text{Amp}}) - (M_{\text{Freq}} \cdot M_{\text{Amp}})] / [(S_{\text{Freq}} - M_{\text{Freq}}) \cdot M_{\text{Amp}}]$, where S_{Freq} denotes spontaneous event frequency; S_{Amp} denotes spontaneous event amplitude; M_{Freq} denotes miniature event frequency and M_{Amp} denotes miniature event amplitude (Hsia, Malenka and Nicoll, 1998).

Immunocytochemistry

Dissociated hippocampal cultures were transfected with either homer1c-dsRED and GluA1-SEP; or gephyrin-mVenus with gamma2-SEP, as described above. Fluorescent activity of dsRED and mVenus is maintained after fixation and in mounting media, and as such were not labelled by amplifying antibodies. However, SEP fluorescence is lost under these conditions and was therefore immunolabelled with the following protocols:

Following 24-hour incubation with experimental autoantibodies, hippocampal cultures were incubated with rabbit anti-GFP (Thermo Fisher A6455) at 1:1000, to uncover the surface expression of SEP-tagged AMPA- or GABA_A receptors, for 30 minutes, at 37°C. Cultures were then washed, fixed, quenched and blocked as described in Chapter II. Subsequently, GluA1-transfected cultures were incubated with goat anti-rabbit alexafluor-488 (Thermo Fisher A11008), at 1:500, for 2 hours at room temperature, under agitation. Gamma2-transfected cultures were similarly labelled with goat anti-rabbit alexafluor568 (Thermo Fisher A11011). Cultures were washed with three times with PBS and ultrapure water prior to mounting in Vectashield anti-fade mounting medium and stored at 4°C until imaging. Immunostainings of endogenous gephyrin were carried out with the following protocols: Following 24-hour incubation with experimental autoantibodies, hippocampal cultures were washed, fixed and quenched as described in Chapter II. Cultures were then permeabilised in an 0.1% Triton-X100 PBS solution for 15 minutes at room temperature. Coverslips were then washed in PBS and blocked as described before, and subsequently incubated with either mouse anti-gephyrin (Synaptic Systems 147 111), at 1:1000 for 2 hours at room temperature, to label gephyrin scaffolds independent of phosphorylation status; or alternatively with mouse anti-gephyrin (Synaptic Systems 147 011), under the same protocols, to label gephyrin scaffolds phosphorylated at S270. Coverslips were then washed and labelled with goat anti-mouse alexafluor488 (Thermo Fisher A11001), for 1 hour at room temperature before washing and mounting and storage as described above.

Results

Autoantibodies mutually dysregulate spontaneous excitatory neurotransmission

We characterised the precise impacts on functional synaptic phenotypes of excitatory neurotransmission in organotypic hippocampal slice cultures. These preparations were used for functional investigations in order to unveil synaptic activity in semi-maintained hippocampal circuit architecture. Spontaneous AMPA receptor-mediated excitatory postsynaptic currents (sEPSC) were revealed by whole-cell patch clamp electrophysiology (Figure 8A-B). Analysis of sEPSC amplitudes revealed a significant reduction after 24-hour exposure to NMDA- or GABA_A receptor monoclonal antibodies, as compared to non-reactive control-monoclonal treated slices (Figure 8C-D). However, there was no observable shift in the either rise or decay kinetics of the sEPSC of either pathogenic antibody, compared to control (Figure 8E-F). Suggesting a loss of AMPA receptors from excitatory synaptic compartments, which is not selective for particular subunit containing channels, instead reflecting a generalised disorganisation of the postsynaptic compartment. A similar reduction in AMPA receptor signalling has been recently demonstrated in an *in vivo* model of NMDA receptor encephalitis (Ceanga *et al.*, 2022), but to our knowledge this is the first demonstration of a congruent deficit in excitatory neurotransmission elicited by GABA_A receptor monoclonal antibodies. Moreover, analysis of sEPSC frequency did not reveal any

significant alterations by either pathogenic autoantibody (Figure 8G-H). However, analysis of event temporal distributions revealed that GABA_A receptor autoantibodies selectively, promote a clustering – or burst-like – event profile as compared to control conditions (Figure 8I).

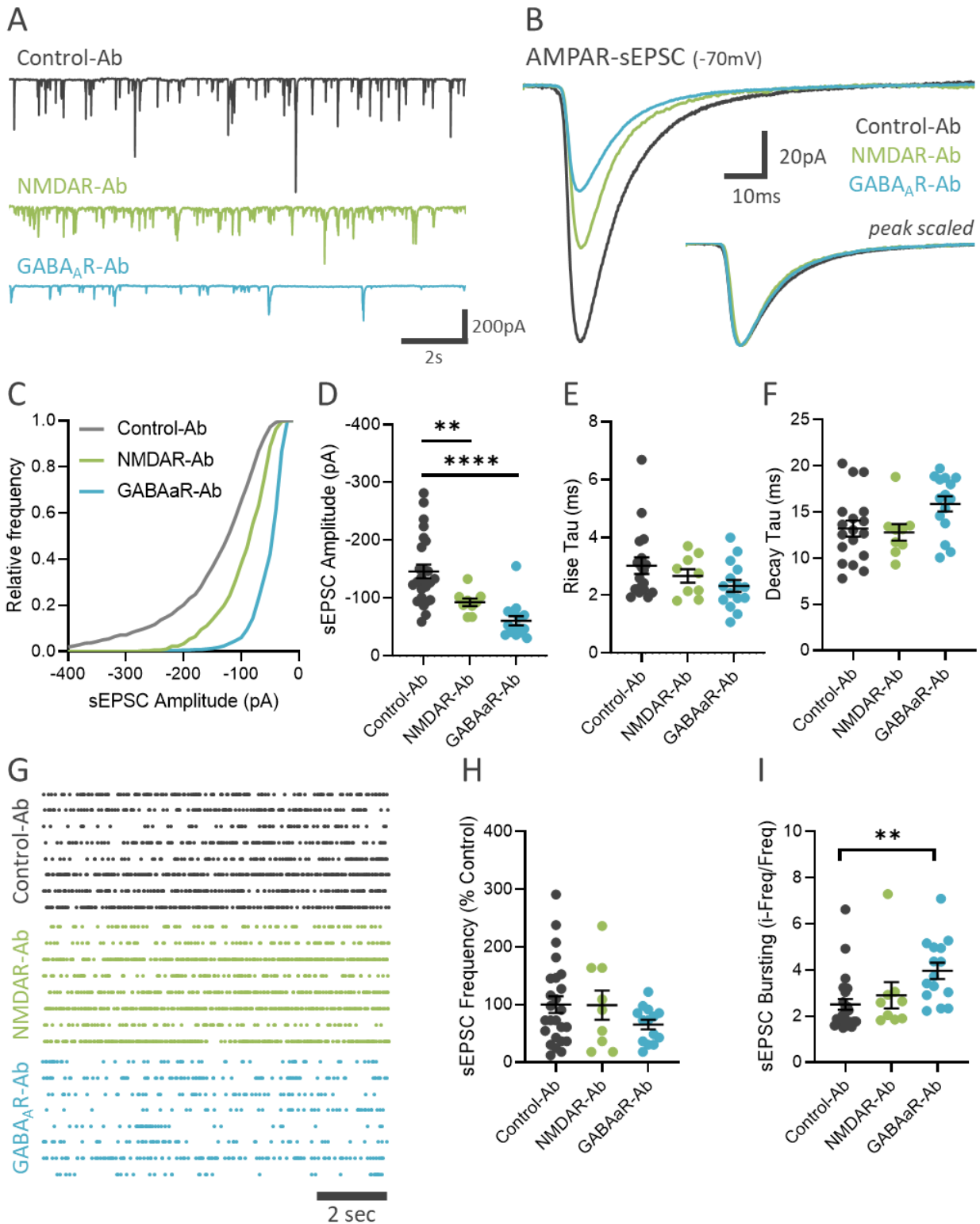


Figure 8: Autoantibodies similarly disrupt excitatory synaptic phenotypes.

(A) Example traces of AMPAR-mediated sEPSC, incubated for 24 hours with monoclonal antibodies against a null antigen (Control-Ab), NMDA receptors (NMDAR-Ab) or GABA_A receptors (GABA_AR-Ab). (B) Average AMPAR-sEPSC from these conditions, insert illustrating these events after peak-scaling. (C) Cumulative frequency distributions (Control-Ab: N = 1020 events; NMDAR-Ab: N = 296 events; GABA_AR-Ab: N = 449 events) and (D) cell mean AMPAR-sEPSC amplitudes (Control-Ab: N = 25 cells; NMDAR-Ab: N = 9 cells; GABA_AR-Ab: N = 15 cells). (E) Cell mean rise and (F) decay kinetics (Control-Ab: N = 18 cells; NMDAR-Ab: N = 9 cells; GABA_AR-Ab: N = 15 cells). (G) Eight example temporal raster plots from each condition where each row denotes an individual cell, with each point representing an automatic detection of an sEPSC. (H) Quantified AMPAR-sEPSC frequency per cell and (I) the bursting pattern of the sEPSC distributions (Control-Ab: N = 25 cells; NMDAR-Ab: N = 9 cells; GABA_AR-Ab: N = 14 cells). Error bars denote the standard error of the mean.

Autoantibodies mutually dysregulate spontaneous inhibitory neurotransmission

Quantification of sIPSC amplitudes revealed a similar common impact on inhibitory signalling (Figure 9A-B). Compared to control, we observed a significant reduction after exposure to either NMDA- or GABA_A receptor monoclonals (Figure 9C-D). Such a reduction in GABAergic signalling is expected by GABA_A receptor antibody exposure, since the mechanisms of action of these antibodies is, at least in part, a direct antagonism of GABA_A receptor channel function. However, the novel identification of a common impact by NMDA receptor autoantibodies is intriguing, demonstrating that the pathological co-regulation of NMDA- and GABA_A receptors we previously identified translated into a functional pathology of synaptic transmission. Additionally, we did not observe any alteration in the kinetics of the sIPSC (Figure 9E-F), indicating that the reductions in current are mediated by uniform reductions in channel function or organisation at the postsynaptic compartment, as opposed to a selective action of specific subunit containing GABA_A receptors. Moreover, both NMDA- and GABA_A receptor autoantibodies did not induce any overall alteration in sIPSC frequency, however we did uncover a slight but significant increase in the burst-like activity of inhibitory synaptic inputs after application of GABA_A receptor monoclonals, selectively (Figure 9G-I). This is consistent with our previous analysis of excitatory transmission, suggesting that GABA_A receptor autoantibodies elicit further disruptions in the temporal profile of synaptic inputs – both excitatory and inhibitory – to CA1 pyramidal neurons.

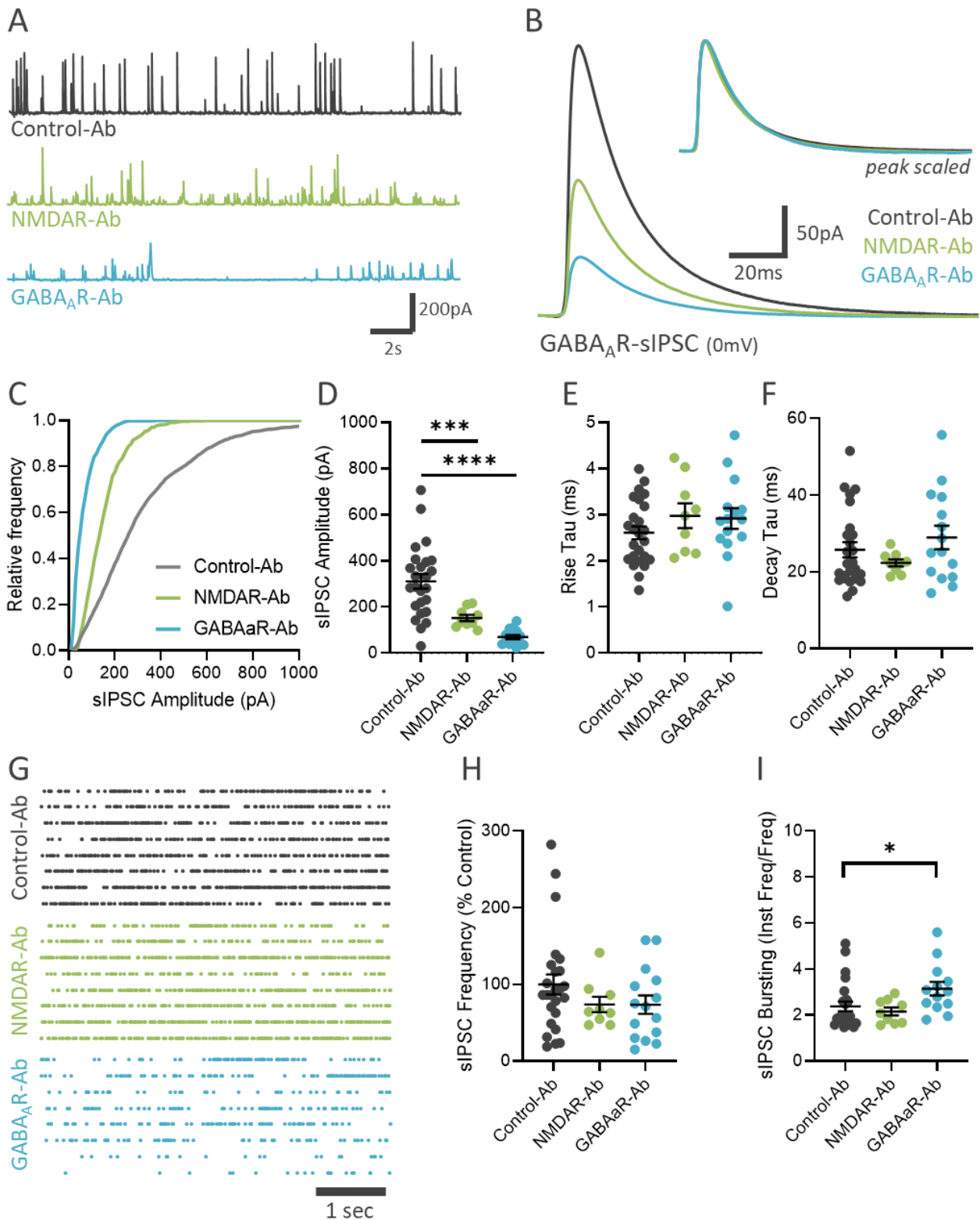


Figure 9: Autoantibodies similarly disrupt inhibitory synaptic phenotypes. (A) Example traces of GABA_A-mediated sIPSC, incubated for 24 hours with monoclonal antibodies against a null antigen (Control-Ab), NMDA receptors (NMDAR-Ab) or GABA_A receptors (GABA_A-Ab). (B) Average GABA_A-sIPSC from these conditions, insert illustrating these events after peak-scaling. (C) Cumulative frequency distributions (Control-Ab: N = 1952 events; NMDAR-Ab: N = 538 events; GABA_A-Ab: N = 873 events) and (D) cell mean AMPAR-sEPSC amplitudes (Control-Ab: N = 25 cells; NMDAR-Ab: N = 9 cells; GABA_A-Ab: N = 15 cells). (E) Cell mean rise and (F) decay kinetics (Control-Ab: N = 25 cells; NMDAR-Ab: N = 9 cells; GABA_A-Ab: N = 15 cells). (G) Eight example temporal raster plots from each condition where each row denotes an individual cell, with each point representing an automatic detection of an sIPSC. (H) Quantified GABA_A-sIPSC frequency per cell and (I) the bursting pattern of the sIPSC distributions (Control-Ab: N = 25 cells; NMDAR-Ab: N = 9 cells; GABA_A-Ab: N = 14 cells). Error bars denote the standard error of the mean.

Autoantibodies disrupt synaptic multiplicity and excitation-inhibition balance

Analysis of mEPSC data revealed a significant reduction in amplitudes, elicited by NMDA receptor monoclonals (Figure 10A-B). However, in this configuration we did not observe any alteration after exposure to GABA_A receptor autoantibodies (Figure 10A-B). Further, mEPSC instantaneous frequency was unaltered by either pathogenic autoantibody (Figure 10C). Additionally, neither NMDA- nor GABA_A receptor autoantibodies elicited significant alterations to mIPSC amplitude (Figure 10E-F). While this was unexpected given our prior observations of significant impacts of these autoantibodies on spontaneous synaptic transmission, previous reports have similarly demonstrated that NMDA receptor autoantibodies did not alter GABAergic mIPSC amplitudes *in vivo* (Ceanga *et al.*, 2022). However, both autoantibodies elicited significant reduction in mIPSC event frequency (Figure 10G). To further explore this data, in combination with our characterisation of spontaneous transmission, we modelled the synaptic glutamatergic and GABAergic multiplicity by random sampling from gaussian distributions, each fitted to our experimentally determined parameters. Generation of 18 stochastically derived sample parameters permitted adequate statistical power for preliminary investigation of theoretically modelled multiplicity values. Analysis identified no theoretical alterations to glutamatergic multiplicity, however GABAergic multiplicity was found to be reduced by exposure to both NMDA- and GABA_A receptor autoantibody exposure (Figure 10D&H). Suggesting that despite the lack expected congruency between spontaneous and miniature postsynaptic recordings, when considered in combination with each other, our results are consistent with a reduction in the density of interneuron-to-principal cell connections. This possibility is directly investigated in subsequent immunostaining experiments.

To further explore the excitatory inhibitory balance of the synaptic inputs, we expressed the mean amplitudes of the AMPA receptor-mediated sEPSC, relative to the mean amplitudes of the GABAergic-sIPSC for each recorded cell. This analysis revealed a significant shift towards hyperexcitation after application of either NMDA- or GABA_A receptor autoantibodies, relative to the Control autoantibody conditions (Figure 10I-J). This demonstrates that although there is a general reduction of synaptic inputs to hippocampal pyramidal cells in the CA1, the deficits in GABAergic transmission are consistently greater in magnitude compared to the impacts on excitatory signalling. This greater disturbance of inhibitory neuro-communication, at the functional level, may play a significant role in the development of seizure activity, commonly observed in the clinical presentation of NMDA- and GABA_A receptor encephalitis (Figure 10K).

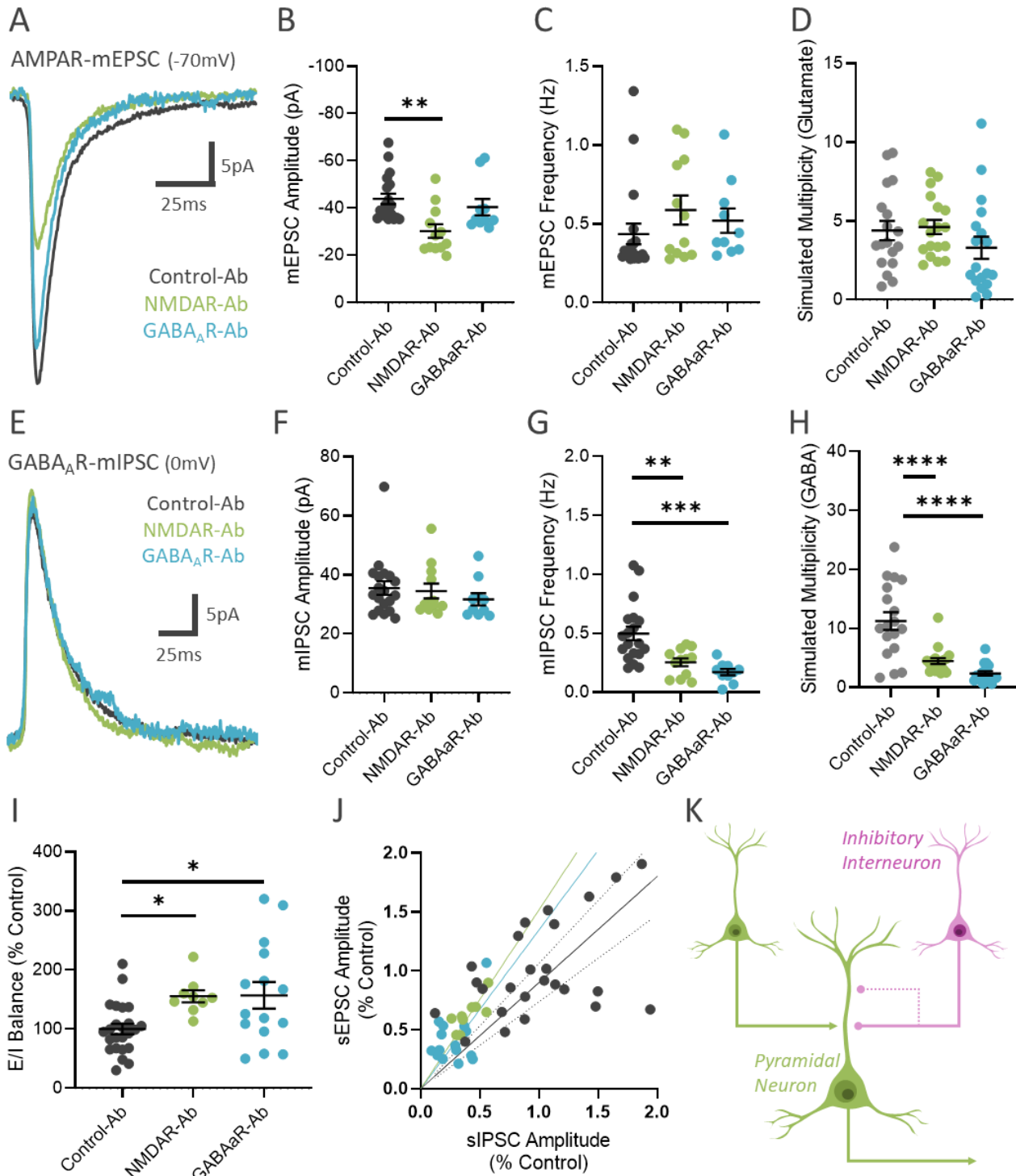


Figure 10: Autoantibodies reduce GABAergic multiplicity and excitation-inhibition balance.

(A) Mean AMPAR-mediated mEPSC after incubation for 24 hours with monoclonal antibodies against a null antigen (Control-Ab), NMDA receptors (NMDAR-Ab) or GABA_A receptors (GABA_AR-Ab). (B) Quantified cell means for mEPSC amplitude and (C) frequency (Control-Ab: N = 19 cells; NMDAR-Ab: N = 12 cells; GABA_AR-Ab: N = 10 cells). (D) Simulated synaptic multiplicity for glutamate (18 simulated datapoints per condition). (E) Mean GABA_AR-mediated mIPSC after incubation for 24 hours with monoclonal antibodies against a null antigen (Control-Ab), NMDA receptors (NMDAR-Ab) or GABA_A receptors (GABA_AR-Ab). (F) Quantified cell means for mIPSC amplitude and (G) frequency (Control-Ab: N = 19 cells; NMDAR-Ab: N = 12 cells; GABA_AR-Ab: N = 10 cells). (H) Simulated synaptic multiplicity for GABA (18 simulated datapoints per condition). (I) Excitation-inhibition ratio, after exposure to monoclonal autoantibodies, normalised to control conditions, calculated from spontaneous synaptic current amplitudes (Control-Ab: N = 25 cells; NMDAR-Ab: N = 9 cells; GABA_AR-Ab: N = 15 cells). (J) Data from (I) represented as a scatter-plot where solid lines represent the linear regression, restrained to pass through the origin, where an increase in gradient would indicate a shift towards hyperexcitation. (K) schematic representation of reduced connectivity between interneurons and principal cells.

Autoantibodies disrupt AMPA receptor synaptic localisation

To further characterise the direct- and cross-synaptic impacts elicited by NMDA- and GABA_A receptor autoantibodies, we examined the macroscopic synaptic content by quantifying the expression and localisation of the AMPA receptor population. To achieve high precision staining of surface proteins, and enable quantifications confined to single dendritic segments, dissociated neuronal cultures were transfected with tagged ionotropic receptor subunits and associated fluorescently tagged synaptic markers. Given our previous demonstration of mutual dysregulation of AMPA receptor signalling in intact hippocampal networks by both pathogenic autoantibodies, we expected a translation into a macroscale disorganisation of AMPA receptors at excitatory synaptic compartments. We observed that exposure to both NMDA- and GABA_A receptor monoclonal antibodies elicited a reduction in total and synaptic AMPA receptor cluster size, relative to Control antibody treated cultures (Figure 11A-C) with no alteration in cluster density (Figure 11E-F), indicating moderate alteration in the organisation of AMPA receptors within synaptic compartments. Given that AMPA receptors display a substantially lower affinity for glutamate, compared to the NMDA receptor population, precise synaptic nanoarchitecture is required to align subsynaptic AMPA receptor clusters with presynaptic release sites. It is possible that the subtle disorganisation we observe here at the macroscopic scale, may be underpinned by a larger dysregulation of nanoscale organisation, which may have profound effects on excitatory AMPA-mediated neurotransmission, despite only a modest reduction in synaptic content. Given that both NMDA- and AMPA receptors are stabilised at synapses – at least in part – by association with scaffolding proteins in the postsynaptic density, we further assessed the possibility that synaptic scaffolding proteins may be impacted after 24 hours of autoantibody exposure. However, we did not uncover any significant alterations in the cluster density, or cluster size of the synaptic scaffold, homer1c – indicating that pathogenic autoantibodies do not modify the scaffold integrity of excitatory synapses (Figure 11D&G).

Given the role of ionotropic activity of the NMDA receptor in synaptic plasticity, we further wanted to assess whether these alterations in synaptic AMPA receptor content could be shaped by reduced function of NMDA receptors, independently of the aberrant surface diffusion induced by autoantibody exposure (Mikasova *et al.*, 2012). To explore this, we exposed neuronal cultures to a partially-blocking concentration (1 μ M) of the NMDA receptor antagonist APV for 24 hours. This approach permitted us to uncouple the reduction in NMDA receptor signalling and impact on membrane diffusion, as APV has been demonstrated to elicit no clear impact on NMDA receptor dynamics (unpublished observations of colleagues). The partially-blocking concentration was selected as to mimic the incomplete reduction of NMDA receptor-mediated signalling induced by autoantibody exposure (Hughes *et al.*, 2010). This pharmacological manipulation failed to recapitulate any alteration in AMPA receptor localisation to synaptic structures, as we observed after NMDA- and GABA_A receptor monoclonal exposure (Figure 11H-N), suggesting that – in the case of NMDA receptor autoantibody

exposure – these impacts are primarily triggered by alteration in the surface dynamics of membrane proteins alone, or in combination with modulation in ion channel activity.

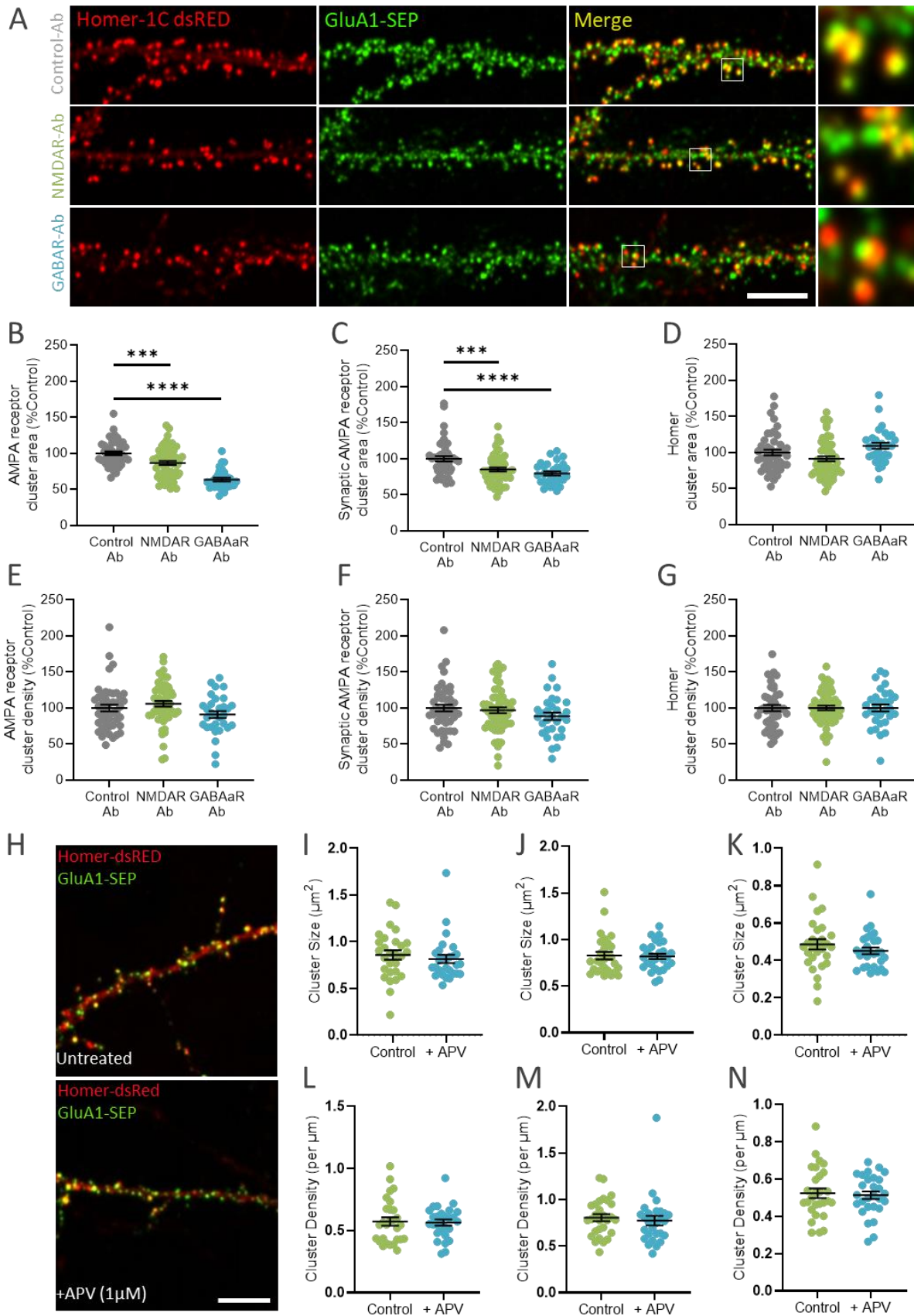


Figure 11: Autoantibodies reduce synaptic localisation of AMPA receptors.

(A) Representative images of Homer1c-dsRED and associated immunostainings for GluA1-SEP, after incubation with autoantibodies for 24 hours. Right panel shows a zoomed section represented in the merge column. (B) Mean AMPA receptor cluster area and (E) density. (C) Mean synaptic AMPA cluster area and (F) density. (D) mean homer cluster area and (G) density (Control-Ab: N = 46 cells; NMDAR-Ab: N = 57 cells; GABA_AR-Ab: N = 31 cells). (H) Representative images of Homer1c-dsRED and immunostaining for GluA1-SEP, after incubation with drug-free control- or APV-supplemented media for 24 hours. (I) Mean AMPA receptor cluster area and (L) density. (J) Mean synaptic AMPA cluster area and (M) density. (K) mean homer cluster area and (N) density (Control: N = 28 cells; APV: N = 28 cells). Error bars denote the standard error of the mean.

Autoantibodies fail to disrupt AMPA receptor synaptic localisation in the absence of astrocytes

Our observations did not uncover any substantial alteration in excitatory postsynaptic scaffolds, at least at the level of Homer-1c and at the resolution that can be achieved with confocal microscopy. However, given that astrocytes have been shown to modulate AMPA- and NMDA receptor signalling during synaptic plasticity, circadian rhythms and neurodevelopmental processes (Ota, Zanetti and Hallock, 2013; Blanco-Suarez *et al.*, 2018; McCauley *et al.*, 2020). Of particular interest, it has been demonstrated that metabotropic glutamate receptors, expressed on hippocampal astrocytes, can elicit the release of ATP which in turn drives a reduction in AMPA receptor surface expression through a P2X receptor-dependant pathway (Lalo and Pankratov, 2022). In light of these findings, we hypothesised that astrocytes may play a role in the pathological coregulation of AMPA receptor expression and organisation in our model system of autoimmune encephalitis. As such, we performed immunostainings for AMPA receptors and homer-1c, as described above, however 24-hour autoantibody exposure was performed in the absence of astrocytes. In this case, analysis of AMPA receptor expression revealed no alterations elicited by either autoantibody, when astrocytes were not present during antibody exposure (Figure 12A-F). Suggesting that astrocytes may play a significant role in the regulation of AMPA receptor synaptic redistribution in this case. It is tempting to speculate that autoantibody exposure may trigger hyperactivity of neuronal networks, and increased glutamate release, which could in turn activate metabotropic glutamate receptors expressed on the surface of surrounding astrocytic cells. However, such a mechanism requires further investigation.

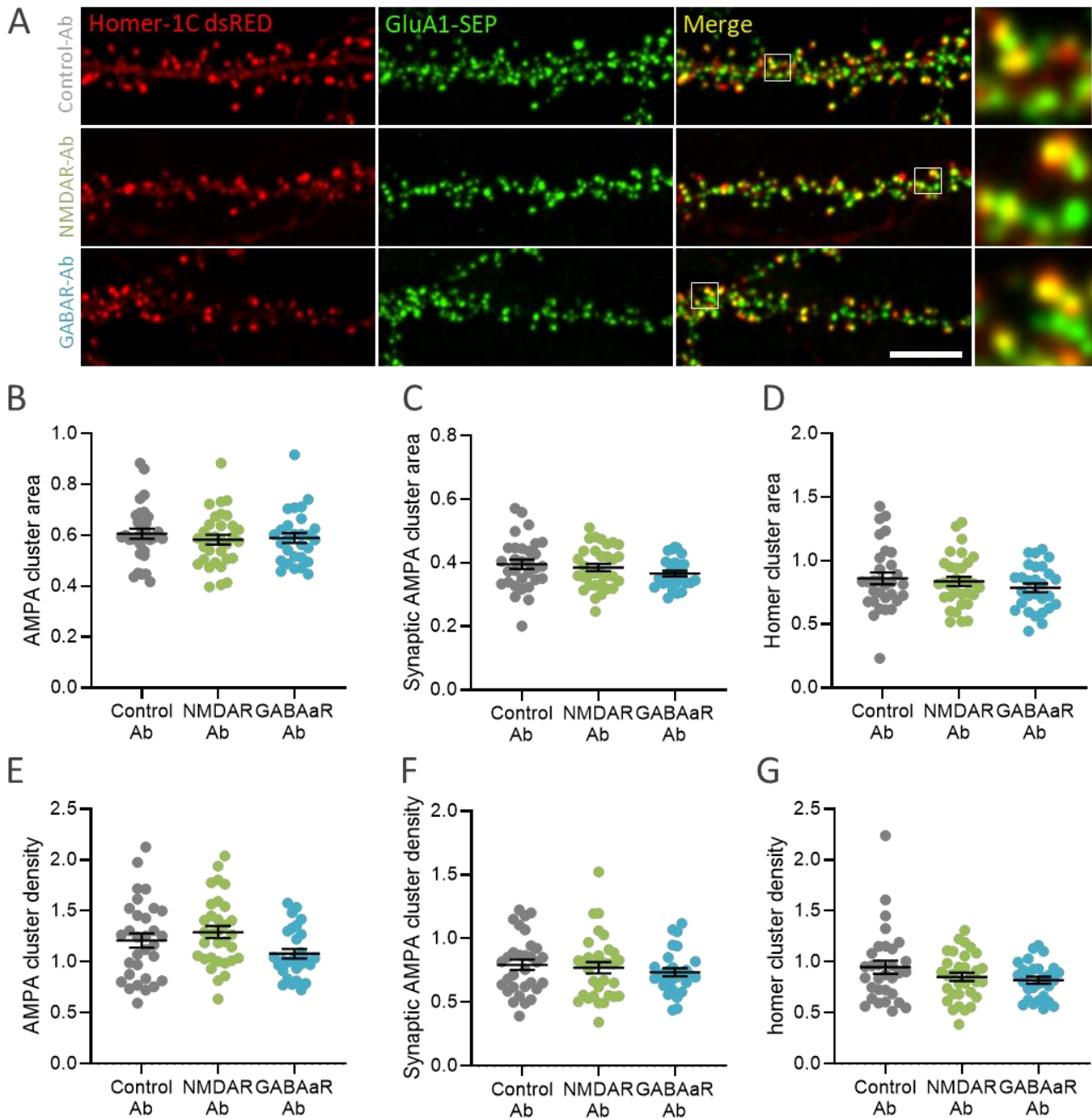


Figure 12: Autoantibodies fail to reduce synaptic localisation of AMPA receptors in the absence of astrocytes.

(A) Representative images of homer-1c-dsRED expression and immunostainings for GluA1-SEP. (B) Mean AMPA receptor cluster area and (E) density. (C) Mean synaptic AMPA cluster area and (F) density. (D) mean homer cluster area and (G) density (Control-Ab: N = 31 cells; NMDAR-Ab: N = 32 cells; GABA_A-Ab: N = 28 cells). Error bars denote the standard error of the mean.

Autoantibodies mutually disrupt inhibitory synaptic content and scaffolding proteins

Having uncovered substantial dysregulation at excitatory synapses by both encephalitis-derived autoantibody samples, we further investigated the content and structuration of inhibitory synaptic compartments. Analysis of GABA_A receptor clustering demonstrated that both patient-derived autoantibodies also reduced the synaptic localisation of GABA_A receptor (Figure 13A). In this case, we uncovered a significant reduction in overall GABA_A receptor cluster density, after exposure to GABA_A receptor monoclonals, without alteration in

cluster area (Figure 13B&E). Exposure to NMDA receptor autoantibodies elicited a similar impact, however this failed to reach the significance threshold (Figure 13B&E). Further, we observed clear reductions in synaptically localised GABA_A receptor cluster density and area, after exposure to either GABA_A- or NMDA receptor autoantibody samples (Figure 13C&F). Taken together with our previous observations of excitatory synaptic disorganisation, these findings demonstrate the striking capability of highly specific patient-derived autoantibodies, directed against single target antigens, to induce neuron-wide disorganisation of surface proteins, spanning across both excitatory and inhibitory synaptic structures, irrespective of the native localisation of the target antigen. Considering our observations of substantial disorganisation of inhibitory synaptic content, and given that synaptic GABA_A receptor diffusion has previously been linked to destabilisation of inhibitory synaptic scaffolding proteins (Battaglia *et al.*, 2018), we investigated the potential for autoantibody-mediated disturbance of the inhibitory synaptic scaffolding protein, gephyrin. In primary hippocampal cultures exposed to either NMDA- or GABA_A receptor autoantibodies for 24 hours, we observed a clear reduction in the density of gephyrin clusters along dendritic segments relative to Control antibody exposed cultures (Figure 13D&G). Given the association of network hyperexcitation and seizure phenotypes in these disorders, such a degradation of inhibitory signalling sites cannot be explained by typical homeostatic regulation. This selective loss of inhibitory synapses adds a further avenue towards synaptically driven hyperexcitation of principal hippocampal neurons, yielding further perturbation of the excitatory-inhibitory balance.

Given the known role of gephyrin phosphorylation sites in controlling the cluster size and density of inhibitory synaptic scaffolds (Tyagarajan *et al.*, 2011; Zacchi, Antonelli and Cherubini, 2014), we explored the phosphorylation status of gephyrin to uncover if this was a driving factor behind the degradation of inhibitory synaptic scaffolds, observed after treatment with autoantibody samples. Specifically, phosphorylation of a serine residue at position 270 (S270) has been demonstrated to induce a reduction in gephyrin cluster densities, through the enhanced recruitment of calpain, a protease that subsequently cleaves the scaffolding protein (Tyagarajan *et al.*, 2011). To explore this further, primary hippocampal cultures were exposed to Control-, NMDA- or GABA_A receptor autoantibodies for 24 hours prior to immuno-labelling for total gephyrin expression or with a phosphorylation specific antibody, which binds selectively to gephyrin scaffolds that have been phosphorylated at S270 (Kuhse *et al.*, 2012). As expected, the density and area of phosphorylated gephyrin puncta were significantly lower compared to total gephyrin, in control conditions (Figure 13H-L). This relates to the native state in which gephyrin phosphorylation is generally low, in order to prevent calpain recruitment and maintain stability of inhibitory synaptic scaffolds. This is in stark contrast to our findings that phosphorylated (S270) gephyrin cluster density and area were similar to total gephyrin cluster levels after exposure to NMDA- or GABA_A receptor monoclonal antibodies (Figure 13I-L). This indicates that both

pathogenic autoantibody samples induce an increase in the phosphorylation of gephyrin scaffolds, promoting their degradation and overall loss of inhibitory synapses from principal cell dendrites.

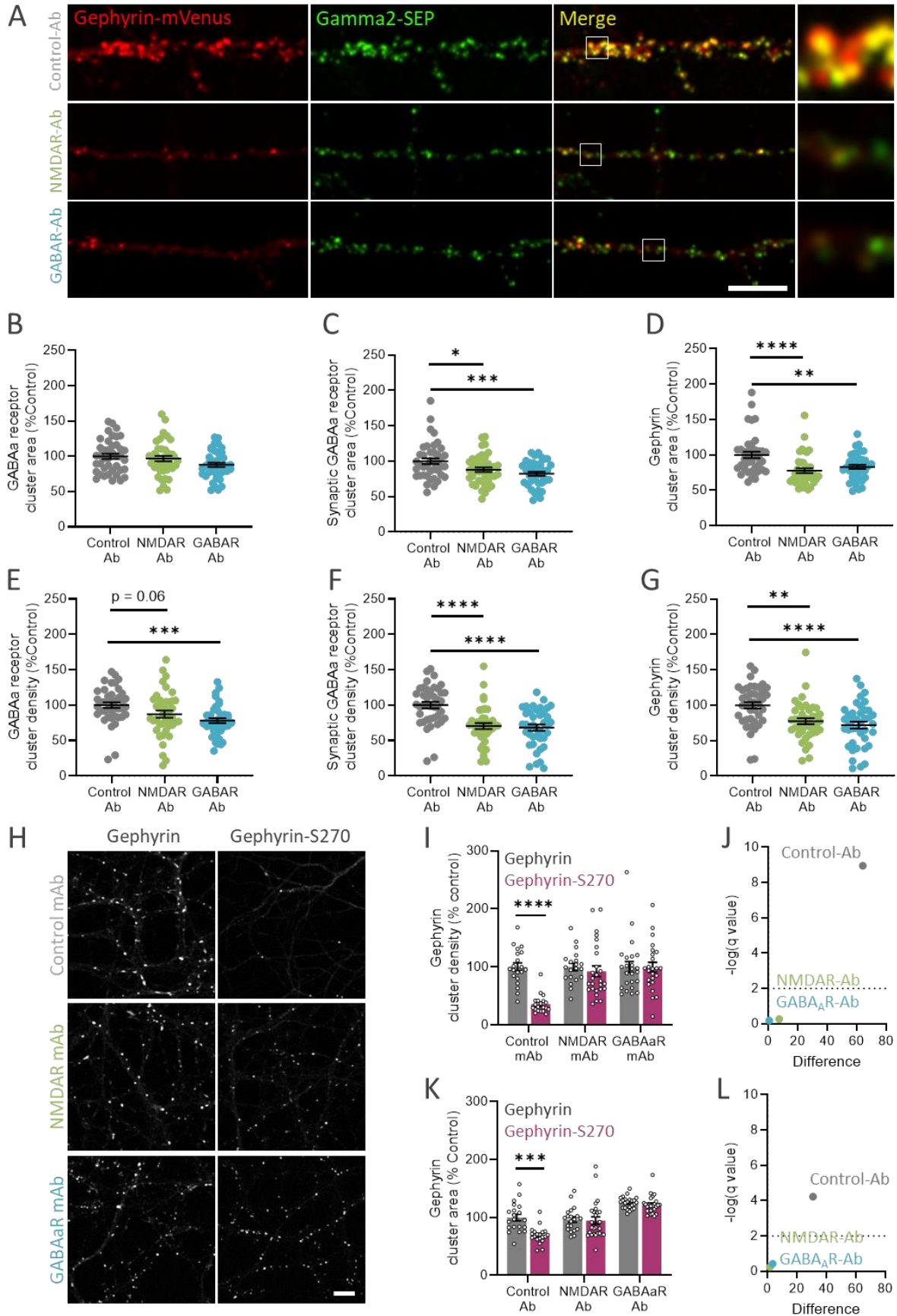


Figure 13: Autoantibodies reduce synaptic localisation of GABA_A receptors and promote gephyrin phosphorylation.

(A) Representative images of gephyrin-mVenus and associated immunostainings for Gamma2-SEP-containing GABA_A receptors, after incubation with autoantibodies for 24 hours. Right panel shows a zoomed section represented in the merge column. (B) Mean GABA_A receptor cluster area and (E) density. (C) Mean synaptic GABA_A cluster area and (F) density. (D) mean gephyrin cluster area and (G) density (Control-Ab: N = 41 cells; NMDAR-Ab: N = 41 cells; GABA_AR-Ab: N = 40 cells). (H) Representative images of gephyrin immunostainings with phospho-unspecific or phosphor(S270)-specific antibody, labelled gephyrin and gephyrin-S270, respectively. (I) Mean gephyrin cluster density and (J) associated volcano plots. (K) Mean gephyrin cluster area and (L) associated volcano plots (Control gephyrin: N = 20 fields; Control gephyrin-S270: N = 20 fields; NMDAR-Ab gephyrin: N = 20 fields; NMDAR-Ab gephyrin-S270: N = 26 fields; GABA_AR-Ab gephyrin: N = 24 fields; GABA_AR-Ab gephyrin-S270: N = 24 fields). Error bars denote the standard error of the mean.

Chapter Summary

The AMPA receptor constitutes the majority of excitatory synaptic transmission, as such, we were interested to quantify the prominent synaptic phenotypes after exposure to NMDA- and GABA_A receptor monoclonal autoantibodies. We identified a striking and mutual reduction in AMPA receptor-mediated currents, after exposure to either pathogenic autoantibody. Imaging of receptor organisation and synaptic localisation further identified a moderate but significant reduction in AMPA receptor localisation to postsynaptic sites. Intriguingly, the larger magnitude impact we observe on current reduction, relative to synaptic depletion could suggest an additional alteration to the nanoscale topography of synaptically resident AMPA receptors. Moreover, we observed a substantial reduction in inhibitory GABA_A receptor-mediated currents after exposure to GABA_A – and also – NMDA receptor autoantibodies. While such a reduction was anticipated in the context of GABA_A receptor autoantibody impacts, due to their direct and well characterised action on channel function, to observe a congruent alteration by NMDA receptor autoantibodies is of particular interest. Encouragingly, similar reductions in inhibitory signalling of autaptic cortical cultures have recently been described after exposure to this same NMDA receptor monoclonal antibody (Andrzejak *et al.*, 2022). Further analysis of GABA_A receptor localisation unveiled that both pathogenic autoantibodies drive a depletion of γ 2-subunit containing channels from inhibitory postsynaptic compartments. Taken together, these data give us a clear indication that autoantibodies directed against single neuronal antigens, hold the potential to elicit pathogenicity through a variety of mechanisms, including directed antigen impacts but also downstream dysregulation of neuronal functions – crossing the gap between excitatory and inhibitory synaptic activity.

To investigate the balance between spontaneous excitatory and inhibitory synaptic inputs, we calculated the relative level of excitatory event amplitudes, normalised to that of GABAergic inputs. This revealed that the balance of synaptic inputs to CA1 pyramidal neurons is shifted in favour of hyperexcitation, despite the generalised reduction in both excitation and inhibition. To fully understand the impact of this alteration, it is critical to characterise the intrinsic excitability of hippocampal neurons, to uncover a possible modulation in the computational processes involved in synaptic integration. Our direct investigation of intrinsic excitability,

of both principal cells and inhibitory interneurons of the CA1 hippocampal subfield, are described in the subsequent chapter.

We further investigated the synaptic phenotypes of CA1 principal cells exposed to autoantibodies, in the absence of network activity. Curiously, these results suggested that while NMDA receptor autoantibodies elicit a reduction in AMPA receptor-mediated excitatory inputs – consistent with spontaneous neurotransmission characterisations – GABA_A receptor autoantibodies did not substantially alter mEPSC amplitudes. Moreover, while we observed significant reductions in sIPSC amplitudes, this was not recapitulated in mIPSC datasets. Yet, we observed a significant reduction in the frequency of mIPSC events. To fully explore this data, we generated theoretical models of synaptic multiplicity, from our experimentally observed distributions of spontaneous and miniature event amplitudes and frequencies. This gave us preliminary insights into the level of excitatory and inhibitory synaptic connectivity onto CA1 pyramidal neurons. As such, our data would suggest that neither NMDA- nor GABA_A receptor monoclonal autoantibodies alter the density of excitatory synapses, but conversely, both autoantibody samples induce a downregulation of inhibitory synapses on these cells. We might speculate that the large amplitude sIPSC we observed are largely driven by action-potential generated inhibitory inputs, which are temporally summed at the cellular level, where autoantibody-mediated destabilisation of inhibitory synapses drives a reduction in amplitude – at least partially – through a degradation of the number of inhibitory connections arriving from any given GABAergic neuron. However, the absence of impact on mEPSC amplitude and frequency by GABA_A receptor autoantibodies remains elusive and may require further validation and investigation.

To investigate potential molecular mechanisms underpinning synaptic destabilisation after autoantibody exposure, we investigated a possible role of synaptic scaffold degradation. Immunostaining data revealed no significant impact of excitatory synaptic scaffolding protein, homer-1c. However, we observe that autoantibody incubation in the absence of astrocytes prevented the reduction of synaptically localised AMPA receptors, otherwise observed in standard dissociated neuronal culture paradigms. This suggests that astrocytes may play a direct role in the pathogenic alterations elicited by autoantibodies, in terms of the alterations to AMPA receptor organisation. The precise mechanism by which this occurs remains unknown, but future experiments may aim to investigate the potential role of astrocyte-derived ATP in promoting P2X receptor-dependant synaptic depression, or for a direct action of autoantibodies on NMDA- and GABA_A receptors located on the surface of astrocytes, although NMDA receptor presence on astrocytic membranes remains a topic of debate (Fraser, Mudrick-Donnon and MacVicar, 1994; Rosewater and Sontheimer, 1994; Skowrońska *et al.*, 2019; Verkhratsky and Chvátal, 2020). Additionally, immunostaining data unveiled a significant decrease in the density of inhibitory synaptic scaffolding protein, gephyrin. Our further investigations support the notion that this degradation of inhibitory synapses is mediated by hyperphosphorylation of particular serine residue (S270), which is known to exert control over gephyrin

cluster density (Kuhse *et al.*, 2012). Intriguingly, similar levels of phosphorylation and degradation of inhibitory synaptic scaffolds were observed after exposure to either GABA_A- or NMDA receptor autoantibodies, suggesting that aberrant intracellular kinase activity may be a common substrate for pathogenicity in autoimmune encephalitis, irrespective of the specific target antigens.

IV

CELLULAR PHENOTYPES

Chapter Introduction

We have identified significant dysregulation across the principal ion channels, mediating the vast majority of excitatory and inhibitory neuronal communication. However, the functional properties governed by neuronal membrane organisation extend beyond synaptic neurotransmission. The correct expression and localisation of many other ion channels, including voltage-gated sodium and potassium channels, and tonic excitatory and inhibitory currents through the membrane contribute to setting the intrinsic excitability of neuronal cells. It has been shown that monoclonal autoantibodies, and cerebrospinal fluid, from patients with leucine-rich glioma inactivated 1 (LGI1) autoimmune encephalitis increase intrinsic excitability of hippocampal pyramidal neurons (Romoli *et al.*, 2019; Kornau *et al.*, 2020; Extrémet *et al.*, 2022). Of interest, LGI1 interacts with voltage-gated potassium channel, Kv1.1, a known regulator of neuronal excitability expressed at the axon initial segment (Seagar *et al.*, 2017; Hivert *et al.*, 2019; Pinatel and Faivre-Sarrailh, 2020). The increased intrinsic excitability of hippocampal neurons exposed to LGI1 autoantibodies has been shown to correlate with a reduction in sensitivity of these cells to Kv1.1 blockade, suggesting that this alteration in intrinsic excitability is through a subsequent impact of LGI1 on the potassium channel (Extrémet *et al.*, 2022). Further, autoantibodies from patients with anti-AMPA receptor autoimmune encephalitis have also been shown to increase hippocampal neuronal excitability and decrease inhibitory neurotransmission, through an as yet unidentified mechanism which may be – in part – homeostatic, resulting from a decrease in AMPA receptor-mediated excitatory transmission (Peng *et al.*, 2015). It is clear that autoantibodies targeting neuronal surface proteins demonstrate the capacity to modulate intrinsic neuronal function, through direct interaction with excitability-defining channels – such as LGI1 and Kv1.1 – or through possible homeostatic regulatory mechanisms – as may be the case for AMPA receptor autoantibody-mediated pathology. As such, in the context of this literature and our prior observations of generalised alterations to excitatory and inhibitory synaptic inputs, we aimed to elucidate a possible impact of NMDA- and GABA_A receptor autoantibodies on the intrinsic activity of hippocampal neurons.

Considering the routes by which autoantibodies directed against NMDA- or GABA_A receptors may modulate intrinsic excitability, we considered a possible alteration in extrasynaptically-mediated tonic conductance through these ion channels directly. It has been demonstrated that ambient GABA, activating extrasynaptic GABA_A receptors and mediating tonic inhibition, is largely derived from synaptic GABA release sites (Glykys

and Mody, 2007). It is therefore tempting to speculate that this correlation between tonic and phasic GABAergic conductance would suggest that in conditions of reduced inhibitory synaptic activity, and in the context of inhibitory synapse degradation – as we observe in neuronal cultures exposed to autoantibodies – would result in a downstream reduction in tonic GABAergic inhibition. Yet, there is ample evidence to suggest that loss of synaptic inhibition, in the context of status epilepticus, either does not alter, or increases, tonic GABAergic conductance in excitatory hippocampal neurons (Scimemi *et al.*, 2005; Zhang *et al.*, 2007; Goodkin *et al.*, 2008; Zhan and Nadler, 2009). It has been suggested that this maintenance of GABAergic inhibition may arise from upregulatory recruitment of $\alpha 4\gamma 2$ -containing channels, at least in dentate granule cells of the hippocampus and thalamic relay neurons of the dorsal lateral geniculate nucleus (Rajasekaran *et al.*, 2010; Nani *et al.*, 2013). While the anti-GABA_A receptor monoclonal we have employed in our studies has been shown to exhibit no reactivity for $\alpha 4\beta 3\gamma 2$ -GABA_A receptors, it is not clear whether such an $\alpha 4\gamma 2$ -dependent mechanism is present in CA1 hippocampal neurons, as no alteration in $\alpha 4$ -coding mRNA or protein expression is observed in the hippocampi of seizure-sensitive gerbil and pentylenetetrazol-kindled rodent models of epilepsy (Kamphuis, De Rijk and Lopes da Silva, 1995; Hwang *et al.*, 2004; Szyndler *et al.*, 2018). It has been demonstrated that tonic inhibition in the hippocampus is generally mediated by δ - and $\alpha 5$ -containing GABA_A receptors, however the reactivity of our monoclonal for such receptors has not been fully characterised. It is therefore possible that the monoclonal GABA_A receptor autoantibody, #113-115, may show similar reductions in tonic GABAergic inhibition, as elicited on phasic neurotransmission, through a direct impact on extrasynaptic receptors. In fact, considering the epitope variability of GABA_A receptor autoantibodies in the polyclonal disease state, it may be even more likely that tonic GABAergic currents will be reduced, considering the increased likelihood for autoantibodies targeting additional receptor subunits.

Additionally, GABA_A receptor expression has been observed at the axon initial segment, and is known to regulate certain properties of intrinsic neuronal function (Rojas *et al.*, 2011; Kerti-Szigeti and Nusser, 2016; Nathanson, Davies and Moss, 2019). Since the axon initial segment is the locus of action potential generation, and that GABA_A receptors are understood to be the only ligand-gated ion channels present in this neuronal compartment, inhibitory synapses located in this region establish an magnified level of control over neuronal excitability (Glickfeld *et al.*, 2009; Leterrier, 2018). Moreover, it has been observed that activation of axon-localised GABA_A receptors locally hyperpolarise the membrane of the axon initial segment and modulate the action potential waveform (Xia *et al.*, 2014). As such, dysregulation of GABAergic function, localised to the axon initial segment, could provide a substantial pathway towards neuronal hyperactivation, possibly extending to alterations in action potential waveform propagation, in the context of GABA_A receptor encephalitis. However, it should be noted that the current debate regarding whether axonal application of GABA is hyper- or depolarising adds significant complexity to our ability to hypothesise on the potential action

of autoantibodies on axonal GABA_A receptors (Pugh and Jahr, 2013; Xia *et al.*, 2014; Zorrilla de San Martin, Trigo and Kawaguchi, 2017).

Finally, we also considered the possible role of NMDA receptor autoantibodies on tonic excitatory conductance. While tonic excitatory currents of small-amplitude have been identified in CA1 pyramidal cells, there is a relative abundance of literature regarding the role of NMDA receptor-mediated tonic currents on setting the intrinsic excitability of the inhibitory neuronal population (Alkondon, Pereira and Albuquerque, 2003, 2011; Le Meur *et al.*, 2007; Mann and Mody, 2010; Povysheva and Johnson, 2012; Riebe *et al.*, 2016). It is expected that the increased presence of magnesium-insensitive GluN2D-containing NMDA receptors on the surface of inhibitory interneurons contributes to the increased dependence of these cells on tonically active channels (Riebe *et al.*, 2016). This is consistent with the hypothesis that low-dose psychotomimetic antagonists, such as ketamine, show preferential blockade of tonically active NMDA receptors on inhibitory cell populations, and this disinhibitory mechanism may underpin pharmacologically-induced psychosis or rapid antidepressant effects (Zorumski, Izumi and Mennerick, 2016; Ingram *et al.*, 2018; Gerhard *et al.*, 2020; Luscher, Feng and Jefferson, 2020). Considering this context, it is possible that NMDA receptor autoantibodies may drive a substantial reduction in the tonic excitatory currents, largely present on inhibitory neuronal populations. Given that such disinhibitory mechanism has been implicated in the development of psychosis by NMDA receptor antagonists, and that NMDA receptor encephalitis is itself associated with congruent neuropsychiatric symptoms, it is intriguing to speculate that a similar action by autoantibodies may pose a functional pathway to both seizure and psychosis phenotypes, commonly observed in the clinical presentation of NMDA receptor encephalitis.

To investigate these open questions, we performed whole-cell current clamp recording of CA1 pyramidal neurons and inhibitory interneurons, to quantify action potential output and intrinsic excitability. Additionally, to investigate spontaneous output of both cell types, we further performed cell-attached voltage-clamp recordings to quantify spontaneous action potential generation, in the context of possible aberrant network activity, in a minimally invasive recording configuration non-requiring manipulation of intracellular solutions.

Methods

Organotypic hippocampal cultures and interneuron labelling

Organotypic hippocampal cultures were generated as described in Chapter II. Where indicated, slice cultures were transfected using an adeno-associated viral strategy, to label a non-specific group of inhibitory interneurons. Viral particles containing the plasmid *pAAV-mDlx-GFP* (purchased from AddGene, Plasmid #83900), were applied directly onto hippocampal slices at DIV 1, during medium exchange. A 20µl droplet of viral particle-containing culture media was placed directly on top of the slice, to enable diffusion into the

tissue. Hippocampal slices were exposed to viral particles until the subsequent medium exchange at DIV 4. Expression of soluble GFP was confirmed by visual inspection for immunofluorescence of sparsely localised cell bodies across hippocampal layers, between DIV 12 and DIV 15.

Electrophysiology

Quantification of tonic GABAergic currents was performed by the same recording configuration described in Chapter III for the recording of miniature GABA_A receptor-mediated inhibitory postsynaptic currents. After 5-minute recording of a stable baseline period, bicuculline was washed into the recording solution, at a concentration of 5 μ M. The amplitude of tonic GABAergic currents was estimated by recording the mean baseline before addition of bicuculline and subtracting the measured baseline after addition and 5-minute wash-in of the drug.

Characterisation of intrinsic cellular excitability of hippocampal pyramidal cells and inhibitory interneurons was performed using a whole-cell current-clamp approach. In these experiments, patch pipettes were filled with intracellular solution composed of 127mM K-gluconate, 8mM KCl, 10mM HEPES, 15mM phosphocreatine, 4mM Mg-ATP and 0.3mM Na-GTP. The recording paradigm consisted of 12 sweeps of 2 second duration. A 500ms variable-amplitude square current step was introduced between 250ms and 750ms on each sweep, outside the current step epoch, cells were injected with 0pA of current. Current step amplitudes ran sequentially from hyperpolarising (-150pA) to depolarising (400pA), in 50pA increments. Traces were recorded using a Multiclamp 700B amplifier and a Digidata 1550B interface controlled by Clampex 10.7 (Molecular Devices), and data were analysed using ClampFit. Input-output curves were generated by extracting the number of action potential discharges inside each applied depolarising current step. Action potentials were identified using a template search algorithm in ClampFit. Resting membrane potential was estimated in each recorded cell by measuring the mean membrane potential during the final 1s of each sweep, such that measurement would not be impacted by variable amplitude current-injection epochs. Threshold for action potential firing was calculated using the first observed action potential in each cell recording. The threshold was defined as the lowest mV value at which the action potential waveform exceeded 20mV per millisecond. Rheobase was defined as the lowest amplitude of an applied current step, in which an action potential was elicited. Input resistance was calculated from each of the three hyperpolarising current steps (-150, -100 and -50pA), using the equation $(\text{Input Resistance}) = (\text{Mean Baseline Voltage}) / (\text{Injected Current})$. Visualisation of inhibitory interneurons was performed in hippocampal slices infected with an AAV inducing soluble cytoplasmic GFP expression under an interneuron specific (mDlx) promoter (see slice culture methods above), where patched cells were identified as GFP-positive under 488nm LED illumination.

Spontaneous action potential firing frequencies were quantified using a cell-attached voltage-clamp configuration. For these experiments, patch pipettes were filled with 150mM NaCl. After formation of a seal

between the cell membrane and patch pipette, in the order of gigaohm resistance, the voltage clamp was set to a potential at which zero current was injected through the pipette. Traces were recorded using a Multiclamp 700B amplifier and a Digidata 1550B interface controlled by Clampex 10.7 (Molecular Devices), and data were analysed using ClampFit. Action potential spikes were extracted from traces using a template search algorithm.

Results

GABA_A receptor autoantibodies selectively increase principal cell excitability

Given that we have observed striking dysregulation of membrane organisation and function at synaptic compartments, we tested whether the action of pathogenic autoantibodies extends to alterations in intrinsic firing of hippocampal principal cells. To explore a range of cell intrinsic parameters, we used a whole cell current clamp protocol, in which CA1 neurons were administered a series of hyper- to depolarising current injections (Figure 14A). Input-output curves were generated, quantifying the number of action potential discharges per current step (Figure 14B). We uncovered an increased propensity for action potential firing after exposure to GABA_A receptor autoantibodies, evident across all depolarising current injections. However, this shift towards intrinsic hyperexcitability in response to stimulation was not observed in neurons exposed to NMDA receptor monoclonals.

We next analysed the resting membrane potential by measuring the mean baseline voltage during the 12 inter-step intervals, during which time there was no current injection to the cell. The mean resting membrane potential in control conditions was estimated at -61mV (Figure 14C). Pre-exposure to NMDA receptor autoantibodies showed no impact on the resting membrane potential, whereas GABA_A receptor antibody exposure elicited a depolarisation of the resting state potential (Figure 14C). Given this shift in the resting potential, we also characterised the threshold for action potential discharge, by identifying the millivolt value at which the action potential waveform exceeded 20mV/ms ($\delta mV/\delta t > 20$). We uncovered no significant difference in the threshold across either NMDA- or GABA_A receptor antibody treated conditions, relative to control (Figure 14D). Rheobase and spike latency were reduced, suggesting a shift in favour of hyperexcitation after exposure to GABA_A receptor antibodies, relative to Control-antibody treated hippocampal slices (Figure 14E-F), which is expected given the depolarised resting potential and unchanged threshold for action potential generation. Taken together, these findings suggest that GABA_A receptor monoclonal autoantibodies increase the intrinsic excitability of principal cells in the hippocampus, an effect not recapitulated by NMDA receptor antibody exposure. Moreover, input resistance was estimated by quantification of the mean baseline reduction, relative to current injection, in response to the three administered hyperpolarising current steps (-150 to -50 pA). Input resistance was exclusively impacted by GABA_A receptor antibody exposure, in which case

we observed an increase relative to control conditions (Figure 14G). However, no change in input resistance was identified in response to NMDA receptor autoantibody exposure. This demonstrates a loss of leak current across the neuronal membrane and suggests an increase in the sensitivity of cellular discharge in response to synaptic inputs. It is possible that this represents a further loss of excitatory cells to downregulate their action potential output through a loss of shunting inhibition, due to the loss of constitutively active extrasynaptic GABA_A receptor populations.

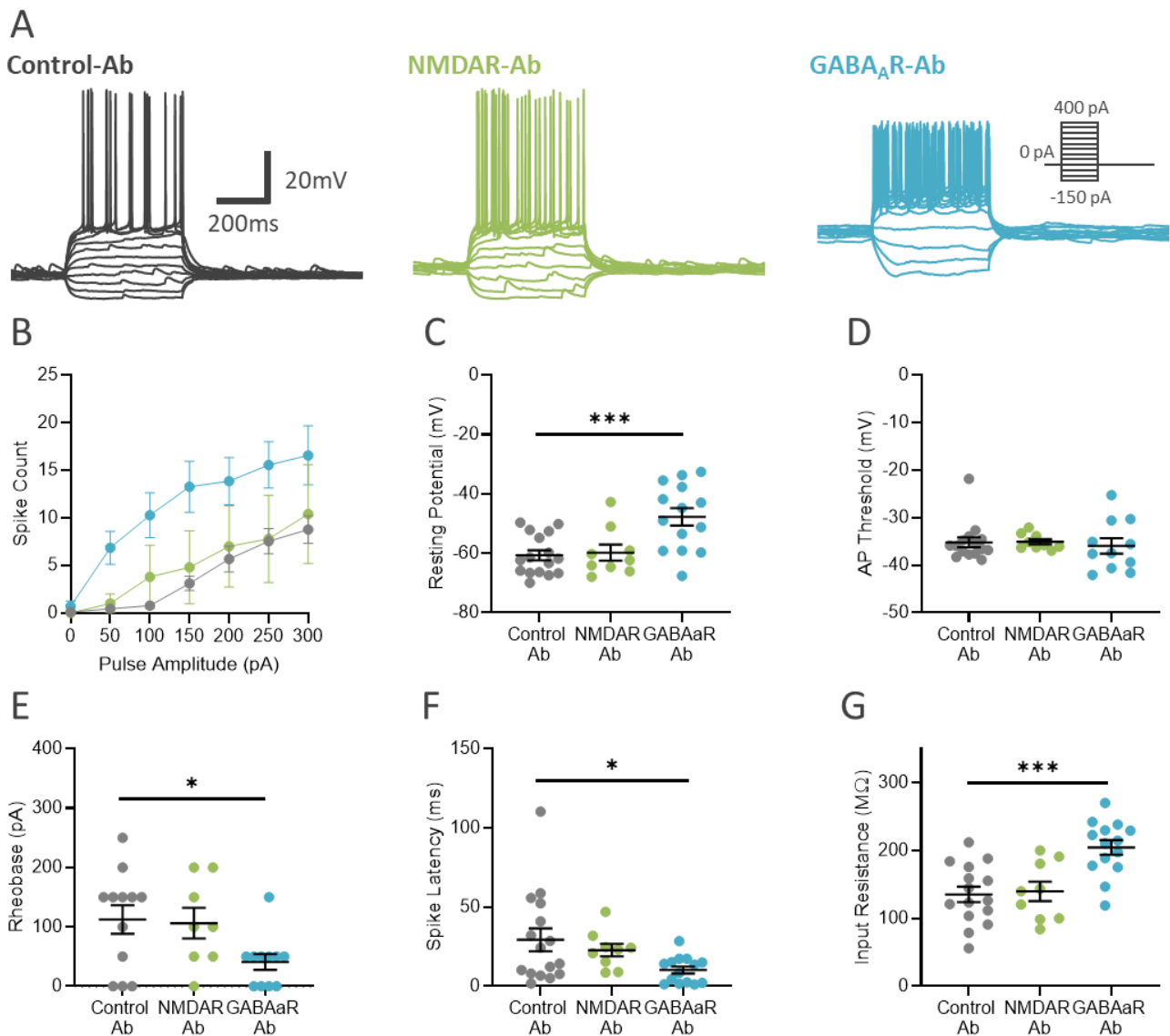


Figure 14: GABA_A receptor autoantibodies increase principal cell excitability

(A) Example traces collected from hippocampal CA1 pyramidal neurons, under a whole-cell current-clamp configuration, exposed for 24 hours to control-, NMDA- or GABA_A receptor autoantibodies. During recording, cells were subjected to 300ms current pulses between -150 and 400pA, in 50pA increments. Cell mean quantifications of (B) input-output curves of action potential frequency against depolarising current injections (Control-Ab: N = 9 cells; NMDAR-Ab: N = 5 cells; GABA_AR-Ab: N = 7 cells), (C) resting membrane potential (Control-Ab: N = 16 cells; NMDAR-Ab: N = 9 cells; GABA_AR-Ab: N = 14 cells), (D) action potential threshold (Control-Ab: N = 15 cells; NMDAR-Ab: N = 9 cells; GABA_AR-Ab: N = 11 cells), (E) rheobase (Control-Ab: N = 12 cells; NMDAR-Ab: N = 8 cells; GABA_AR-Ab: N = 11 cells), (F) spike latency (Control-Ab: N = 16 cells; NMDAR-Ab: N = 9 cells; GABA_AR-Ab: N = 14 cells) and (G) input resistance (Control-Ab: N = 15 cells; NMDAR-Ab: N = 9 cells; GABA_AR-Ab: N = 14 cells). Error bars denote the standard error of the mean.

Tonic GABAergic currents and Action potential waveform

The presence of a tonic inhibitory current, mediated by extrasynaptic GABA_A receptors can influence neuronal excitability through modulation of membrane potential and input resistance (Li, Yu and Jiang, 2013). Given our observation that GABA_A receptor autoantibodies alter both of these parameters of intrinsic excitability, we directly investigated the presence and amplitude of tonic GABAergic inhibition in CA1 pyramidal cells. We observe a moderate tonic GABAergic current, of approximately 20pA under control conditions, which is significantly reduced and virtually abolished by exposure to NMDA- and GABA_A receptor autoantibodies, respectively (Figure 15A-B). Intriguingly, this complete loss of tonic inhibition after exposure to GABA_A receptor autoantibodies may suggest that – at least in the case of our monoclonal – antibodies targeting the GABA_A receptor are indeed capable of binding and antagonising extrasynaptically localised channels. Further, while tonic inhibition is maintained in NMDA receptor antibody treated slices, the magnitude of this current is significantly reduced, however, we observed no alterations in resting potential or input resistance in these conditions. The reduction in extrasynaptic GABA_A receptor function after NMDA receptor autoantibody exposure may reflect a dilution of the extrasynaptic GABA_A receptor population with non-tonically active channels that have been displaced from synaptic compartments, requiring the assumption that internalisation of channels is upregulated to maintain a constant pool of extrasynaptic receptors. Such a loss of tonic inhibitory currents after either autoantibody exposure may further render destabilisation of neuronal function, through a reduced capacity to counteract the hyperexcitability driven by modulated synaptic inputs.

Since the presence of GABA_A receptors on the axon initial segment demonstrate the capacity to shape the action potential waveform, in addition to altering intrinsic excitability, we investigated the mean action potential amplitude and half-width from our current clamp recordings. This analysis revealed a selective modulation in action potential waveform by GABA_A receptor autoantibodies, which was not recapitulated by NMDA receptor autoantibody exposure (Figure 15C-D). Incubation with GABA_A receptor antibodies specifically reduced the amplitude and halfwidth of action potential discharges (Figure 15E-F), indicating a modulation in the functioning of GABA_A receptors at the axon initial segment. Such alterations have also been suggested in human ictogenesis and directly observed in rodent models of epilepsy (Merricks *et al.*, 2015, 2021; Codadu *et al.*, 2019).

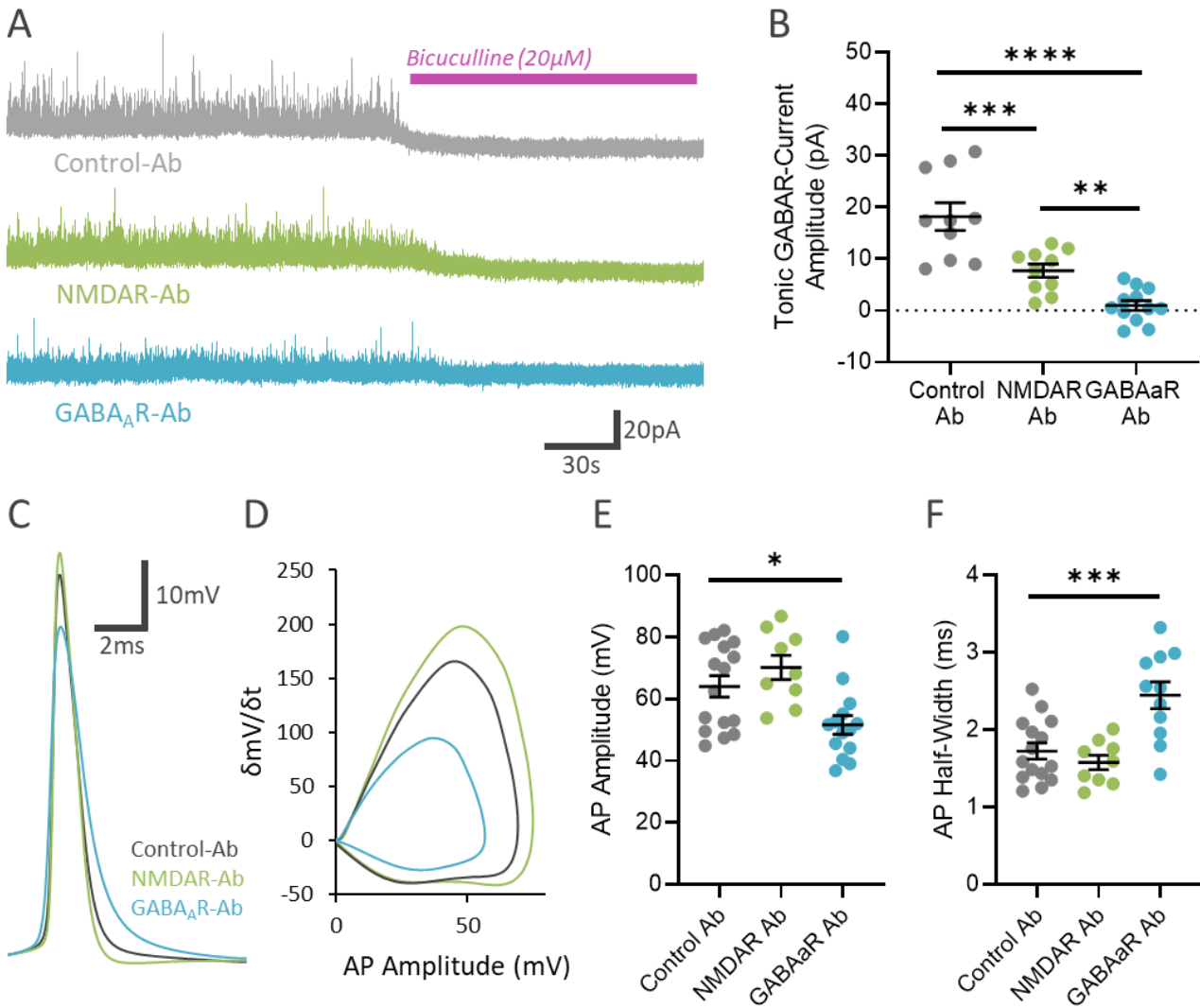


Figure 15: GABA_A receptor autoantibodies abolish tonic GABAergic conductance and alter action potential waveforms
 (A) Example traces collected from hippocampal CA1 pyramidal neurons, under a whole-cell voltage-clamp configuration, exposed for 24 hours to control-, NMDA- or GABA_A receptor autoantibodies. During recording, slices were perfused with bicuculline (20µM) to uncover tonic GABAergic conductance through the shift in recording baselines. (B) Cell mean quantifications of the amplitude of uncovered tonic GABA_A receptor-mediated current (Control-Ab: N = 10 cells; NMDAR-Ab: N = 10 cells; GABA_AR-Ab: N = 12 cells). (C) Example action potential waveform discharges collected under whole-cell current-clamp after 24-hour exposure to autoantibody samples and (D) mean phase-plane plots of action potential discharges, representing the gradient of the action potential waveform ($\delta mV/\delta t$) against the action potential amplitude. (E) Quantified mean action potential amplitudes and (F) half-width current (Control-Ab: N = 16 cells; NMDAR-Ab: N = 9 cells; GABA_AR-Ab: N = 12 cells). Error bars denote the standard error of the mean.

NMDA receptor autoantibodies selectively drive inhibitory cell dysfunction

Considering the postulation that inhibitory interneuron dysfunction is central to the pathology of NMDA receptor encephalitis, we investigated the possibility that hippocampal interneurons may display alteration in their intrinsic properties after exposure to either NMDA- or GABA_A receptor monoclonals (Huang and Xiong, 2021; Hunter, Jamet and Groc, 2021). We administered a series of hyper- to depolarising current steps to inhibitory hippocampal cells in the CA1 subfield (Figure 16A), revealing a significant reduction in the propensity for action potential generation in response to depolarising current injection, selectively in hippocampal slice cultures exposed to NMDA receptor autoantibodies (Figure 16B). However, this shift towards hypo-excitation was not observed in hippocampal interneurons exposed to GABA_A receptor antibodies (Figure 16B), indicating that interneuron dysfunction is a discriminatory phenotype associated with NMDA receptor encephalitis which may contribute to subgroups of symptomology not shared between these two encephalitic diseases. Moreover, tonic NMDA receptor-mediated currents have been demonstrated to contribute to the setting of depolarised resting membrane potentials in some interneuron populations, we therefore investigated whether autoantibody-induced NMDA receptor expression deficits were sufficient to alter the resting membrane potential of hippocampal interneurons (Hanson *et al.*, 2019). We identified a modest but significant hyperpolarisation of the resting membrane potential in interneurons exposed to NMDA receptor autoantibodies, relative to Control conditions (Figure 16C). Since we did not observe any change in the threshold for action potential generation in these cells after exposure to either NMDA- or GABA_A receptor monoclonals, relative to Control monoclonal treated hippocampal slices (Figure 16D), we anticipate that this deflection in resting potential will serve to increase the requirement for excitatory input, before successful elicitation of neuronal firing in inhibitory cell populations. This expectation was supported by our observation of an increased rheobase in interneurons exposed to NMDA receptor antibodies (Figure 16E). Again, these autoantibody-driven modulations in intrinsic excitability were confined to NMDA receptor autoantibodies, and not recapitulated after exposure to GABA_A receptor monoclonal samples, suggesting that interneuron dysfunction may be a selective phenotype associated with NMDA receptor encephalitis. Intriguingly, however, we did observe some selective alterations mediated by GABA_A receptor autoantibodies on inhibitory interneurons, namely a reduction in spike latency and input resistance (Figure 16F-G). While the former might suggest a mild increase in excitability, the latter would indicate a reduction in sensitivity to synaptic inputs. It is challenging to rationalise the possible mechanisms and functional consequences of these observations, particularly since the modulation of input resistance is of opposing direction to that observed in pyramidal cell populations. Yet, while antibody-mediated GABAergic dysfunction on interneurons does perturb some elements of neuronal function, it seems clear that this does not translate to generalised alteration in the intrinsic excitability of this cell population.

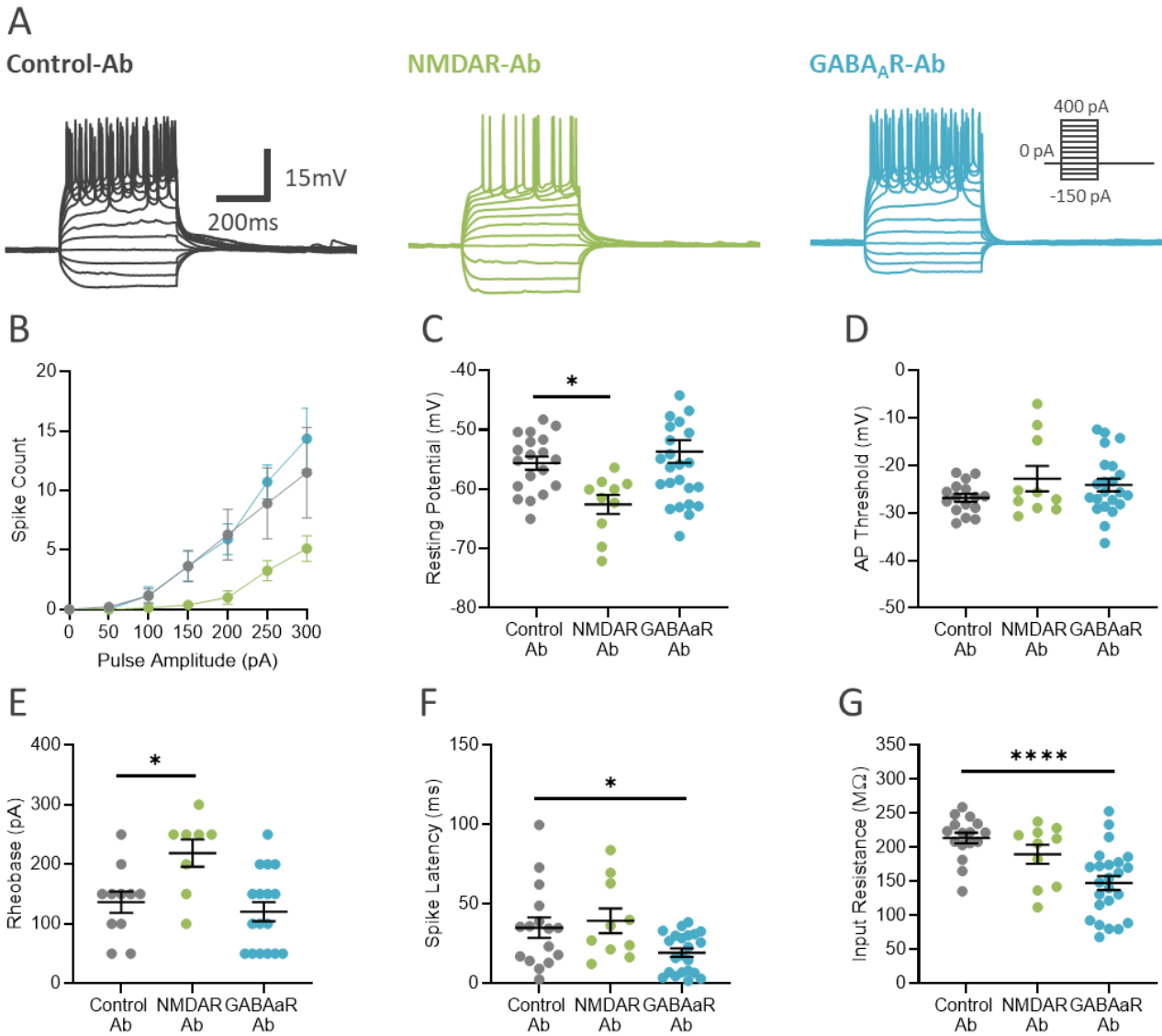


Figure 16: NMDA receptor autoantibodies reduce GABAergic cell excitability
 (A) Example traces collected from hippocampal CA1 GABAergic neurons, under a whole-cell current-clamp configuration, exposed for 24 hours to control-, NMDA- or GABA_A receptor autoantibodies. During recording, cells were subjected to 300ms current pulses between -150 and 400pA, in 50pA increments. Cell mean quantifications of (B) input-output curves of action potential frequency against depolarising current injections (Control-Ab: N = 14 cells; NMDAR-Ab: N = 8 cells; GABA_AR-Ab: N = 11 cells), (C) resting membrane potential (Control-Ab: N = 19 cells; NMDAR-Ab: N = 10 cells; GABA_AR-Ab: N = 23 cells), (D) action potential threshold (Control-Ab: N = 16 cells; NMDAR-Ab: N = 10 cells; GABA_AR-Ab: N = 21 cells), (E) rheobase (Control-Ab: N = 11 cells; NMDAR-Ab: N = 8 cells; GABA_AR-Ab: N = 17 cells), (F) spike latency (Control-Ab: N = 16 cells; NMDAR-Ab: N = 10 cells; GABA_AR-Ab: N = 21 cells) and (G) input resistance (Control-Ab: N = 16 cells; NMDAR-Ab: N = 10 cells; GABA_AR-Ab: N = 24 cells). Error bars denote the standard error of the mean.

Autoantibodies differentially modulate spontaneous action potential outputs

Currently, we have observed that synaptic inputs are uniformly dysregulated on principal neurons by both NMDA- and GABA_A receptor autoantibodies, and conversely that intrinsic cellular properties are distinctly modulated on GABAergic and glutamatergic neurons after exposure to NMDA- and GABA_A receptor antibodies, respectively. In light of these findings, we investigated the spontaneous cellular outputs from both

excitatory and inhibitory hippocampal cells in the CA1. Using a cell-attached voltage-clamp approach, quantify cellular spiking with a minimally invasive recording configuration. We identified a substantial loss of GABAergic cellular firing after exposure of hippocampal slice culture to NMDA receptor monoclonals, relative to control conditions (Figure 17A-B). This reduction in inhibitory interneuron activity was not observed after exposure to GABA_A receptor autoantibodies, suggesting that this silencing of GABAergic activity is a unique pathology related to NMDA receptor encephalitis, and is likely driven by the selective deficits in intrinsic cellular excitability in these cell populations. Moreover, analysis of action potential discharges in principal cell populations uncovered a clear increase in excitatory neuron outputs, after exposure to both NMDA- and GABA_A receptor monoclonals, relative to control conditions (Figure 17C-D). Taken in the context of our existing findings, we would postulate that this increase in excitatory output is primarily driven by a shift towards hyperexcitation of phasic synaptic inputs, that is compounded either by an increase in intrinsic excitability by GABA_A receptor antibodies; or by the ablated inhibitory networks in the case of NMDA receptor antibody exposure, which then fails to control excitatory connections between principal cells, resulting a hypersynchronous network phenotype. However, measures of overarching network activity and synchrony remain to be directly investigated.

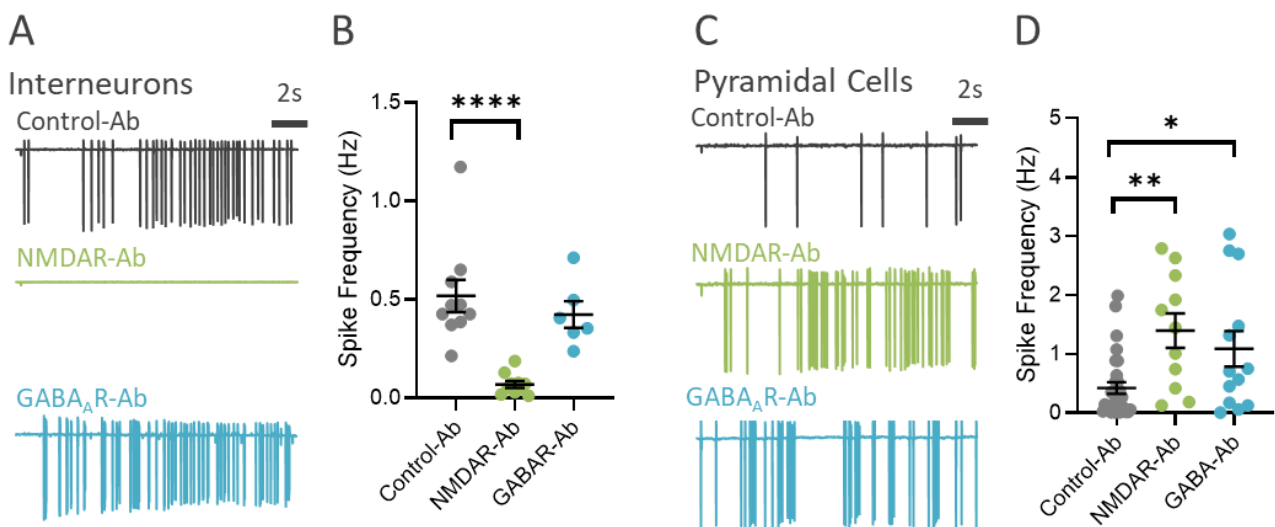


Figure 17: Autoantibodies show differential impact on spontaneous action potential discharge frequency.

(A) Example traces collected from hippocampal CA1 GABAergic neurons, under a cell-attached voltage-clamp configuration, exposed for 24 hours to control-, NMDA- or GABA_A receptor autoantibodies. (B) Cell mean quantifications of action potential frequency (Control-Ab: N = 10 cells; NMDAR-Ab: N = 7 cells; GABA_AR-Ab: N = 6 cells). (C) Example traces collected from hippocampal CA1 pyramidal neurons, under a cell-attached voltage-clamp configuration, exposed for 24 hours to control-, NMDA- or GABA_A receptor autoantibodies. (D) Cell mean quantifications of action potential frequency (Control-Ab: N = 29 cells; NMDAR-Ab: N = 11 cells; GABA_AR-Ab: N = 13 cells). Error bars denote the standard error of the mean.

Chapter Summary

We have characterised the intrinsic excitability of distinct hippocampal cell populations, namely the glutamatergic pyramidal neurons and GABAergic interneurons, after exposure to either NMDA or GABA_A receptor monoclonal autoantibodies. These investigations unveiled a specific action of GABA_A receptor autoantibodies on the intrinsic properties of excitatory pyramidal neurons in the CA1 hippocampal subfield. Specifically, we observed increased action potential output in response to depolarising current injections, depolarisation of resting membrane potential, decreased rheobase and spike latency and an increase in input resistance, after exposure to GABA_A receptor monoclonal antibodies. All of these findings are consistent with an increase in excitability. Notably however, none of these parameters were found to be modulated by exposure to NMDA receptor autoantibodies. As such, these data strongly suggest a selective functional pathogenesis in the disease state of GABA_A receptor encephalitis, whereby autoantibodies drive a hyperexcitation of the pyramidal neurons directly.

Given the possible impact of autoantibodies to alter intrinsic excitability through extrasynaptic and axonally localised GABA_A receptors, we investigated tonic GABAergic conductance and action potential waveforms to elucidate these possible routes to pathogenesis, respectively. Analysis of tonic conductance revealed an abolition of tonic GABAergic inhibition on principal cells, which was partially recapitulated by NMDA receptor monoclonal antibody exposure. We anticipate that this loss of inhibition can promote cellular hyperactivity, possibly through a depolarisation of the resting potential. Intriguingly, the presence of tonically-active GABA_A receptors located in close proximity to excitatory synapses are thought to induce shunting of excitatory synaptic currents and impair the activation of postsynaptic NMDA receptors (Shen *et al.*, 2010; Smith, 2013). As such, reductions in tonic GABA_A receptor-mediated conductance may play a direct role in altering the thresholds for synaptic plasticity, and consequently lead to an alteration of cognitive functions, dependent on NMDA receptor-mediated plasticity. This aspect is particularly intriguing considering the high prevalence of cognitive impairments associated with GABA_A receptor encephalitis, which regularly take the form of deficits in learning and memory, a key feature under the control of hippocampal NMDA receptor function (Spatola *et al.*, 2017). Our investigations also uncovered a selective action of GABA_A receptor autoantibodies on action potential waveforms, specifically eliciting a reduction in amplitude and increased duration. This reduced amplitude and prolongation of the action potential implies that GABA_A receptor autoantibody exposure elicits an alteration in the functional properties of voltage-gated sodium and potassium channels. Specifically, reduced sodium influx during depolarisation and slowed potassium outflux during the repolarisation phase. The mechanism by which GABA_A receptor autoantibodies mediate these impacts remain unclear, but we may speculate that autoantibodies can reduce the expression or function of axonal GABA_A receptors. As such a reduced chloride conductance resulting from a loss of shunting inhibition may lead to depolarisation of the axonal membrane potential (Glickfeld *et al.*, 2009; Xia *et al.*, 2014). As such, this depolarisation could lead to

a slowing of the repolarisation phase during potassium efflux, as indeed it has been shown inversely that activation of hyperpolarising GABA_A receptors at the axon leads to an acceleration of action potential durations (Xia *et al.*, 2014).

Investigation of intraneuronal excitability revealed a selective action of NMDA receptor autoantibodies, in which GABAergic activity was markedly reduced. This deficit was observed across several parameters pertaining to excitability, including negative shift of input-output plots, hyperpolarisation of the resting potential and an increase in rheobase. The precise mechanisms by which autoantibodies targeting the NMDA receptors elicit these impacts remains to be fully elucidated but we would expect that tonic excitatory conductance, mediated by extrasynaptic NMDA receptors are likely involved in these processes. It is understood that the enriched presence of magnesium insensitive GluN2D-containing channels on GABAergic neurons are responsible for an increased level of tonic conductance in this cell population, as compared to pyramidal neurons (Riebe *et al.*, 2016; Hanson *et al.*, 2019). Moreover, partial pharmacological block of this tonic current has been shown to provoke a reduction in excitability of interneurons in the CA1, resulting in disinhibition and network hyperactivation (Riebe *et al.*, 2016). Given that NMDA receptor autoantibodies target the GluN1 subunit and will therefore bind indiscriminately to GluN2 subunit-containing channels, it is conceivable that autoantibody action on interneurons alters their excitability directly by ablating excitatory tonic conductance. This is particularly interesting considering that psychoactive open channel blockers are expected to elicit psychosis-like behaviours, largely through a similar blockade of NMDA receptor-mediated tonic conductance. As such it is tempting to speculate that neuropsychiatric symptoms associated with NMDA receptor encephalitis, but not GABA_A receptor encephalitis, may be underpinned by a similar disinhibitory mechanism. Additionally, we have hypothesised elsewhere that autoantibodies in the disease state of autoimmune encephalitis, more generally, may display preference for action on extrasynaptic receptor populations – due to the increased antigen availability outside of the synaptic cleft (Hunter, Jamet and Groc, 2021). Here, we argued that the high protein density and narrow width of the synaptic cleft can act as a hindrance for antibody penetration into synaptic structures, limiting antibody binding in these compartments. Within this hypothetical model, NMDA receptor autoantibodies may show preferential reduction in tonic over phasic excitation, lending greater significance to the pathogenicity mediated by tonic current abolition. Further studies should aim to directly investigate the impact of NMDA receptor autoantibodies on tonic NMDA receptor-mediated conductance, on both excitatory and inhibitory neuronal populations.

Finally, we characterised the spontaneous activity of pyramidal and inhibitory neurons in the CA1 subfield, to uncover how the alterations to synaptic inputs and intrinsic properties may alter the overall output of these cells within hippocampal networks. As expected, we observed a substantial silencing of inhibitory cellular outputs after exposure to NMDA receptor autoantibodies, that was not recapitulated by GABA_A receptor

antibody exposure – an effect which presumably stems from the reductions in intrinsic excitability. Notably, recordings from excitatory neurons, however, revealed a significant increase in activity after exposure to either pathogenic antibody sample. In this case, we expect this hyperactivity to be underpinned by a dual-hit on cellular function, whereby synaptic inputs are shifted in favour of hyperexcitation (as discussed in Chapter III), and then further compounded by a direct GABA_A receptor antibody-specific increase in excitability; or a further reduction of local inhibitory networks exposed to NMDA receptor monoclonals. These results together would suggest that congruent action of both autoantibodies on the synaptic phenotype, and discrepant action on cellular excitability converge to pyramidal cell hyperexcitation in hippocampal circuits. To fully uncover the possibility of shared antibody-mediated impact on network activity, the overarching phenotype of pyramidal cells in hippocampal networks is directly explored in the subsequent Chapter.

V

NETWORK PHENOTYPES

Chapter Introduction

Thus far, we have identified common synaptic alterations by both NMDA- and GABA_A receptor autoantibodies, whereby synaptic inputs are generally downregulated with a high magnitude impact on phasic inhibition, thereby shifting excitation-inhibition balance in favour of excitation. Additionally, we observe divergence in autoantibody action, at the level of intrinsic excitability. While GABA_A receptor autoantibodies drive a selective increase in glutamatergic cell excitability, NMDA receptor autoantibodies elicit a converse impact on inhibitory cell populations. As such, it is imperative to characterise the overarching dysfunction elicited by these antibodies on overall network function. Based on the clinical disease profiles, and our data indicating a dual-hit mechanism of pathogenic autoantibodies in promoting pyramidal cell outputs, either through direct hyperexcitation or disinhibitory mechanisms, we would hypothesise that both autoantibodies will converge at the circuit-scale, to induce hyperactivity of hippocampal networks.

Investigations employing a calcium imaging approach have previously shown that the GABA_A receptor monoclonal antibody that we have employed in our studies can elicit network hyperactivation of primary cortical-striatal cultures after 24-hour incubations (van Casteren *et al.*, 2022). Additionally, both calcium imaging and multi-electrode array (MEA) recording techniques have been used to demonstrate a disinhibition-mediated hyperactivity of primary cortical networks after 24-hour exposure to the monoclonal anti-NMDA receptor antibody we have used in this project work (Andrzejak *et al.*, 2022). However, no current studies have assessed network dysfunction elicited by NMDA- and GABA_A receptor autoantibodies in parallel, and as such the *in vitro* network phenotypes evoked by exposure to either autoantibody cannot be directly compared. This is particularly striking given our observations that network hyperactivity may derive from distinct cellular mechanisms, possibly giving rise to subtle differences in the precise dynamics of network activity. To address this, we performed calcium imaging experiments of cultured organotypic hippocampal networks exposed for 24 hours to either NMDA- or GABA_A receptor autoantibodies. Such investigations will allow us to examine the impact of these autoantibodies on hippocampal network activity, a principal locus of pathogenesis in autoimmune encephalitis and commonly implicated in the development of both epileptogenesis and cognitive dysfunction (Thom, 2014; Heine *et al.*, 2015; Huberfeld, Blauwblomme and Miles, 2015; Bettio, Rajendran and Gil-Mohapel, 2017; Romoli *et al.*, 2019; Lissner *et al.*, 2021).

The CA1 hippocampal subfield has been implicated in both spatial and temporal processing of memory (Langston *et al.*, 2010; Drieskens *et al.*, 2017; Geiller, Priestley and Losonczy, 2023). Specifically, bilateral inactivation of the CA1 hippocampal subfields of Wistar rats has been shown to abrogate integrative episodic memory performance, involving reductions in recognition of objects, their locations and temporal associations (Drieskens *et al.*, 2017). The emerging role of the CA1 in memory is particularly intriguing, since the knowledge of relatively sparse connectivity between principal cells in this region – in contrast to that of the CA3 – has historically rendered less interest in the computational power of this hippocampal subfield (Geiller, Priestley and Losonczy, 2023). Moreover, many studies have unveiled a pivotal role of GABAergic inhibitory interneurons in the formation, consolidation and processing of hippocampal-dependant memory (Allen *et al.*, 2011; Lovett-Barron *et al.*, 2014; Artinian *et al.*, 2019; de Salas-Quiroga *et al.*, 2020; Oliveira da Cruz *et al.*, 2020; Delorme *et al.*, 2021). The functional role of the CA1 in memory is particularly intriguing, given the high association with cognitive dysfunction observed in patient cohorts of both NMDA- and GABA_A receptor encephalitis (Spatola *et al.*, 2017; Dalmau *et al.*, 2019; X. Liu *et al.*, 2019; Guo, Gelfand and Geschwind, 2020). The possible relevance of CA1 circuit alterations is further purported given the high association between CA1 imaging abnormalities and human intractable temporal-lobe epilepsy (Maccotta *et al.*, 2015; Goubran *et al.*, 2016; Moghaddam *et al.*, 2021; Pai *et al.*, 2021; Chau Loo Kung *et al.*, 2022). It has further been suggested CA1 atrophy shows predictive value regarding epilepsy-induced long-term memory impairment in humans, through a disruption consolidation processes (Mukaino *et al.*, 2022). Taken together, this would suggest that the CA1 hippocampal subfield network dysfunction may be a candidate locus for cognitive dysfunction and epileptogenesis by NMDA- and GABA_A receptor autoantibodies.

While calcium imaging approaches permit us to examine network activity and dynamics in intact hippocampal slices, maintaining more physiological circuit architecture; the implementation of MEA recording techniques allow further characterisation of network activity – with the higher precision of electrophysiological recording. Currently, there is substantial disparity regarding the impact of NMDA receptor autoantibodies on neuronal network activity of primary neuronal cultures, investigated with MEA recording strategies. For instance, one study has demonstrated that patient cerebrospinal fluid elicits a decrease in overall network activity without alteration in network synchrony (Jantzen *et al.*, 2013). Conversely, it has been demonstrated elsewhere that NMDA receptor autoantibodies drive an increase in both spike and burst rates of primary cortical cultures after 24 hours of exposure (Andrzejak *et al.*, 2022). Moreover, both of these studies failed to observe any alteration in the synchronicity of network activity, while two recent independent investigations have demonstrated that NMDA receptor autoantibodies increase hippocampal synchrony *in vivo* (Wright *et al.*, 2021; Ceanga *et al.*, 2022). To address some of this discrepancy in our own hands, we investigated changes in network activity by calcium imaging in hippocampal slice cultures exposed to NMDA- and GABA_A receptor autoantibodies and performed additional preliminary characterisations of antibody-induced alterations to

dissociated hippocampal culture activity with MEA recording techniques. Given our prior findings, we would anticipate that both autoantibodies will induce hyperexcitability at the network scale, with a possible alteration in synchronicity elicited by NMDA receptor autoantibodies selectively, through their targeted dysregulation in the cellular outputs of GABAergic interneuron populations.

Methods

Calcium Imaging

Organotypic hippocampal cultures were setup as described in Chapter II and virally transfected using an AAV, inducing the expression of calcium reporter, GCaMPVI, under the excitatory neuron specific promoter for CaMKII. Infection was performed as described in Chapter IV, and after 24-hour exposure to either Control-, NMDA- or GABA_A receptor monoclonal autoantibodies, the CA1 subfield of hippocampal slice cultures was imaged using a spinning disk confocal microscope (Nikon Ni-E with spinning Yokogawa X1), at a frame rate of approximately 3Hz. Slice cultures were maintained in warmed (37°C) BrainPhys neuronal culture media throughout the imaging procedure, where time lapse recordings in a single Z-plane were acquired for 7 minutes. Acquired image stacks were then analysed using a set of custom-built scripts for ImageJ software. Briefly, somatic regions of interest (ROIs) were selected by creating a $\delta F/F$ image stack, in which somatic calcium transients were identified as the fluorescence of each pixel was normalised to the mean fluorescence value of the prior five frames. Mean fluorescence intensity was then measured in each ROI across the 7-minute recording interval, and fluorescence fluctuations were determined by creating $\delta F/F$ traces, where δF = Mean fluorescence intensity at frame n ; and F = mean fluorescence intensity of the prior 5 consecutive frames ($n-6$ to $n-1$ frames). Spikes were then identified by running a spike detection algorithm, which searched the $\delta F/F$ trace for incidents where point values on the $\delta F/F$ function exceeds 3 times the standard deviation of the full $\delta F/F$ trace. Spike detections were manually inspected in a subset of ROIs in each recording to ensure adequate and equivalent detections across acquisitions. Correlation index was determined through pairwise analysis of all cell pairs in an identified network, according to the methods described by Wong *et al.* (1993).

Multielectrode Array Recordings

Dissociated hippocampal cultures were setup as described in Chapter II and plated on 6-well multielectrode array chips from Multi Channel Systems (Reference: 60-6wellMEA200/30iR-Ti-rcr). Cells were plated at a higher density of 500×10^3 cells per ml, due to the relative lack of neuronal adhesion proximal to electrode sites. After initial plating in a low volume of culture media to aide adhesion, cultures were supplemented 6 hours later with additional culture media. The dissection and culture media were the same as described in Chapter II. Chips were maintained in a humidity-controlled incubator, at 37°C and 5% CO₂, with full culture media exchanges performed twice per week until recording. For acute drug-exposure experiments, DIV14 cultures

were recorded for baseline activity before addition of either, control drug-free culture media, 50 μ M APV, 20 μ M NBQX, 20 μ M BCC or 2 μ M TTX. After 2 minutes, cultures were again recorded for 8 minutes. For autoantibody experiments, 8-minute baseline recordings were collected from DIV14 cultures, after which 2 μ l of pre-diluted autoantibody stocks were directly added to the culture media. After 24-hour incubation with antibody samples, 8-minute recordings were repeated. Files were analysed with Multi Channel Systems analysis software, in which traces were filtered before threshold-based spike detection. Spike sums per well were then calculated and normalised to baseline recording conditions.

Results

Autoantibodies similarly induce hippocampal network hyperactivity

In order to uncover the network-scale phenotype of hippocampal CA1 principal cells, resulting from the identified deficits in synaptic transmission, we employed a calcium imaging approach as a proxy for action potential discharges. Principal cells of organotypic hippocampal cultures were virally transfected with the calcium sensor, GCaMPVI, to visualise somatic calcium influx, enabling us to visualise cellular firing within the context of hippocampal circuitry (Figure 18A). The total number of somatic regions identified did not vary between Control-, NMDA- and GABA_A receptor autoantibody-treated conditions (Figure 18B). As predicted, we observed an increase in the frequency of calcium transients at network level (Figure 18C), indicating that in our system, exposure of hippocampal circuits to either NMDA- or GABA_A receptor monoclonal antibodies alone is sufficient to drive network hyperactivation. To further understand if this hyperactive state is driven by a generalised increase in principal cellular activity, or by a select subgroup of hyperactive neurons that in turn evoke network effects, we analysed the relative frequency distributions and maximum firing frequency of individual cellular within each recorded slice (Figure 18D-E). We demonstrate that the cumulative distributions of cellular activity are uniformly right-shifted in cultures exposed to NMDA- and GABA_A receptor antibody samples, indicating an overall increase in cellular activity (Figure 18D). Additionally, however, we do not observe any alteration in the maximum firing frequency, after exposure to either patient-derived autoantibody samples (Figure 18E), suggesting that this increase is relatively homogeneous modulation across principal cells in the CA1, as opposed to increased activity in a subpopulation of neurons.

NMDA receptor autoantibodies induce hippocampal network hypersynchrony

Recent reports have indicated that NMDA receptor autoantibodies induce a hypersynchronous network state between excitatory neurons in hippocampal circuits (Ceanga *et al.*, 2022). To investigate this phenomenon in our model system and to explore whether this may also be the case for networks exposed to GABA_A receptor monoclonals, we calculated the mean correlation index (Wong, Meister and Shatz, 1993) from pairwise comparisons between all cells in the recorded networks (Figure 18F). We uncovered a significant increase in

the mean cell correlation index, after exposure to NMDA receptor autoantibodies selectively. This increase in synchronous cell firing was not recapitulated after exposure to GABA_A receptor monoclonals, suggesting that – although both autoantibodies drive hyperexcitation of hippocampal networks – the underpinning network phenotype is subtly dissimilar in these two disease states. However, analysis of simultaneously occurring cellular firing, here referring to calcium transients identified within the same imaging frame (300ms), revealed that both NMDA- and GABA_A receptor antibodies increase the rate of simultaneous detections; suggesting that GABA_A receptor autoantibodies may modulate paired spiking patterns between ensembles of neurons that is not captured by an alteration in the correlation index (Figure 18G). Given these alterations in network activity, specifically the induction of hyper-synchronicity in the case of NMDA receptor monoclonal antibody exposure, we also investigated the percentage of pairwise comparisons that yielded a positive correlation index, across all pairs of neurons within each network. This analysis revealed a significant increase in the percentage of positively correlated spike trains after exposure to NMDA receptor autoantibodies, as compared to Control antibody treated networks (Figure 18H). This demonstrated that in addition to an overall increase in the synchronicity, NMDA receptor monoclonals also broaden the number of neurons recruited into hypersynchronous subpopulations of hippocampal neurons. Again, this observation was not seen in organotypic cultures exposed to GABA_A receptor autoantibodies, indicating that hyper-synchronicity is an NMDA receptor autoantibody-specific route towards network hyperactivation.

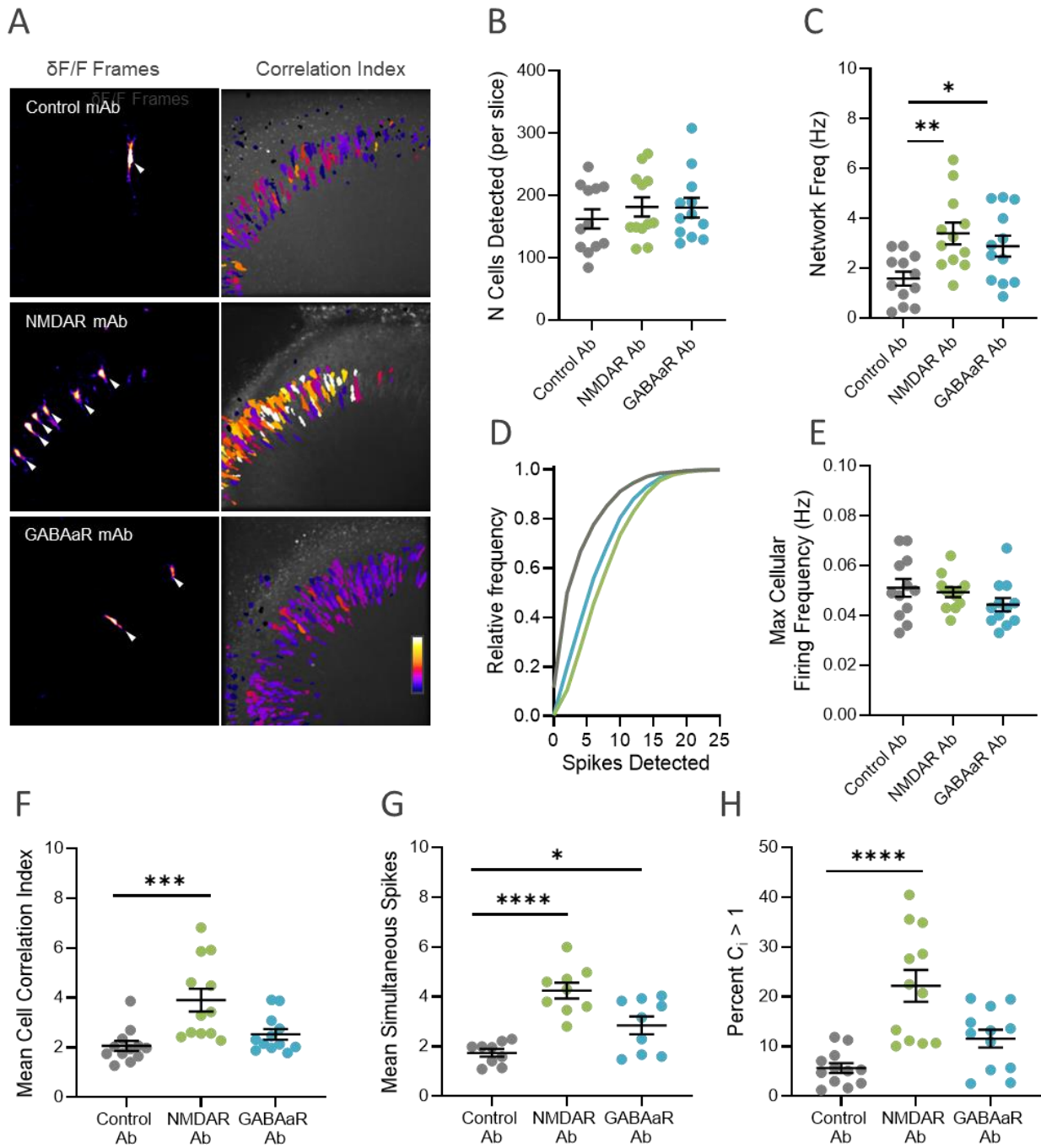


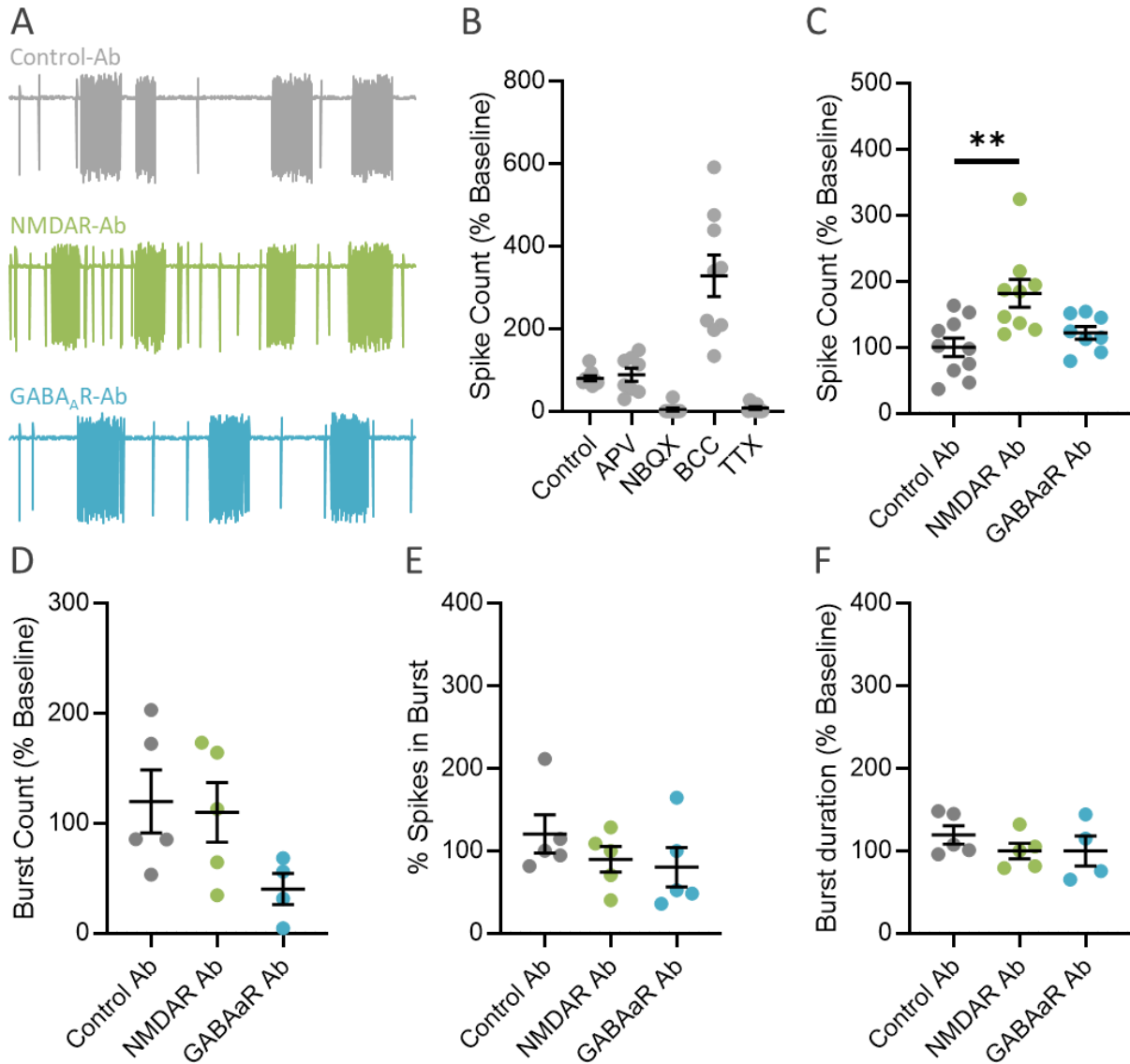
Figure 18: Autoantibodies increase network activity with differential impacts on synchrony.

(A) Representative image frames of $\delta F/F$ stacks, showing somatic calcium transients (highlighted with white arrowheads) (B) Cell mean quantifications of number of cells per network detected across conditions (Control-Ab: N = 12 networks; NMDAR-Ab: N = 12 networks; GABA_AR-Ab: N = 12 networks). (C) Mean network frequency, determined by summing the total number of somatic calcium transients across recording period. (D) Cumulative cell frequency distributions (Control-Ab: N = 1946 cells; NMDAR-Ab: N = 2177 cells; GABA_AR-Ab: N = 2163 cells). (E) Cell mean max firing frequency, mean per slice network recorded conditions (Control-Ab: N = 12 networks; NMDAR-Ab: N = 12 networks; GABA_AR-Ab: N = 12 networks). (F) Quantified mean cellular correlation index, (G) simultaneous spike rate and (H) percentage of pairwise connections with a positive correlation index conditions (Control-Ab: N = 12 networks; NMDAR-Ab: N = 12 networks; GABA_AR-Ab: N = 12 networks). Error bars denote the standard error of the mean.

Autoantibodies show differential impacts on dissociated cellular cultures

To further characterise the impact of NMDA- and GABA_A receptor autoantibodies on network activity, we employed an MEA recording strategy to permit more precise and direct detection of action potential discharges and network bursting activity (Figure 19A). The manipulation of spiking activity was investigated in our experimental culture setup, through acute exposure to ionotropic receptor antagonists. After recording of baseline activity, cultures were exposed to either APV, NBQX, bicuculline (BCC) or TTX, antagonists of NMDA-, AMPA-, GABA_A- and voltage-gated sodium channels, respectively. While a single run of this pharmacological manipulations is insufficient to perform statistical analysis, from qualitative inspection of the data we observed the expected reduction in spike counts after application of NBQX and TTX (reduced to 4% and 9% of baseline, respectively), and an increase in network activity after application of BCC (increased to 410%) (Figure 19B). Curiously, however, we did not observe any striking alteration in network activity after application of APV (110% of baseline). However, it is possible that the loss of NMDA receptor-dependant signalling in these cultures may impact network activity over longer timescales. These observations led us to believe that network activity of our cultures at DIV14 can indeed be modulated by alterations in ionotropic channel function, and as such was an adequate system to further investigate the impacts of NMDA- and GABA_A receptor autoantibody samples.

Autoantibody incubation experiments revealed that exposure of dissociated hippocampal neurons to NMDA receptor monoclonals, for 24 hours, elicits an increase in the action potential output, however this was not recapitulated by exposure to GABA_A receptor autoantibody samples (Figure 19C). This remains inconsistent with our calcium imaging datasets, in which we observed a significant increase in network activity after exposure to either NMDA- or GABA_A receptor antibodies. We further investigated network-based bursting in the cultures that demonstrated robust network-bursting activity (Control: 5/10; NMDA receptor antibody: 5/9; GABA_A receptor antibody: 5/8). Of these cultures, we identified no alteration in the number of network bursts, the percentage of spike detections localised within burst periods or network burst duration (Figure 19D-F). Given that network bursting is a common tool to investigate synchrony, we would have anticipated a selective alteration by NMDA receptor autoantibody exposure. However, the reason behind this apparent inconsistency remains an intriguing open question.



Chapter Summary

Seizure phenotypes and cognitive dysfunction, underpinned by pathological circuit-level activity of neuronal networks, form a principal basis for the disease aetiology of both NMDA- and GABA_A receptor autoimmune encephalitis. As such, we aimed to characterise the impact of hippocampal network exposure to either NMDA- or GABA_A receptor autoantibodies, on the overarching activity of CA1 circuits. Our calcium imaging experiments unveiled a convergent action of both pathogenic autoantibodies, eliciting an increase in the overall output of CA1 pyramidal neurons, indicative of a hyperactive network state. In the context of our existing data, we would expect this to be the result of similarly perturbed synaptic inputs, shifted in favour of hyperexcitation, and additional selective impacts on the excitatory and inhibitory cellular excitability. Specifically, the increased excitability of principal cells by GABA_A receptor autoantibodies, renders a direct hyperactivation of these cells at the network-level; whereas ablation of inhibitory drive after exposure to NMDA receptor autoantibodies limits the capability of the inhibitory system to exert control over recurrent hippocampal excitation. Such hippocampal hyperactivation is consistent with the knowledge that autoimmune encephalitides are commonly associated with temporal lobe epilepsy (Irani, Bien and Lang, 2011; Serafini *et al.*, 2016; Kuehn *et al.*, 2020). Given the high density of principal cell interconnectivity and recurrent circuitry in hippocampal structures, it is tempting to speculate that destabilisation and hyperactivation of networks within these structures may pose a principal locus for seizure generation in patients (Silberberg *et al.*, 2005; Sheikh *et al.*, 2019; Pan, Puranam and McNamara, 2022).

Severe epileptic phenotypes are regularly comorbid with cognitive decline, yet the precise determinants of epilepsy-related cognitive dysfunction remain to be elucidated, there are a variety of functional mechanisms in which hippocampal hyperactivation may yield deficits in cognitive processing (Zhu *et al.*, 2020). The hippocampus is widely associated with a wide variety of cognitive functions, particularly in tasks requiring associative learning, memory and spatial navigation (Preston and Eichenbaum, 2013; Eichenbaum, 2017; Lisman *et al.*, 2017; Soltesz and Losonczy, 2018). The architecture of hippocampal structures allows the sequential processing of information, principally arising from entorhinal cortical projections to the dentate gyrus, then further processed by CA3 and CA1 subfields before returning back to the entorhinal cortex. As such, the CA1 comprises a final computational locus of hippocampal processing, before output to downstream brain regions (Vakilna *et al.*, 2021). Thus, fine coordination in the activity of pyramidal CA1 output cells is likely critical for healthy cognitive processes. As such, excessive activation in the CA1 hippocampal subfield could disrupt normal circuit computations by increasing noise of the basal network activity; however, such a hypothesis requires further investigation. Moreover, alteration to baseline hippocampal activity can alter the thresholds for synaptic potentiation and depression, through processes collectively termed metaplasticity (Abraham, 2008; McHail and Dumas, 2015; Çalışkan and Stork, 2018). As such, it is plausible that increased activity of CA1 hippocampal network may provoke further deficits in the induction of long-term potentiation,

a pivotal molecular substrate for learning and memory (Lynch, 2004; Nicoll, 2017). Intriguingly, it has been shown that anti-NMDA receptor autoantibodies derived from patients undergoing first-episode psychosis, or NMDA receptor encephalitis, can abolish the induction of long-term potentiation of CA1-Schaffer collateral synapses (Zhang *et al.*, 2012; Jézéquel, Johansson, *et al.*, 2017). It has also been demonstrated that functional strengthening of inhibitory synaptic responses onto CA1 pyramidal neurons precedes potentiation of excitatory synapses during contextual learning (Sakimoto *et al.*, 2021). Together, this could suggest that the action of both NMDA- and GABA_A receptor autoantibodies may alter the induction of plasticity, convergently altering cognitive processing. However, alteration to hippocampal synaptic plasticity in the context of GABA_A receptor encephalitis remains to be explored.

Our calcium imaging experiments further unveiled a selective alteration by NMDA receptor autoantibodies, eliciting a hypersynchronous network state. GABAergic interneurons are known to exert control over pyramidal neuronal populations, through feed-forward and feedback connections. Feedback inhibition arises from pyramidal cell driven excitation of local interneuron populations, which in turn inhibit local pyramidal neurons, promoting sparse single-unit firing through a filtering out of neurons receiving weak excitatory inputs (McKenzie, 2018). Additionally, a role for direct lateral inhibition has recently been proposed to play a role in CA1 information processing (Nakajima *et al.*, 2021). Given that we have uncovered a selective impact of NMDA receptor autoantibodies in dampening the activity of CA1 inhibitory interneurons, and a subsequent rise in hippocampal synchrony, we may speculate a possible link between interneuron dysfunction and hypersynchronous discharges arising from a reduction in feedback and lateral inhibition. While our experiments examined the action of interneurons broadly, identified by GFP expression under a ubiquitous *dlx*-promotor, it would be interesting to investigate the action of NMDA receptor autoantibodies on GABAergic subpopulations. For instance, parvalbumin- and somatostatin-positive interneurons are known to regulate hippocampal feedback inhibition over differential timescales (Cornford *et al.*, 2019).

Finally, we also performed MEA recordings to determine the impact of NMDA- and GABA_A receptor autoantibodies on the network activity of dissociated hippocampal cultures. While the increase in activity was maintained in this system upon exposure to NMDA receptor autoantibodies, we failed to observe any alteration after GABA_A receptor antibody incubation. Moreover, we did not observe any changes in network burst parameters, indicative of changes to synchronicity, after exposure to either pathogenic autoantibody. As such, the results obtained from these experiments are somewhat inconsistent with our prior findings. This apparent disparity may arise from the high degree of variability of MEA datasets, where independent neuronal networks show highly divergent levels of baseline activity and synchrony – thereby masking more subtle impacts of autoantibodies. Alternatively, it is possible that some impacts of autoantibodies on hippocampal networks arise from perturbations in a circuit architecture-dependent manner.

VI

DISCUSSION & CONCLUSIONS

Summary and Interpretations

Our initial investigations focussed on the excitatory and inhibitory synaptic phenotypes of hippocampal cultures exposed to either NMDA- or GABA_A receptor antibodies for 24 hours. We uncovered convergent macroscale organisational consequences of autoantibody exposure, reducing the synaptic localisation of both AMPA and GABA_A receptors –constituting the vast majority of excitatory and inhibitory synaptic transmission, respectively. As such, pathogenic autoantibody exposure – irrespective of the target antigen – impedes the correct physiological localisation of ion channels to synaptic compartments. Ultimately, our observations demonstrate that autoantibody exposure triggers a generalised disruption in ion channel organisation across both excitatory and inhibitory synapses, indicating that the pathogenic mechanisms underpinning NMDA- and GABA_A receptor autoimmune encephalitis extend beyond direct and isolated impacts on their target antigens. This is particularly notable considering the rapid increase in antigen identifications associated with autoimmune encephalitides. To date, 18 distinct subtypes of autoimmune encephalitis have been classified, however, in 2022, approximately 55 autoantibodies had been identified in patients directed against unique neuronal proteins and receptors (Graus and Dalmau, 2022). Typically, research efforts have primarily focussed on characterisation of autoantibody-mediated impacts exclusively on their target antigen. Our data demonstrates that long-term exposure to autoantibodies, even in the highly selective monoclonal model, is capable of eliciting downstream impacts across the neuronal surface. Further, such “off-target” impacts may be vital to the development of disease, as we anticipate that such an induction of protein or receptor crosstalk can present a viable substrate for pathogenesis directly. This is well exemplified in our investigations of NMDA receptor encephalitis, in which the direct impact on the antigen is to drive an excitatory hypofunction, yet the clinical presentation of this disease is commonly associated with seizure phenotypes and a hyperglutamatergic state. Our characterisation of the macroscopic synaptic phenotypes illustrates that while NMDA receptor autoantibodies indeed drive a reduction in excitatory synaptic content, there is an additional impact on inhibitory synaptic compartments, of substantially higher magnitude. As such, this relatively disproportionate impact on the inhibitory system, may be a principal driver of pathogenesis, at least that which pertains to epileptogenesis and hyperglutamatergic states.

We further aimed to identify some putative mechanisms underpinning the direct and synaptic-cross talk effects of NMDA- and GABA_A receptor autoantibodies. Of particular interest, analysis of synaptic scaffold

integrity unveiled a unique vulnerability of inhibitory synaptic structures to autoantibody-mediated degradation. We have identified a putative intracellular mechanism, by which the selective depletion of inhibitory synapses appears to be mediated by hyper-phosphorylation and degradation of the inhibitory synaptic scaffolding protein, gephyrin. This selective loss of GABAergic synaptic puncta is particularly striking given that seizure forms a prominent symptom of both NMDA- and GABA_A receptor encephalitis. We anticipate that this additional destabilisation of inhibitory synapses – beyond the shared reduction in AMPA receptor localisation observed also at excitatory postsynaptic compartments – renders a greater magnitude reduction in phasic inhibitory control, relative to excitatory inputs. While there are a few identified kinases, known to regulate gephyrin puncta density through phosphorylation of a serine residue at position 270, including GSK3 β , CDK5 and ERK (Tyagarajan *et al.*, 2011, 2013; Kuhse *et al.*, 2012; Zhou *et al.*, 2021); additional data collected by Dr Mar Petit-Pedrol identified that GSK3 β appears to be upregulated in hippocampal cultures exposed to either NMDA- or GABA_A receptor autoantibodies (unpublished data, in manuscript in Annex A). Moreover, recent mass spectrometry data from hippocampal tissue from an *in vivo* rodent model of NMDA receptor encephalitis, also identified a reduction in gephyrin protein after chronic intraventricular infusion of NMDA receptor monoclonal, #003-102 (Ceanga *et al.*, 2022). Notably, GSK3 β is constitutively active, through autophosphorylation of a tyrosine within its activation loop, Y216, and its activity is downregulated by upstream molecular pathways (Lochhead *et al.*, 2006). As such, it is tempting to speculate that the disruption of intracellular signalling cascades resulting from reduced function of NMDA receptors – by their synaptic displacement – may lead to uncontrolled autophosphorylation-induced hyperactivation of GSK3 β . Intriguingly, further data from the lab has demonstrated that both NMDA- and GABA_A receptor autoantibodies similarly deplete NMDA receptors from synaptic compartments (unpublished data from Dr Mar Petit-Pedrol, in manuscript in Annex A). As such, the increased activity of GSK3 β after exposure to GABA_A receptor autoantibodies, may similarly derive from altered signalling cascades. In a study of GSK3 β -induced apoptosis in both cortical and hippocampal neurons, moderate GluN2B-containing NMDA receptor signalling has been shown to reduce GSK3 β activity, providing a possible link between NMDA receptor activity and suppression of GSK3 β , which may be lost after exposure to pathogenic autoantibodies (Habas *et al.*, 2006). Alternatively, GSK3 β has been implicated in the internalisation of GABA_A receptors, after strong activation of dopamine receptors (Li, Wang and Gao, 2012). While it remains unclear whether GABA_A receptor autoantibody exposure provokes internalisation – or simply membrane redistribution – of GABA_A receptors, if indeed their internalisation is upregulated, in a comparable fashion to that of NMDA receptors in the context of NMDA receptor encephalitis, it is possible that the upregulation of GSK3 β serves to enable increased internalisation of the GABA_A receptors. In such a case, the increase in activity of GSK3 β may induce off-target impacts on gephyrin scaffolding proteins. Ultimately, while we observe clear alterations to the inhibitory synaptic scaffolds, the precise molecular processes underpinning this destabilisation remain to be determined.

However, we would strongly suggest that future studies may consider GSK3 β activation and phosphorylation of gephyrin scaffolds as a candidate mechanism for this antibody-induced disruption of inhibitory synaptic structures.

While aberrant intracellular signalling cascades may underpin the part of the destabilisation of inhibitory synapses, mediating the loss of GABA_A receptor retention in postsynaptic compartments, we further identified that astrocytes may play a role in the destabilisation of AMPA receptors from excitatory synapses. Our immunostaining experiments revealed that under standard culture conditions – with the presence of astrocytes – that 24-hour incubation with either NMDA- or GABA_A receptor autoantibodies reduced the colocalisation of GluA1-containing AMPA receptors with excitatory synaptic scaffolding protein, homer-1c. However, when experiments were replicated in Banker cultures, and autoantibody exposure was performed in the absence of astrocytes, we observed no alteration in this colocalisation by either pathogenic autoantibody. Whether the abrogation of this impact is dependent on the release of astrocytic compounds, or through a structural mechanism of tripartite synapses is unknown. However, future experiments could be performed, in which antibody exposure is carried out in Banker configuration cultures, still in the presence of the astrocyte feeder layers. If the impacts are maintained in this case, it would suggest that the synaptic depletion of AMPA receptors is dependent on the release of astrocyte-derived factors. However, if the loss of alteration in AMPA receptor localisation is maintained in these settings, it would suggest that functional connection of astrocytic processes around synaptic compartments is more likely to play a role in this process. Of interest, it has been shown that the presence of astrocytes in hippocampal cell cultures increases synaptic current influx, arising from GluN2B-containing NMDA receptors (Hahn, Wang and Margeta, 2015). As such, it is possible that in our Banker culture experiments, synapses are relatively “pre-depleted” of NMDA receptors, and as such the downstream impacts of autoantibody exposure may be more limited due to reduced downmodulation of NMDA receptor-mediated currents. Intriguingly, this astrocyte-dependant upregulation of GluN2B-containing NMDA receptor currents has been shown to depend on activation of protein kinase C (PKC), providing an additional link to altered intracellular signalling cascades, which may form the core of immune-mediated cross-synaptic dysfunction. To address this possibility, future studies could aim to confirm this reduction of synaptic NMDA receptor function in Banker cultures, and additionally test for autoantibody-mediated impacts on AMPA receptor localisation in GluN2B knockdown systems. Notably, it is also well understood that astrocytes express GABA_A receptors, and a potential for a low abundance of NMDA receptors has also been suggested (Fraser, Mudrick-Donnon and MacVicar, 1994; Tateishi *et al.*, 2006; Skowrońska *et al.*, 2019; Verkhatsky and Chvátal, 2020). Yet, the impact of autoantibodies – which presumably bind directly at the astrocytic cell surface – remains unknown. Future studies should critically aim to uncover a potential role of direct antibody-mediated dysfunction of astrocytic cellular populations.

To further explore this phenomenon, we have examined the functional synaptic phenotypes of excitatory and inhibitory neurotransmission, resulting from antibody-induced synapse disorganisation. Analysis of spontaneous AMPA receptor-mediated excitatory postsynaptic currents revealed that both NMDA- and GABA_A receptor monoclonals reduce excitatory input onto CA1 hippocampal pyramidal neurons. Remarkably, despite the relatively moderate macroscale disorganisation of the AMPA receptor population at excitatory synapses, the greater magnitude of functional reduction in excitatory current amplitudes would suggest that autoantibodies elicit a substantial disruption of nanoscale synaptic architecture, possibly through a destabilisation of nanocolumns – in which AMPA receptor clusters are closely aligned with presynaptic glutamate release sites. It would therefore be interesting to characterise the subsynaptic nanoscale organisation of the remaining synaptic AMPA receptor population after exposure to autoantibodies using super-resolution techniques, as our data strongly suggests that – in addition to macroscale disorganisation, revealed at the diffraction-limited resolution of confocal microscopy – integrity of subsynaptic nanodomain clustering is also impaired by autoantibody exposure. Moreover, we observed that GABA_A receptor-mediated inhibitory signalling is substantially reduced by both NMDA- and GABA_A receptor antibodies. Consistently, the magnitude of reduction in inhibitory transmission was greater than that observed in excitatory transmission, yielding a clear shift in the excitation-inhibition balance of synaptic inputs, in favour of hyperexcitation. While such a shift may have been anticipated in the case of GABA_A receptor monoclonal action, with direct antagonism of channels, it is compelling to observe a congruent impact by NMDA receptor autoantibodies after 24 hours of exposure. Ultimately, these homogenous impacts, by both pathogenic antibody samples, across excitatory and inhibitory synaptic structures, clearly indicates that there are convergent routes contributing to pathogenesis, even across distinct autoimmune encephalitic syndromes. Evidently, while substantial characterisation of direct-antigen effects is highly valuable; examination of wider neuronal organisation and function – beyond direct-antigen driven impacts – are necessary to elucidate the extensive pathogenic avenues towards brain dysfunction.

To contextualise the substantial aberrations that we have observed at the level of the neuronal surface organisation and functional synaptic inputs, we consequently characterised the intrinsic properties of CA1 hippocampal pyramidal cells and GABAergic interneurons. We have revealed that the perturbation of synaptic inputs is further compounded by shifts in intrinsic cellular excitability of pyramidal neurons exposed to GABA_A receptor antibodies. Interestingly however, while this shift towards hyperexcitability was not observed in principal cells exposed to NMDA receptor monoclonals, we subsequently uncovered an opposing reduction in the excitability of inhibitory cell populations. While it is possible that divergence in antibody-mediated pathology at this level of neuronal function may result in the segregated symptoms of disease, namely the strong neuropsychiatric component of NMDA receptor encephalitis – a phenotype otherwise associated with reduced glutamatergic drive on GABAergic interneuron populations – and not typically observed in cases of

GABA_A receptor encephalitis. However, this conjecture would require substantial further investigation. It may be of interest to investigate psychosis-associated behavioural phenotypes in rodent models of encephalitis, and characterise the therapeutic potential of interneuron activity manipulation, for example by administration of positive modulators of K_v3.1 voltage-gated potassium channels, EX15 and RE01, which have been demonstrated to increase hippocampal interneuron excitability (Boddum *et al.*, 2017). Moreover, we identified a unique impact of GABA_A receptor autoantibodies, altering the action potential waveform in CA1 pyramidal neurons. While the precise mechanisms by which this occurs remains to be fully elucidated, such observations would be consistent with an alteration in the function of axonally expressed GABA_A receptors, which have otherwise been shown to modulate action potential amplitude and duration (Xia *et al.*, 2014). Such alterations are demonstrative of the fact that distinct subcellular populations of receptors – synaptic, extrasynaptic, somatic and axonal GABA_A receptors, for instance – can constitute largely disparate neuronal functions, such as phasic, tonic and shunting inhibition. As such, the plethora of distinct ionotropic functions, mediated by such receptor populations, needs to be carefully considered when considering the overall impact of immune-targeting of surface proteins, as there is likely not a single unifying action of antibody-mediated effects underpinning highly orchestrated neuronal dysfunction.

In the context of autoimmune encephalitis, it is however conceivable that this bidirectional action on opposing neuronal systems, increased excitation or decreased inhibition, yields the same endpoint of increased hippocampal activity at the network scale. To directly investigate this possibility, we have characterised the network level effects of autoantibody exposure, using calcium imaging and MEA approaches. Calcium imaging data demonstrated a common overarching hyperactivation of hippocampal pyramidal cells, presumably culminating from the dysregulation across the synaptic and cellular functions. However, we do note subtle disparities in the network phenotypes, whereby NMDA receptor autoantibodies exclusively drive hippocampal networks to a hypersynchronous state, a feature not observed after GABA_A receptor antibody exposure. This observation of hypersynchrony is in alignment with recent advances by others in the field, regarding the pathogenicity of NMDA receptor autoantibodies (Wright *et al.*, 2021; Ceanga *et al.*, 2022). However, we were unable to replicate these findings in dissociated hippocampal networks, cultured on multielectrode arrays. However, this could be due to a variety of experimental challenges, stemming largely from high variability in baseline network activity, even present between cultures generated from the same animal tissue and cultured in the same MEA chips. Alternatively, this disparity may arise from altered ability of autoantibodies to modulate synaptic, cellular or circuit-based functions in the absence of physiological hippocampal architecture. Given that the development of hippocampal structures provides a basis for highly precise axonal targeting of interneuron-to-pyramidal cell connections (Stark *et al.*, 2014; Pelkey *et al.*, 2017); the loss of such organised functional connectivity in dissociated networks may inherently limit the impacts of autoantibodies, through the removal of hyperactivity-generating circuitry.

Ultimately, this thesis work has uncovered a set of functional pathways which may contribute to the disease aetiology of NMDA- and GABA_A receptor autoimmune encephalitis. Surprisingly, our initial investigations unveiled a common impact of these highly distinct autoantibodies on both excitatory and inhibitory synaptic phenotypes, leading to a shift in the phasic excitation-inhibition balance. We contextualised these aberrant synaptic inputs by investigating the intrinsic excitability of both pyramidal and GABAergic neuronal populations. This revealed a divergent action of NMDA- and GABA_A receptor autoantibodies, whereby GABA_A receptor antibodies reduce the excitability of pyramidal neurons directly, possibly via an action on extrasynaptic GABA_A receptors, reducing tonic GABAergic conductance and leading to a decrease in shunting inhibition. We also note a selective impact of these autoantibodies to modulate the action potential waveform, which likely stems from GABAergic dysfunction at the axon initial segment, which is well placed to elicit changes in overall neuronal excitability. Further, we identified a converse action of NMDA receptor autoantibodies, reducing the intrinsic excitability of inhibitory neuronal populations, adding further perturbations to CA1 inhibitory tone. This loss of inhibitory network activity may render the CA1 pyramidal cell populations vulnerable to recurrent hyperexcitation, stemming – in part – from CA3 microcircuits, which can no longer be adequately controlled by local inhibitory drive. Finally, we observed that the overarching impact of these synaptic and cellular dysfunctions leads to hippocampal hyperactivation of excitatory neurons in the CA1 subfield, with an additional impact of NMDA receptor autoantibodies in the elicitation of hypersynchronous network states.

Implications

Our identification that NMDA receptor autoantibodies can preferentially alter the intrinsic excitability of GABAergic interneuron populations, as compared to local pyramidal cells, reveals a fascinating aspect of how these antibodies influence the disease phenotype. This finding highlights the diverse functional roles of NMDA receptors within different cell types, which ultimately shape the overall manifestation of the disease. Understanding these specific roles is crucial in comprehending the occurrence and progression of the disease. One aspect worth considering is how the functional role of NMDA receptors dynamically changes throughout neurodevelopment and synaptogenesis. During early stages of neurodevelopment, the ratio of surface-expressed GluN2A:2B-containing NMDA receptors exerts significant control over the rates of synaptogenesis and spine mobility (Gambrill & Barria, 2011). This observation suggests that the expression of NMDA receptors during this critical time window may have profound implications for synaptic development and maturation. Taking this into account opens additional perspectives on the pathogenesis of NMDA receptor encephalitis in patients experiencing the disease during early life. The disruption caused by autoantibodies targeting NMDA receptors at this stage could impede the healthy development of synapses and maturational processes. This may lead to long-lasting consequences, as proper synapse formation and maturation are crucial for establishing functional neuronal networks and cognitive abilities. Therefore, recognizing the dynamic functional roles of NMDA receptors and their vulnerability to autoantibodies during neurodevelopment provides insights into potential pathogenic mechanisms underlying NMDA receptor encephalitis. Further exploration of these pathogenic routes and their impact on synapse development may offer opportunities for early interventions and targeted therapeutic approaches that aim to mitigate the long-term consequences of the disease, particularly in individuals affected during early life.

Additionally, our investigations have revealed a wide range of effects on neuronal function across multiple scales, including synaptic, cellular, and network levels. It is remarkable to observe that single monoclonal autoantibodies display the ability to elicit such a diverse array of impacts on neuronal activity. The debate surrounding the use of monoclonal versus biological samples in autoimmune encephalitis research continues within the scientific community. However, our findings strongly suggest that monoclonal autoantibodies can indeed drive various neurological deficits, thereby recapitulating a significant subset of disease mechanisms present in a polyclonal disease environment. The ability of monoclonal autoantibodies to induce such widespread and diverse effects on neuronal function underscores their potency in disrupting the intricate balance of excitatory and inhibitory systems. The findings also raise intriguing questions about the interplay between monoclonal and polyclonal autoantibodies in the disease process. While monoclonal autoantibodies can reproduce several aspects of the disease phenotype, it is likely that a polyclonal environment, where multiple autoantibodies with different specificities are present, would further amplify the complexity and severity of the disease. The combined effects of diverse autoantibodies targeting various antigens could lead

to a broader spectrum of neurological deficits and contribute to the heterogeneity observed in patients with autoimmune encephalitis. Understanding the impact of monoclonal autoantibodies and their relationship to polyclonal responses is crucial for unravelling the mechanisms underlying autoimmune encephalitis. It highlights the need for comprehensive investigations that consider both monoclonal and polyclonal contexts to gain a more complete understanding of disease pathogenesis.

Furthermore, our findings that both NMDA and GABA_A receptor autoantibodies have the capacity to disrupt the surface organization of non-antigen receptors provide compelling evidence for the existence of complex coregulation among neuronal proteins in autoimmune encephalitic syndromes. This suggests that the pathogenic mechanisms underlying these disorders should not be viewed as isolated channelopathies, where autoantibodies solely impact their specific target antigens. Instead, these diseases should be considered as intricate processes with broader and sometimes convergent routes to pathogenesis. Traditionally, much of the research on neurological disorders has focused on studying the defects associated with individual proteins, such as those seen in genetic models of epilepsy or neurodevelopmental conditions. However, our observations emphasize the importance of considering the intricate interplay and coordinated regulation of multiple neuronal proteins. The high degree of orchestrated co-regulation between these proteins can, in itself, become a substrate for pathogenesis. By recognizing the interconnected nature of neuronal proteins and their complex regulatory networks, we gain a deeper understanding of how disruptions in one component can have widespread effects on the overall functioning of the brain. Autoimmune encephalitic syndromes, including those involving NMDA and GABA_A receptors, highlight the significance of this co-regulation and suggest that perturbations in one component can lead to dysregulation of the entire system.

These insights also have important implications for our approach to studying and treating autoimmune encephalitic syndromes. Instead of solely focusing on individual antigens, a comprehensive understanding of the intricate interactions and co-regulation among neuronal proteins could provide valuable insights into the underlying mechanisms of these disorders. Moreover, it opens avenues for developing therapeutic strategies that target not only specific antigens but also the broader regulatory networks involved in disease pathogenesis. By addressing the complex interplay of neuronal proteins, we may be able to develop more effective and targeted interventions for autoimmune encephalitic syndromes.

Limitations

While monoclonal antibodies have been valuable tools in the study of various autoimmune encephalitis, it is important to acknowledge their limitations and recognise that their use may potentially impose limitations on the extension of our results to the human disease state. While the recombinant monoclonal antibodies used in our investigations are designed to specifically target a single epitope of the NMDA- or GABA_A receptor, autoimmune encephalitis is a complex disorder involving a multitude of autoantibodies targeting variable epitopes of these receptors, and in many cases also variable proteins. As such, relying solely on monoclonal antibodies may fail to capture the full spectrum of autoantibody-mediated disease processes involved in the pathogenesis of autoimmune encephalitis. This limitation could therefore lead to an incomplete understanding of the variety of possible pathogenic mechanisms. Additionally, in the context of autoimmune encephalitis, patients may exhibit a range of autoantibodies that target different antigens within the central nervous system. Thus, relying solely on monoclonal antibodies could result in overlooking important autoantibody targets that may contribute to the disease pathology. To overcome this limitation, alternative approaches, such as polyclonal antibody samples or multi-target monoclonal pools may need to be considered to provide a more comprehensive characterisation of the autoantibody repertoire.

Moreover, the disease state of autoimmune encephalitis encompasses a diverse range of clinical phenotypes and patient cohorts. For example, while NMDA receptor encephalitis disproportionately impacts women in young adulthood, both NMDA- and GABA_A receptor encephalitis have been observed to onset across the lifespan. Furthermore, disease triggers also show substantial heterogeneity. While often associated with underlying cancers or subsequent to viral infection; it is possible that variable triggers of disease may launch subtle differences in the autoimmune immune response. For instance, it is believed that herpes simplex infection can lead to NMDA receptor encephalitis, due to cross reactivity between a viral antigen and the GlunN1 subunit. Conversely, in cases in which ovarian teratoma is identified, it has been shown that tumorous tissue expresses a high density of NMDA receptors directly. Yet, single monoclonal antibodies may not adequately represent this heterogeneity, as autoantibodies generated in response to direct antigen presentation outside the nervous system, versus an off-target cross-reactivity may be substantially different, in terms of epitope, affinity and impact on their antigen. Consequently, the results obtained using monoclonal antibodies may not be generalizable to the entire population of autoimmune encephalitis patients. However, the development of larger monoclonal libraries, and comparisons between the actions of such autoantibodies, with careful consideration of the clinical presentation and history of the patients from whom they were derived may be of highly valuable use.

In conclusion, while monoclonal antibodies have been instrumental in the study of autoimmune encephalitis, they possess inherent limitations that may hinder the comprehensive investigation of this complex disorder. Recognizing these limitations is crucial for interpreting research findings and underscores the need for

incorporating complementary approaches to complement the insights gained from monoclonal antibody studies. Encouragingly however, by employing a multidisciplinary approach, it is possible to overcome these limitations and contribute to a more holistic understanding of autoimmune encephalitis.

Moreover, our investigations have uniquely employed *in vitro* techniques, including both dissociated and organotypic hippocampal cultures. A principal consideration and possible limitation of such *in vitro* investigations is the lack of physiological context and complexity, as compared to that of *in vivo* systems. While autoimmune encephalitis constitutes a neurological disease, with pathological processes confined to the central nervous system, we should consider the possibility that the lack of *in vivo* studies within our experimental datasets may fail to capture some intricate interactions and complexities that exist in the context of the whole organism. This is notable in the study of autoimmune encephalitis since inflammatory processes and disease triggers are likely to originate outside the brain during onset. However, these mechanisms and phases of the disease are not considered in our work, and as such this likely causes little to no confounds in our interpretations. A further limitation specific to the use of dissociated neuronal cultures is the lack of physiological tissue architecture and three-dimensional organization. This may be particularly vital for the study of autoimmune encephalitis, regarding heightened seizure susceptibility. In fact, our discrepant results regarding network hyperactivation of hippocampal circuits cultured in organotypic and primary culture preparations strongly suggest that hippocampal architecture plays a key role in epileptogenesis. Additionally, all electrophysiological investigations were performed in organotypic culture paradigms, and as such we would argue that a large body of our investigations and datasets – at least partially – account for such a limitation. It is also greatly encouraging to note that some principal findings that we have observed *in vitro*, including the loss of inhibitory synaptic scaffolding protein, gephyrin, at the synaptic scale; through to the network level alteration in NMDA receptor autoantibody-mediated hypersynchrony, has also been replicated in a recent *in vivo* model of NMDA receptor encephalitis (Ceanga *et al.*, 2023). Ultimately, combining *in vitro* and *in vivo* methodologies, animal models, and clinical investigations will surely provide a more comprehensive understanding of the complex biological processes implicated in autoimmune encephalitic conditions, facilitating the translation of scientific discoveries into clinical applications.

Perspectives

Our research has focused on understanding the impact of NMDA and GABA_A receptor autoantibodies on neuronal and network function within the hippocampus. The choice to primarily examine hippocampal pathophysiology stems from the observation that NMDA receptor autoantibodies often exhibit a preference for targeting hippocampal and limbic structures (Wagner *et al.*, 2020). However, the extent of autoantibody binding in cortical regions is more limited, and the role of cortical dysfunction in the context of encephalitis remains less elucidated. This is particularly intriguing considering the high prevalence of cognitive dysfunction and the well-known association between the prefrontal cortex and complex cognitive processes. Given these considerations, it would be highly valuable to expand our investigations to include additional brain regions in order to identify common or divergent actions of autoantibodies throughout the brain. Exploring other regions beyond the hippocampus could shed light on the broader impact of these autoantibodies and help establish a more comprehensive understanding of the pathophysiology of autoimmune encephalitis. In particular, the study of cortical regions could provide insights into the potential mechanisms underlying cognitive dysfunction and the intricate interplay between autoantibodies and cortical circuits. By examining the effects of autoantibodies in various brain regions, we may uncover shared pathological mechanisms throughout brain structures. Additionally – and conversely – we may uncover region-specific vulnerabilities or protective factors that contribute to the differential impact of autoantibodies throughout the brain. This knowledge could have significant implications for both diagnosis and treatment strategies in autoimmune encephalitis. Such expansion of our investigations to additional brain regions would require careful consideration of the unique characteristics and functional properties of each region. For example, different brain regions exhibit highly distinct neuronal circuitry, connectivity patterns, and receptor expression profiles, which may influence their response to autoantibody-induced disruptions. Therefore, a comprehensive approach that encompasses multiple brain regions would provide a more holistic understanding of the complex interactions between autoantibodies and the brain. As such, while our research has primarily focused on the hippocampus due to the preferential targeting of NMDA receptor autoantibodies, the role of cortical dysfunction remains less clear despite the high prevalence of cognitive impairment. To address this gap in knowledge, expanding our investigations to include additional brain regions would enable us to identify common or divergent actions of autoantibodies throughout the brain. This comprehensive approach holds the potential to enhance our understanding of the pathophysiology of autoimmune encephalitis and uncover novel therapeutic targets to mitigate the devastating effects of these disorders.

Furthermore, we have exclusively utilised rodent-derived neuronal systems in our experimental designs. While rodent models can provide valuable insights into neuronal function and disease mechanisms, there may be subtle differences in the expression and functionality of NMDA and GABA_A receptors between rodents and humans. To address this, advancements in stem cell technology offer a promising avenue for investigations of

the autoantibody-mediated impacts on human-derived neuronal systems. The use of such systems, generated from induced pluripotent stem cells (iPSCs), allows for a more direct examination of autoantibody-mediated effects in a human-specific context. Such experiments are of great importance, as they may provide insights into potential species-specific differences and help validate the findings obtained from rodent models. As such, human-derived neuronal systems may also better reflect the complexity and intricacies of human brain function, contributing to a more accurate understanding of the pathological processes underlying autoimmune encephalitis.

Moreover, the field of human-derived organoids has undergone significant advancements in recent years, thereby enabling the study of autoantibody-mediated pathology in three-dimensional tissue structures. Organoids provide a more physiologically relevant platform by mimicking the architecture and cellular diversity found in human brain tissue, and such an approach could offer compelling insights into the characterisation of epileptogenic mechanisms. By incorporating organoids into future investigations, we might gain a deeper understanding of the impact of autoantibodies in a more realistic and complex tissue context. It is important to note, however, that both human-derived neuronal systems and organoids still have their significant limitations. Variability among iPSC lines, differentiation protocols, and culture conditions can introduce challenges in generating consistent and reproducible results. The immaturity of some human-derived systems compared to adult human brain tissue further adds to the complexity. By incorporating these approaches, we can explore potential species-specific differences, validate our findings, and gain a more accurate understanding of autoantibody-mediated pathology in the context of human brain function. Despite the challenges and variability associated with these systems, the integration of multiple approaches holds substantial value in advancing our knowledge of autoimmune encephalitis and may facilitate the development, and testing, of targeted therapeutic strategies.

Our investigations into synaptic phenotypes yielded intriguing findings regarding the mutual coregulation of excitatory and inhibitory synaptic organization and structuration after exposure to either NMDA- or GABA_A receptor autoantibodies. Notably, both antibodies were found to reduce synaptic content of AMPA and GABA_A receptors, as well as reduce the density of inhibitory synapses. Our subsequent experiments provided preliminary insights into the underlying mechanisms of these alterations; suggesting that antibody-induced changes in AMPA receptor organization may be, at least in part, dependent on astrocytic signalling. However, further experiments are necessary to elucidate the precise mechanisms underlying this process. Moreover, our studies suggest that the effects on GABAergic synapses may be dependent on phosphorylation. Specifically, phosphorylation-dependent mechanisms may be involved in the disorganisation of gephyrin and subsequent alterations in GABAergic synaptic density. However, future experiments are needed to explore the specific kinase pathways responsible for gephyrin hyperphosphorylation and to further dissect the underlying molecular processes. To gain deeper insights into these mechanisms, a combination of

experimental approaches can be employed. For instance, the use of kinase inhibitors in conjunction with antibody exposure could help identify the specific kinase pathways involved in the observed hyperphosphorylation of gephyrin. This targeted inhibition approach would allow for a more precise understanding of the signalling cascades contributing to synaptic and intracellular alterations mediated by autoantibodies. Additionally, single-cell proteomics of neuronal cells and astrocytes could provide valuable information regarding potential upregulation of secreted factors in astrocyte populations following exposure to NMDA and GABA_A receptor autoantibodies. This approach may enable the identification and characterisation of specific molecules released by astrocytes in response to autoantibody-induced processes. Such insights may shed light on the precise mechanistic roles of astrocytes in the disorganisation of synaptic AMPA receptors.

Finally, the recovery phase of autoimmune encephalitis continues to pose a significant clinical challenge, as many patients experience persistent neurological symptoms despite a positive response to immunotherapy in reducing autoantibody titres. Patients commonly display ongoing symptoms for months, and some may not fully regain their baseline health status. As such, understanding the recovery of neuronal function following transient exposure to autoantibodies is an area of significant research interest. One possibility is that autoantibody-mediated dysfunction in synaptic, cellular, and network activity can persist even after the clearance of autoantibodies from the system. The highly dysregulated synaptic plasticity observed in conditions like NMDA receptor encephalitis may lead to long-lasting alterations in information processing within neuronal circuits, particularly in regions like the hippocampus. These alterations may not readily return to their pre-exposure physiology, resulting in persistent changes in neuronal function even in the absence of circulating autoantibodies. Additionally, the brain's response to repeated seizures during the acute phase of encephalitic illness can contribute to the perpetuation of the disease cycle and hinder recovery. Seizures can cause damage to neurons, disrupt synaptic connections, and induce further inflammation in the brain. These structural and functional changes may lead to the formation of epileptic foci, hyperactive regions in the brain that generate seizures spontaneously. Even after the clearance of autoantibodies, the presence of epileptic foci can sustain abnormal electrical activity and hinder the restoration of normal neuronal function. Investigating which elements of neuronal dysfunction persist after the clearance of autoantibodies from the tissue is of great interest. Understanding the sustained effects of autoantibody exposure on synaptic, cellular, and network activity can provide valuable insights into the mechanisms underlying persistent symptoms and help guide the development of therapeutics for individuals in the recovery phase of encephalitic disease. By identifying the specific components of neuronal dysfunction that persist, researchers may be able to develop targeted interventions to promote neural recovery and improve outcomes for patients.

VII

APPENDICES & BIBLIOGRAPHY

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A

PUBLICATIONS & MANUSCRIPTS

Review article published in *Neurobiology of Disease*, Volume 147, January 2021.

Autoimmunity and NMDA receptor in brain disorders: Where do we stand?

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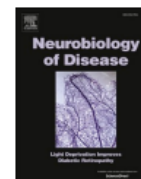
Abstract

Over the past decades, the identification of autoimmune encephalitis in which patients express autoantibodies directed against neurotransmitter receptors has generated great hope to shed new light on the molecular mechanisms underpinning neurological and psychiatric conditions. Among these autoimmune encephalitides, the discovery of autoantibodies directed against the glutamatergic NMDA receptor (NMDAR-Ab), in the anti-NMDAR encephalitis, has provided some key information on how complex neuropsychiatric symptoms can be caused by a deficit in NMDAR signalling. Yet, NMDAR-Abs have also been detected in several neurological and psychiatric conditions, as well as in healthy individuals. In addition, these various NMDAR-Abs appear to have different molecular properties and pathogenicities onto receptors and synaptic functions. Here, we discuss the current view on the variety of NMDAR-Abs and, in particular, how these autoantibodies can lead to receptor dysfunction in neuronal networks. Since our mechanistic understanding on patients' NMDAR-Abs is still in its infancy, several complementary processes can be proposed and further in-depth molecular and cellular investigations will surely reveal key insights. Autoantibodies represent a great opportunity to gain knowledge on the etiology of neuropsychiatric disorders and pave the way for innovative therapeutic strategies.



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Review

Autoimmunity and NMDA receptor in brain disorders: Where do we stand?

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ABSTRACT

Over the past decades, the identification of autoimmune encephalitis in which patients express autoantibodies directed against neurotransmitter receptors has generated great hope to shed new light on the molecular mechanisms underpinning neurological and psychiatric conditions. Among these autoimmune encephalitides, the discovery of autoantibodies directed against the glutamatergic NMDA receptor (NMDAR-Abs), in the anti-NMDAR encephalitis, has provided some key information on how complex neuropsychiatric symptoms can be caused by a deficit in NMDAR signalling. Yet, NMDAR-Abs have also been detected in several neurological and psychiatric conditions, as well as in healthy individuals. In addition, these various NMDAR-Abs appear to have different molecular properties and pathogenicities onto receptors and synaptic functions. Here, we discuss the current view on the variety of NMDAR-Abs and, in particular, how these autoantibodies can lead to receptor dysfunction in neuronal networks. Since our mechanistic understanding on patients' NMDAR-Abs is still in its infancy, several complementary processes can be proposed and further in-depth molecular and cellular investigations will surely reveal key insights. Autoantibodies represent a great opportunity to gain knowledge on the etiology of neuropsychiatric disorders and pave the way for innovative therapeutic strategies.

One sentence summary: Current view on patients' autoantibody against NMDAR.

1. Introduction

In the vertebrate central nervous system, excitatory glutamatergic neurotransmission constitutes the vast majority of intercellular communication (Traynelis et al., 2010). Among the glutamatergic receptors underpinning fast transmission, NMDA receptors (NMDARs) play a key role in synaptic adaptation processes (Lau and Zukin, 2007; Paoletti et al., 2013). Furthermore, dysfunctions of NMDAR signalling have been involved in the etiology of several major neurological and psychiatric disorders. NMDARs are heterotetramers composed of a combination of GluN1, GluN2, and GluN3 subunits. These subunit families contain several variants: the single GluN1 subunit with eight splice isoforms, four distinct GluN2 subunits (A-D), and two GluN3 subunits (Paoletti et al., 2013). The assembled NMDAR has a large extracellular domain, transmembrane loops, and an intracellular tail. The agonist, i.e. glutamate, binds to GluN2 subunits whereas the co-agonist, i.e. glycine or D-serine, binds to GluN1 subunits. The GluN2 subunits provide specific biophysical and pharmacological properties to the receptor. In addition to their biophysical specificities, NMDAR subtypes exhibit different trafficking properties that have been

thoroughly reviewed (Horak et al., 2014; Lau and Zukin, 2007; Lussier et al., 2015; Paoletti et al., 2013). Schematically, there is a large pool of GluN1 subunits in the endoplasmic reticulum that await assembly with GluN2 subunits. The correctly assembled receptors are intracellularly trafficked through the dendrite, carried along microtubules by molecular motor kinesins. After insertion into the plasma membrane, NMDARs laterally diffuse and explore large domains of neuronal dendrites (Ladepêche et al., 2014). Upon entry into postsynaptic compartment, the NMDAR becomes anchored and stabilized by protein-protein interactions with various scaffold partners. In the extrasynaptic compartment NMDAR are endocytosed and eventually recycle to the plasma membrane through exocytosis, keeping the membrane pool rather constant. The surface dynamics of NMDAR is not only important to regulate the synaptic pool, it also plays a key role in the establishment of long-term synaptic plasticity in an unconventional process (Groc and Choquet, 2020).

Understanding the dysfunction of the NMDAR signalling in various brain disorders has captured a lot of attention over the past decades (Hardingham, 2019; Myers et al., 2019; Nakazawa et al., 2017). From genetic mutations observed in patients with neurological and

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psychiatric conditions to drugs that target the NMDAR and trigger neuropsychiatric symptoms, several putative mechanism(s) underpinning NMDAR signalling dysfunction have been proposed. The direct role of NMDAR dysfunction in human brain disorders has recently gained strong support with the discovery of autoantibodies directed against the NMDAR (NMDAR-Abs). [Dalmau et al. \(2007\)](#) identified a patient cohort who presented initially with a viral-like prodromal phase. This condition was followed sharply by psychotic symptoms bearing a striking resemblance to that of a first psychotic episode. It is then concurrent with subtle indicators of underlying neurologic pathology including dyskinesia ([Dalmau et al., 2010](#); [Dalmau et al., 2007](#)). Later stages of the disease progression comprise a seizure phenotype and a worsening autonomic dysfunction often leading to fatality if untreated. It was later identified that the root of this disorder was by the pathogenic action of patient-generated NMDAR-Abs directed against the extracellular domain of the receptor ([Dalmau et al., 2008](#); [Gleichman et al., 2012](#); [Hughes et al., 2010](#)). The initial psychiatric phase is considerably heterogeneous between patients, experiencing symptoms of psychosis (auditory and visual hallucination), depression, mania, eating disorder and addiction ([Graus et al., 2016](#)). Alarminglly, a large fraction of these

patients were initially admitted to psychiatric institutions as opposed to neurological wards ([Graus et al., 2016](#); [Lejuste et al., 2016](#); [Pollak et al., 2020](#)). The question of whether different clinical expression of anti-NMDAR encephalitis exists is a current debate. For instance, NMDAR-Abs have been found in up to 20% of patients diagnosed with schizophrenia ([Jezequel et al., 2018](#)), suggesting that these autoantibodies can be expressed in patients with psychosis and/or in patients with anti-NMDAR encephalitis with prominent psychiatric features. Ongoing investigations will surely shed lights on this debate. Today, less than twenty different autoimmune encephalitic disorders had been identified in which autoantibodies are directed against synaptic receptors (i.e NMDAR, GABA receptor) or neuronal cell surface proteins (i.e LGI1, CASPR2) ([Crisp et al., 2016](#)). Brain-targeting antibodies have also been identified in non-encephalitic disorders. It is estimated that up to 70% of patients with autism spectrum disorder have systemic circulation of autoantibodies against brain targets. Also, in patients with systemic lupus erythematosus and a concurrent neuropsychiatric component (NPSLE), it has been shown that anti-double-stranded DNA antibodies (dsDNA-Abs) have cross-reactivity with NMDARs ([Brimberg et al., 2015](#)). Adding still further complexity, brain-targeting autoantibodies

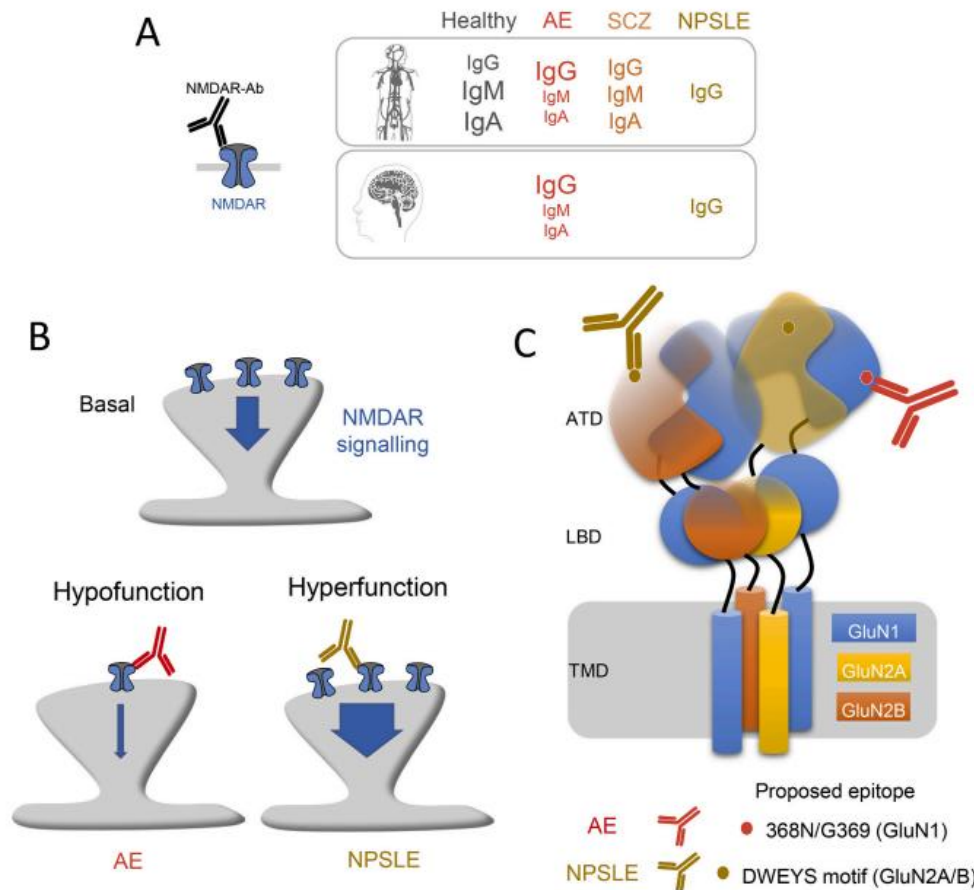


Fig. 1. Illustrations of the diversity of NMDAR-Abs. A) NMDAR-Abs are detected in the CSF (upper panel) and/or the serum (lower panel) of healthy subjects and neuropsychiatric patients (AE, SCZ, NPSLE). NMDAR-Abs are represented by a large diversity of immunoglobulin (Ig) classes with different seroprevalence represented by a lower and higher font size. B) Representation of a post-synaptic compartment and the modulation of NMDAR signalling (blue arrow) by NMDAR-Abs from different diseases. NMDAR-Abs have different impacts on NMDARs compared to basal conditions. In anti-NMDAR encephalitis, a hypofunction (represented by a smaller arrow) is induced by NMDAR-Abs while a hyperfunction (bigger arrow) occurs in NPSLE patients with NMDAR-Abs. C) Representative structure of NMDAR in 3D with proposed epitopes of NMDAR-Abs. The epitope of NMDAR-Abs from encephalitic patients is located at the 368 N/369G region of the GluN1 subunit. In NPSLE, the NMDAR epitope for NMDAR-Abs is located on GluN2A/B subunits, at an extracellular DWEYS motif. Abbreviations: anti-NMDAR encephalitis (AE); schizophrenia (SCZ); neuropsychiatric systemic lupus erythematosus (NPSLE); amino terminal domain (ATD); ligand-binding domain (LBD); transmembrane domain (TMD). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

have also been identified in the systemic circulation of healthy individuals, suggesting that - in at least some cases - such autoantibodies lack any observable pathogenicity. Given the association of NMDAR-reactive autoantibodies across many neuropsychiatric disorders, and its high prevalence in autoimmune encephalitis and NPSLE, we will dedicate our focus to the actions of two mechanistically opposing NMDAR-Abs, those found in encephalitis and NPSLE. As there is considerable heterogeneity in the mechanism of action for these NMDAR-Abs within different neurological conditions, we will discuss these various mechanisms of action and propose emerging working models.

2. Diversity in NMDAR autoantibody types and pathogenicity

The concept that autoantibodies contribute to the etiology of specific neurological and psychiatric conditions is almost a century old (Jezequel et al., 2018). Yet, since the identification of NMDAR-Abs in anti-NMDAR encephalitis more than a decade ago, the number of studies reporting NMDAR-autoimmunity in brain disorders has been exponentially growing (Dalmau et al., 2019; Ehrenreich, 2018; Jezequel et al., 2018). The study of NMDAR-Abs has primarily focussed on neuropsychiatric diseases, since they were initially found in the serum and CSF of women presenting with prominent psychiatric symptoms and underlying ovarian teratoma (Dalmau et al., 2019). In anti-NMDAR encephalitis, the NMDAR-Abs are mainly of the IgG subtype, when compared to IgM and IgA subtypes which make up less than 10% (Fig. 1A). In patients suffering from psychotic disorders, including schizophrenia and bipolar disorder, the fraction of seropositive patients is variable between studies, with estimates ranging from few percent up to 20% (Jezequel et al., 2018). The immunoglobulin subtypes in seropositive psychotic patients also greatly varies between studies, with some reporting a high proportion of IgG, and others, a predominance of IgM/A subtypes (Doss et al., 2014; Ehrenreich, 2018; Hammer et al., 2014) (Fig. 1A). The presence of NMDAR-Abs in a number of patients with variable dementias has also been reported; including progressive supranuclear palsy, corticobasal syndrome, Parkinson's disease-related dementia, primary progressive aphasia, ischemic stroke and amyotrophic lateral sclerosis (Dalmau et al., 2019; Ehrenreich, 2018; Jezequel et al., 2018). In 2001, NMDAR-Abs were reported in patients with NPSLE. This autoimmune disease is first characterised by the presence of dsDNA-Abs. It has been proposed that a pentapeptide sequence in the extracellular portion of the NMDAR GluN2A/2B subunits is a molecular mimic of double-stranded DNA. This cross-reactivity between dsDNA-Abs and NMDAR GluN2A/2B subunits have been associated with cognitive defects in NPSLE patients, with approximately 30% of patients showing seropositivity for NMDAR-Abs against these GluN2 subunits (DeGiorgio et al., 2001) (Fig. 1A). It should be noted that the presence of NMDAR-Abs in NPSLE has been questioned (Varley et al., 2020), calling for further in-depth investigations.

To add another layer of complexity, NMDAR-Abs have also been found in healthy individuals (Dahm et al., 2014; Doss et al., 2014; Hammer et al., 2014; Jezequel et al., 2017a; Pan et al., 2019), with a seroprevalence that has been proposed to increase with age and is slightly higher in male compared to female populations (Hammer et al., 2014).

This extensive diversity in NMDAR-Abs subtypes and disease prevalence raises, among many others, two key questions: (i) whether currently-available techniques are reliable and reproducible measures of antibody prevalence, and (ii) whether all NMDAR-Abs have similar effects on their targets. Considering the former, various assays are used worldwide (Sinnmaz et al., 2015). Recently, it has been shown that identical samples tested in different centres with different assays (e.g. live versus fixed heterologous cells) produced opposite outcomes (Jezequel et al., 2017b). It thus remains urgent to develop sensitive and reproducible methods to detect the presence, or absence, of NMDAR-Abs in the circulation of patients with different conditions. Secondly,

considering the heterogeneity of antibody actions, the best characterised pathogenic mechanism comes from studies of anti-NMDAR encephalitis. Schematically, anti-NMDAR IgGs induce a massive hypofunction of synaptic NMDARs, leading to a decrease in NMDAR signalling (Dalmau et al., 2017) (Fig. 1B). This antibody effect is achieved by an alteration of the lateral displacement of synaptic NMDARs. Similarly, NMDAR-Abs from patients with psychotic disorders, induce an NMDAR hypofunction with comparably aberrant surface dynamics (Jezequel et al., 2017a). These major alterations of NMDAR signalling prevent NMDAR-dependent long-term synaptic plasticity and induce deficits in memory and cognitive functions (Jezequel et al., 2018; Planaguma et al., 2016; Planaguma et al., 2015). NMDAR-Abs (IgG) from NPSLE patients induce a positive allosteric modulation of the NMDAR, leading to a NMDAR hyperfunction, and subsequent cell death (Chan et al., 2020) (Fig. 1B). Paradoxically, at the behavioural level, NMDAR-Abs from NPSLE patients also impair memory, promote psychotic symptoms and epileptic phenotypes, similarly to the antibody pathogenicity observed in anti-NMDAR encephalitis (Chan et al., 2020; Kowal et al., 2006). NMDAR-Abs from healthy individuals lack most of the molecular pathogenicity reported in anti-NMDAR encephalitis, NPSLE or any other NMDAR-autoimmune disorder (Jezequel et al., 2018). In addition, NMDAR-Abs from an autism spectrum disorder patient also failed to alter NMDAR synaptic trafficking (Grea et al., 2017). Still, the diversity of NMDAR-Abs pathogenicity goes even further. The production and isolation of monoclonal NMDAR-Abs from encephalitic patients unveiled that affinity is heterogeneous, with 2-order of magnitude difference in binding constants between monoclonal autoantibody groups (Kreye et al., 2016; Ly et al., 2018; Wenke et al., 2019). Furthermore, several distinct epitopes may be targeted by different NMDAR-Abs. The NMDAR-Abs of encephalitic patients were initially proposed to target the specific N368/G369 region in the ATD domain of GluN1 subunit (Gleichman et al., 2012; Gresa-Arribas et al., 2014) (Fig. 1C). Immunization of mice with a short GluN1 peptide around the N368/G369 produce NMDAR-Abs and cognitive deficits associated to rodent models of anti-NMDAR encephalitis (Wagnon et al., 2020). However, this site appears to be recognised by only half of patient NMDAR-Abs (Castillo-Gomez et al., 2016; Gresa-Arribas et al., 2014), strongly suggesting the presence of additional epitopes. In this line, an extracellular peptide sequence (RNPSDK) located on the S2 domain of the GluN1 subunit have been proposed to be targeted by a monoclonal NMDAR-Abs from anti-NMDAR encephalitis (Amrutkar et al., 2012). Using competition assays, it has also been shown that there is no epitope overlap on native NMDAR expressed in live neurons, between NMDAR-Abs isolated from patients with anti-NMDAR encephalitis versus psychotic disorder (Jezequel et al., 2017a). For NPSLE, the epitope targeted by NMDAR-Abs is the D/E,W, D/E,Y,S/G motif (DWEYS for short) located at the cleft of the amino-terminal domain (ATD) clamshells of GluN2A (sequence DWDYS) and GluN2B (sequence EWDYG) subunit (Faust et al., 2010), although only GluN2A-NMDARs appear to be functionally affected by antibody binding (Chan et al., 2020) (Fig. 1C). It could be mentioned here that antibodies raised against the extracellular GluN1 subunit region (amino acids 654–800; contributes to the glycine binding domain) were found to be seizure-protective and neuroprotective against excitotoxic challenges in rodent models (Young, 2020). Finally, antibodies raised against the extracellular GluN1 subunit region (amino acids 168–187) do not produce brain cell and behavioural alterations (Wagnon et al., 2020). Altogether, it is evident that NMDAR-Abs are significantly heterogeneous in their pathogenic (e.g. anti-NMDAR encephalitis, autoimmune psychosis, NPSLE), protective, or neutral (e.g. healthy individuals) capacity and binding properties, supporting the view that anti-NMDAR antibody diversity is accompanied by a corresponding variety in their mechanisms of action.

3. NMDAR-Ab-induced NMDAR signalling deficit: through which cellular pathway?

The synaptic signalling through the NMDAR population can be altered by changes in the ionotropic function of the receptor or its trafficking to/from the synapse. The development of single nanoparticle tracking unveiled that the synaptic NMDAR pool is dynamic and that NMDARs laterally exchange between synaptic and extrasynaptic compartments. Within the postsynaptic compartment, NMDARs are stabilized and anchored through protein-protein interactions; for instance, by the interaction with EphB2Rs due to negative charge on GluN1

extracellular domain and positive charge on EphB2R (Fig. 2A) (Washburn et al., 2020). In anti-NMDAR encephalitis, autoantibodies target the GluN1 subunit in surface NMDARs, causing a selective and reversible decrease in receptor surface density and synaptic localization (Dalmau et al., 2008; Hughes et al., 2010; Mikasova et al., 2012). The mechanism of this surface loss requires fully intact IgG and is observed in presence of an NMDAR antagonist (Hughes et al., 2010). Using a single nanoparticle tracking approach in hippocampal neurons, it was further shown that NMDAR-Abs, from encephalitic patients, impact the surface dynamics of synaptic and extrasynaptic NMDARs in opposing directions (Mikasova et al., 2012). For synaptic NMDARs, NMDAR-Abs destabilize NMDARs

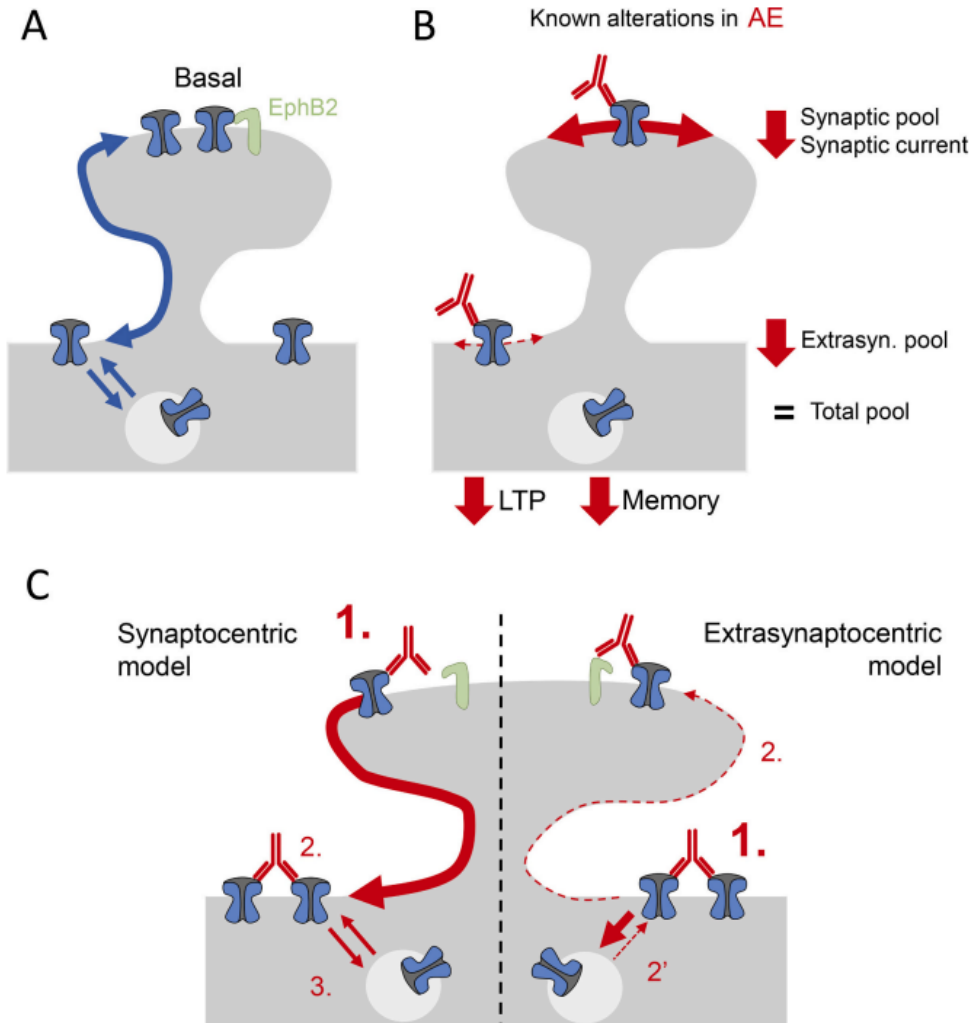


Fig. 2. Facts and models for the molecular mechanism behind NMDAR signalling in health and disease. A) In basal conditions, NMDARs are linked to anchor proteins, such as EphB2. The pool of NMDARs is dynamic, displaying surface trafficking between synaptic and extrasynaptic compartment, providing an equilibrium between the two pools of NMDARs. NMDARs go through endocytosis and exocytosis. B) The facts known in pathological conditions with NMDAR-Ab from AE patients are represented in this figure. It starts with the observation of a massive loss of synaptic and extrasynaptic NMDAR, while the intracellular pool remains the same. The surface trafficking of NMDAR is altered: NMDARs are more dynamic at the synapse and less dynamic at the extrasynaptic compartment where crosslinking events of extrasynaptic NMDARs leads to receptor internalisation. At the synapse, a disruption between NMDAR and EphB2 interaction is observed. These facts lead to global NMDAR hypofunction with decrease of synaptic NMDAR current and LTP as well as impact on the behaviour in rodent such as memory impairment. C) Two models can be built to explain these facts. If NMDAR-Ab can massively target the synapse, the key point (1) in NMDAR-Ab mechanism of action in the synaptocentric model is the disruption of interaction between NMDAR and EphB2. NMDAR is no longer anchored to the synapse, leading to its delocalization in the extrasynaptic part where it's crosslinked (2) and internalized (3). In the extrasynaptocentric model, it is considered that NMDAR-Ab mainly targets extrasynaptic NMDAR due to a better access than within the synaptic cleft. The key point is the crosslinking of extrasynaptic NMDAR by NMDAR-Ab (1). To refill the extrasynaptic pool, there is a massive NMDAR surface trafficking of NMDAR from the synapse to the extrasynaptic compartment (2). Then, the NMDAR that translocates to the extrasynaptic compartment is trapped and internalized (3). NMDAR-EphB2 disruption still occurs but its effect is minimal compared to the synaptocentric model.

by preventing their association with EphB2 receptor, increasing their lateral diffusion and dispersion towards the extrasynaptic compartment. Whereas, for extrasynaptic NMDARs, diffusion was massively reduced in the presence of patient IgGs, consistent with previously described antibody-induced cross linking of surface receptors (Fig. 2B) (Dupuis et al., 2014; Groc et al., 2008; Heine et al., 2008).

In anti-NMDAR encephalitis, it has been proposed that NMDAR synaptic hypofunction is primarily due to a deficit in anchoring of the receptor at postsynaptic sites (Hughes et al., 2010; Mikasova et al., 2012). Indeed, it has been observed that synaptic expression of NMDARs decreases in the presence of NMDAR-Abs, and that this process is associated with the disruption of the NMDAR-EphB2R interaction, an increase of synaptic NMDAR dynamics and altered nanoscale organization within the synapse. A similar phenotype has been reported with NMDAR-Abs from patients diagnosed with psychotic disorders (Jezequel et al., 2017a). In mice infused with CSF from patients with anti-NMDAR encephalitis, administration of ephrin-B2 (ligand of EphB2R) prevents the pathogenic effect of NMDAR-Abs (Planaguma et al., 2016). A positive allosteric modulator of the NMDAR has also been shown to prevent the pathogenic effect of NMDAR-Abs on the synaptic NMDAR content in rodents (Mannara et al., 2020). Furthermore, rodents immunized with NMDAR extracellular domain or amino acid sequences consistently develop NMDAR-dependent cellular and behavioural dysfunctions (Jones et al., 2019; Wagnon et al., 2020). The conventional approach, where focus is dedicated principally to the synaptic component of antibody action, has thus developed an expectantly "synaptocentric" model of the disease (Fig. 2C). However, such a deficit in synaptic NMDAR expression can also feasibly originate from a primarily extrasynaptic antibody action. Indeed, NMDARs traffic through the extrasynaptic compartment to reach the synapse and maintain a stable pool of synaptic receptors. Hence, a robust deficit in NMDAR trafficking outside the synapse would inevitably decrease the synaptic pool over time and also lead to a hypofunction of synaptic NMDAR transmission. Given that NMDAR-Abs demonstrate NMDAR cross-linking competence at extrasynaptic sites, the possibility that antibody-induced synaptic hypofunction instead originates from the disturbance at the extrasynaptic compartment should not be discarded. While not necessarily the exclusive mechanism of action, this hypothesis would constitute an alternative "extrasynaptocentric" model of pathogenesis (Fig. 2C). Compellingly, considering that the synaptic cleft is close in size to NMDAR-Abs (around 20 nm and 12 nm, respectively) and the substantially enriched molecular density, one could assume that NMDAR-Abs can more efficiently target extrasynaptic NMDARs, as compared to the synaptic population. This is further supported by studies showing that different monoclonal NMDAR-Abs can target preferentially extrasynaptic or presynaptic NMDARs rather than postsynaptic ones (Sharma et al., 2018; Wagner et al., 2020). In this scenario, NMDAR-Abs will primarily cross-link extrasynaptic NMDARs, leading to a deficit in diffusion and synaptic incorporation, which by extension reduces the pool of synaptic NMDARs. Some NMDAR-Abs may also penetrate the synapse and destabilize the synaptic pool directly, but in this model such action would represent a simple a confounding effect, insufficient by itself of causing the behavioural pathology seen in encephalitic patients (Fig. 2C). Importantly, the interest to localise predominant antibody action to either synaptic or non-synaptic sites, is not a trivial one. Since NMDAR synaptic hypofunction constitutes the core etiology in major psychotic disorders, understanding the precise locus of NMDAR disorganization is of major translational interest. Following the synaptocentric dogma, the optimal therapeutic design strategy will consist of stabilizing NMDARs, favouring their retention at the synapse. However, in the exact contrary direction, the extrasynaptocentric model would promote the concept of NMDAR destabilisation as a therapeutic route, to negate the influence of crosslinking and favour their trafficking towards synaptic compartments. Ultimately, two molecular hypotheses suggesting two opposing therapeutic solutions. As such, defining the locus of origin for antibody-induced synaptic deficit is of prime importance for

the future development of molecular interventions for neuropsychiatric conditions. Similarly, comparing the effect of NMDAR-Abs from patients with anti-NMDAR encephalitis or psychotic disorders provide further insights to the heterogeneity of antibody actions. As in encephalitis, NMDAR-Abs from patients with psychotic disorders also destabilize the synaptic NMDAR content, through a disruption of EphB2R interactions (Jezequel et al., 2017a). While the psychiatric component of anti-NMDAR encephalitis bears a striking resemblance to that of other psychiatric disorders, the additional symptomology of encephalitis, namely cognitive deficits, dyskinesia and seizure phenotypes, may be explained by differences in the principal subcellular site of antibody action. Conversely to encephalitic autoantibodies, NMDAR-Abs from patients with psychosis do not alter the extrasynaptic NMDAR content (Jezequel et al., 2017a, 2017b), further demonstrating that a refined focus on the extrasynaptic compartment could yield some important insights on the pathogenesis of NMDAR-Abs from different brain disorders. It is conceivable that the main mechanism of action of NMDAR-Abs, at least in the case of anti-NMDAR encephalitis, deviate from the currently accepted model of this disease, and instead follow this novel extrasynaptocentric model.

4. Cellular targets of NMDA-Abs

To date, the majority of antibody pathogenesis has been hypothesised solely on pyramidal cell populations. However, the inhibitory interneuron networks remain an underexploited area of investigation that we expect to be strikingly relevant to the disease mechanisms of NMDAR-Abs action. Although the possibility that interneuron dysfunction may contribute to NMDAR-Abs' pathogenesis has been proposed (Masdeu et al., 2016), direct experimental investigations into this possibility are simply lacking. The interneuron population represents a relative minority in the hippocampus, yet the local inhibitory drive provided by this network forms a major contribution to the larger scale circuit behaviour of hippocampal networks and beyond (Pelkey et al., 2017). Particularly, hippocampal interneurons have been implicated in both psychotic disorder, where their ability to successfully regulate network oscillatory behaviour is compromised (Heckers and Konradi, 2015; Marin, 2012) and in epilepsy, where a loss of inhibitory drive renders hippocampal networks susceptible to hyper-activation states and seizure generation (Marx et al., 2013). These findings, combined with our understanding of anti-NMDAR encephalitis and NPSLE presenting with major symptoms of psychosis and seizure, suggest that a substantial contribution to disease pathogenesis, may originate with a perturbation of hippocampal interneuron function. Recent investigations in the field of psychotic disorders have suggested that NMDAR hypofunction, specifically on interneuron populations, may be a key driver of psychosis phenotypes. Certain NMDAR antagonists are long-since known to acutely replicate poly-symptomatic psychotic disorders in humans, including hallucination, affect flattening and paranoia (Javitt, 2004; Nakazawa et al., 2017). Beyond this behavioural homology, antagonist administration also reproduces the rise in extracellular glutamate of the medial prefrontal cortex seen in psychosis patients, and increases the activity of cortical principal cells in rodents (Moghaddam and Javitt, 2012). Given that the application of antagonists, instead, promotes excitatory cell action and glutamate release, it is hypothesised that a principal route of psychotic disorders is mediated by a disinhibition across brain structures including the frontal cortex and limbic system. Further research has aimed to identify the source of this disinhibition, after the observation that local interneuron action is not a viable route to antagonist-induced activity increase in cortical structures (Amat-Foraster et al., 2019). However, local disinhibition has been demonstrated in the CA1 subfield of the hippocampus, and to effectively disinhibit the prefrontal cortex through disruption of downstream signalling (Jodo et al., 2005). Applying this reasoning to the study of anti-NMDAR encephalitis, we might speculate that antibody-induced receptor hypofunction on interneurons is a principal mechanism for the

generation of psychotic symptoms observed in the initial presentation of this disorder (Fig. 3). These findings are also congruent with the proposition that autoantibody actions are specific on hippocampal and limbic regions in encephalitic patients, albeit through an as yet unidentified mechanism (Dalmou et al., 2008). Conversely, the positive allosteric modulation of GluN2A-NMDARs by NMDAR-Abs in NPSLE, generates a similar phenotype, despite the directly opposing functional consequence of antibody binding (Chan et al., 2020). In this case, we expect that the pathogenesis is mediated by direct hyperactivation of the GluN2A-enriched principal cell populations (Fig. 3). These divergent autoimmune diseases, with paradoxically similar phenotypic disturbance, only demonstrate the need to further elucidate and expand our current models of anti-NMDAR antibody action, both at cellular and circuit levels.

4.1. Cell intrinsic properties of hippocampal interneurons

Parvalbumin expressing (PV+) interneurons exhibit local control over hippocampal excitation through a rapid and repetitive firing pattern. This interneuron population has gained significant traction in the field of psychosis, as this uniquely fast-spiking phenotype is known to exert control over high frequency gamma oscillations, that are also disrupted in psychotic patient electroencephalographs (Marin, 2012). This suggests PV+ interneurons as a good candidate for the disinhibition model of psychosis in anti-NMDAR encephalitis. However, the question remains how encephalitic NMDAR-Abs, which presumably bind indiscriminately to NMDARs may demonstrate a targeted effect on interneurons, without a brain-wide activity depression by an equivalent effect on principal cells. One possibility is that the NMDAR itself serves a different functional purpose when expressed in interneurons or principal cells. Notably, interneuron populations demonstrate more depolarised resting membrane potentials than the surrounding pyramidal cell populations (Lacaille et al., 1987), and it has been further elucidated that the PV+ interneuron population is among the most depolarised of all cell types in the hippocampal structure, usually remaining around -55 mV (Tricoire et al., 2011). Despite this more depolarised resting potential, all interneuron families have an approximately equivalent threshold for action potential generation as pyramidal cells, at around -35 mV (Tricoire et al., 2011), setting apart the PV+ interneuron subtype as they normally rest relatively close to spike threshold. It has been shown that PV+ interneurons display a significant enrichment of the magnesium-insensitive GluN2D-containing NMDAR (Akgul and McBain, 2016). The expression of this receptor subtype is known to contribute to resting membrane potential in early developmental stages in the cortex (Hanson et al., 2019). It is possible that a similar tonic current on PV+ interneurons, may represent a unique pathological avenue for encephalitic disease, whereby the internalisation of NMDARs on interneurons drives down the resting potential away from threshold, ablating the fast-spiking phenotype and inhibitory drive. This remains to be examined in the context of exposure to encephalitic NMDAR-Abs, however it has encouragingly been demonstrated that genetic ablation of NMDARs on PV+ fast-spiking interneurons does significantly disrupt the characteristic firing pattern of this interneuron family (Carlen et al., 2012). This differential subunit enrichment on interneurons is also consistent with the opposing action of NMDAR-Abs in NPSLE. These antibodies bind both GluN2A and GluN2B subunits, however the increased open probability via positive allosteric modulation is selective for the GluN2A-containing receptor (Chan et al., 2020). While GluN2A-NMDARs are also expressed on hippocampal interneurons, the expression is significantly lower as compared to that on principal cells in the hippocampal structure (Akgul and McBain, 2016). Hence, the relative reduction of GluN2A subunit expression on interneuron populations renders inhibitory activity unchanged in response to antibody exposure in NPSLE. However, the high GluN2A-NMDAR expression at pyramidal cell synapses is likely to shift the overall network behaviour in favour of direct hyperexcitation (Fig. 3). This is certainly interesting considering that the

phenotype of NPSLE is similar to that observed in anti-NMDAR encephalitis. Demonstrating that even directly opposing action of antibody pathogenesis, hypo- versus hyper-function, can result in a comparable phenotypic outcome through discrete mechanisms, further highlighting the necessity to characterise the possible effects on NMDAR function on both excitatory principal cells and the concurrent balancing effect on local inhibitory networks.

4.2. Differential glutamatergic neurotransmission on hippocampal interneurons

Alongside the variable functional roles of NMDARs expressed on principal and interneuron cell types, glutamatergic transmission is itself mediated differently between these neural populations (Pelkey et al., 2017). Interneurons in the CA1 area of the hippocampus which receive Schaffer collateral inputs, maintain a GluN2B-NMDAR predominance at the synapse across all stages of development, differing from the pyramidal cell population which upregulates synaptic incorporation of GluN2A-NMDARs after early postnatal development (Matta et al., 2013). Interestingly, the relative contributions of NMDA and AMPA receptor populations at these synapses is approximately equal, again differing from the pyramidal cell, where the AMPA receptor number greatly exceeds the NMDAR population (Matta et al., 2013). Taken together, the maintenance of the GluN2B-NMDAR, with an endogenously increased current transfer and prolonged decay constant when compared to GluN2A-NMDARs, and higher ratio of NMDA-to-AMPA expression at interneuron synapses, indicates an increased reliance on NMDAR pools for hippocampal interneurons. It is therefore possible that a homogenous action of NMDAR-Abs in encephalitis, might induce a greater disruption to synaptic (tonic) function on interneurons over principal cells (Fig. 3). Considering this, in addition to the increased vulnerability of interneurons by the presence of tonic NMDAR currents, yields a significantly heightened susceptibility to perturbation by NMDAR-Abs. Genetic ablation of GluN2B subunits from interneurons in the CA1 subfield of the hippocampus causes a localised decrease in AMPA-mediated miniature excitatory postsynaptic currents in interneuron populations in the *stratum oriens* (Kelsch et al., 2014). It was further demonstrated that this loss of excitatory drive onto inhibitory networks, not only developed into a local disinhibition of the CA1, but induced epileptiform activity (Kelsch et al., 2014). Given this specific role of GluN2B-NMDARs on the hippocampal interneuron population, we might consider a route to seizure pathology in anti-NMDAR encephalitis, may be mediated by surface loss of GluN2B from interneuron synapses.

Regarding NPSLE, this disparity between the synaptic composition of NMDAR subtypes on principal cells and interneurons again suggests a selective shielding of interneurons from the hyperexcitation elicited by antibody binding; further shifting the overall balance towards hyperexcitation that is mediated by a relatively isolated impact at the pyramidal cell synapse (Fig. 3). We may further consider that this unbalancing of physiological excitatory-inhibitory action will develop into epileptogenic seizure generation in NPSLE, and prevent the correct level of interneuron control over network oscillations caused by the mismatch in cell perturbations which may contribute to the psychotic elements in this disease. However, one must note that this hypothesis is based on knowledge from antibody binding to NMDARs expressed in a heterologous system (Chan et al., 2020). It is therefore possible that while antibody binding to GluN2B-NMDARs does not induce any functional consequence to the channel, alterations to surface expression level or surface diffusion may well introduce a possible impact on this receptor population. This point is recapitulated by our knowledge that NMDAR-Abs in encephalitis do not produce a direct modulation of channel function, but instead elicit their effect via indirect perturbations to channel internalisation and altered stabilisation states in the cell membrane (Jezequel et al., 2018).

Ultimately, there is a current challenge posed by directly opposing

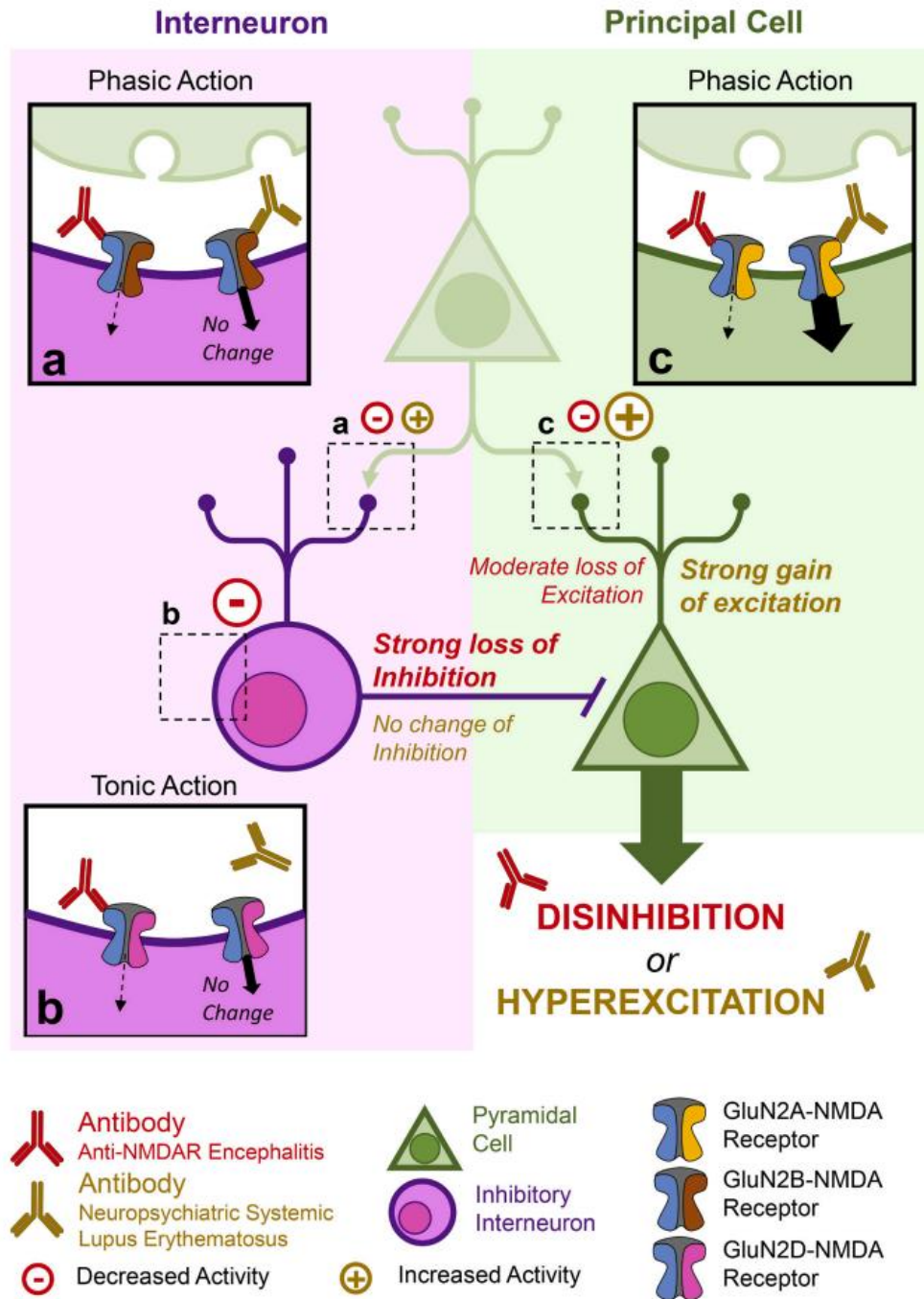


Fig. 3. Schematic model of network disturbance by discrete NMDA receptor autoantibodies. The centre panel denotes a simple schematic network comprised of two pyramidal cells (green) and an interneuron (purple); the left on this panel demonstrates a model of disinhibition hypothesised to underpin network dysfunction in anti-NMDAR encephalitic patients (AE, red), the right describing a model of hyperexcitation in lupus patients (NPSLE, blue). The phasic action on interneuron synapses is shown in (A), where AE antibodies (red) induce a GluN2B-NMDA receptor hypofunction and lupus antibodies (blue) bind but elicit no change in these receptors. B) The impact of antibody exposure on the tonic current of interneurons, where AE antibodies again induce further hypofunction of the GluN2D-NMDARs but lupus antibodies show no action or binding on this receptor subtype. C) The action at a glutamate synapse onto a pyramidal cell dendrite enriched in GluN2A-NMDAR expression, where the action of antibodies from AE induce a synaptic hypofunction; and NPSLE antibodies promote an opposing hyperfunction of synaptic NMDAR signalling. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

actions of autoantibodies in anti-NMDAR encephalitis and NPSLE, whereby the respective receptor hypofunction and hyperfunction generates a paradoxically similar phenotype between these two conditions. We would argue that the inclusion of inhibitory interneuron networks into the current disease models, not only forms a more comprehensive hypothetical model for NMDAR-Ab action, but is required to address this current challenge. In this case, exposure to NMDAR-Abs in encephalitis will result in divergent degrees of reduction in NMDAR signalling and perturbation of both principal cell and inhibitory interneuron action. Such a mismatch in the functional output between principal and interneuron networks will cause a synergistic disruption to the homeostatic excitatory-inhibitory balance. This reframing of encephalitic antibody action suggests that we should consider disinhibition as a major possible component to pathology of anti-NMDAR encephalitis, whereby relative network hyperfunction is a resulting feature of targeted - or dominant - NMDAR hypofunction on inhibitory interneuron networks. Such a hypothesis is in line with the observed increased action of cortical principal cells of rodent models of psychosis (Moghaddam and Javitt, 2012). Moreover, this disinhibition hypothesis of encephalitis is also more intuitively consistent with the proposed action of NMDAR-Abs in NPSLE. Here, we propose that antibodies act directly and exclusively on principal cell populations to drive a hyperexcitation and a similar unbalancing of the excitatory inhibitory drive as observed in encephalitis. Hence, while these NMDAR-Abs act through two distinct routes, we propose that the overall network perturbation and altered ratio of excitatory and inhibitory action is the driving factor for the observed phenotypic similarity of these two conditions.

5. Do NMDAR-Ab similarly hit different brain structures?

Since the identification of anti-NMDAR encephalitis, it has been noted that the binding of patient NMDAR-Abs appears substantially enriched in hippocampal and limbic structures as compared to cortical regions (Dalmau et al., 2017). This apparent hippocampal targeting has been corroborated by the observation that encephalitic patients display a marked reduction in NMDAR expression in hippocampus and memory impairments present as a primary symptom of disease in many cases (Dalmau et al., 2017). It has also been shown that hippocampal structures display a significant reduction in volume as a result of encephalitic disease and that such a reduction in volume and structural integrity across hippocampal subfields was predictive of the degree to memory impairment, cognitive deficit and disease severity in these patients (Finke et al., 2016). As such, it has remained an open question to validate the supposed targeting of patient autoantibodies to hippocampal NMDAR populations and to further elucidate a mechanism by which such a specificity in binding may arise. As previously discussed, GluN2 subunit expression is regionally and temporally controlled, such that principal cell expression of GluN2A and GluN2B forms the major fraction of channels throughout the brain, while the relative ratio is altered during an early postnatal switch, and potentially into adulthood (Paoletti et al., 2013). The less numerous interneuron populations are enriched in GluN2D-NMDARs and many show a reduction in expression of GluN2A as compared to the principal cell population (Matta et al., 2013; Perszyk et al., 2016). GluN2C expression is confined mostly to cerebellar structures, however there is also evidence for enriched expression also on interneuron populations throughout the thalamus (Alsaad et al., 2019). While early work on anti-NMDAR encephalitis speculated a possible targeting of GluN2B-NMDARs, such expression profiles do not seem to bare any clear relationship to antibody specificity to the hippocampus. Moreover, the antigenic GluN1 subunit is also known to exist in eight distinct splice variants, determined by two possible deletions to the intracellular domain of the receptor and further by the exclusion or inclusion of a 21 amino acid sequence (N1 cassette) in the ATD, termed GluN1a and GluN1b, respectively (Paoletti et al., 2013). While the functional behaviour of these splice variants and their regional expression at the broad structural level during development has

been well characterised (e.g. (Liu et al., 2019; Sengar et al., 2019), there is comparatively less known regarding the endogenous subcellular organization and neuron subtype-specific expression profiles of these variants (Paoletti et al., 2013). Encouragingly however ho however, it has recently been demonstrated that excitatory cells and interneuron populations within the mouse cortex demonstrate differing levels of exon inclusion, such that interneurons are enriched for GluN1b relative to their principal cell counterparts, this difference was most striking in the PV+ interneuron population (Huntley et al., 2020). We recognise that it is unlikely that variation within the intracellular C-terminal domain will have any effect on antibody action. However, we note that the localisation of the N1 cassette is of particular interest: at the interface between the GluN1-ATD and the GluN1/GluN2B ligand binding domains, and with relative proximity to a proposed epitope within the N368/G369 region of the GluN1 ATD (Gleichman et al., 2012; Regan et al., 2018). The inclusion of this sequence may therefore provide some subtle conformational differences between GluN1a and GluN1b splice variants in combination with GluN2 subunits that will shift binding efficacy for the antibody in favour of particular splice variants that are regionally targeted to hippocampal populations. Additionally, while we often consider channel function to be conferred solely by the inclusion of particular GluN2 subunits, it is important to consider that channel function can also be modulated reciprocally by the GluN1 subunit splice variant present in the channel (Rumbaugh et al., 2000). Such factors may underpin the preferential effect of patient NMDAR-Abs for hippocampal and limbic regions in the brain. In addition to potential differences in subunit and splice variant expression, differing mechanisms for receptor turnover, internalisation and anchoring in hippocampal neurons may represent further mechanisms for the observed impact on hippocampal populations, but these remain to be further explored. While the imaging studies from encephalitic patients showing hippocampal degeneration is compelling for the hippocampal selectivity of NMDAR-Abs, it is however worth considering that reduced hippocampal volumes are not uncommon in epileptic pathologies where the hippocampus is expected to be the source of seizure generation (Finke et al., 2016). We might argue that antibody function across the brain will perturb local networks and neuronal functioning, while the microarchitecture of the hippocampus – being rich in inhibitory interneurons and recurrent CA3 microcircuits – yields a propensity for sustained hyper-activation and epileptogenesis. In this case, the underlying cause of specific loss of hippocampal volumes may not be indicative of antibody targeting to hippocampal neurons, but simply that the result of antibody action in this region is such a significant disruption to homeostasis that the increase in activation becomes neurotoxic. Depletion of hippocampal volumes in this manner would sequentially explain the memory deficits observed in patients.

A further mechanism for antibody selectivity for NMDAR populations in limbic structures may reside in posttranslational modifications of the subunit proteins in these regions, however this remains to be explored. Typically, such modifications include receptor phosphorylation, ubiquitination and palmitoylation. Phosphorylation is known to occur extensively on the long intracellular tail of the GluN2B subunit, and such modification is understood to alter receptor currents, expression and diffusion in the membrane (Sanz-Clemente et al., 2013). However, it is difficult to conceptualise how such a modification, which is by nature intracellular, may impact antibody binding at the extracellular site. However, we might consider that antibody binding may work synergistically with phosphorylation processes that play a role in the lateral mobilisation of receptors and downstream internalisation mechanics. Similarly, ubiquitination as another intracellular modification that is important for protein degradation, may not have any substantive influence over antibody action in the internalisation of receptors, however it is tempting to postulate that there may exist some differences on the degradative and receptor turnover processes that make hippocampal neuron populations intrinsically less able to compensate for antibody-mediated receptor internalisation and surface

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loss. Again this would suggest however, that antibody action is not hippocampal selective per se, but instead that processes downstream of antibody action are the basis for the apparent perturbation of limbic structures. Differential glycosylation of the GluN1 extracellular domain between brain regions may constitute an additional possibility, although evidence is still lacking. Finally, the extracellular space of the brain parenchyma is a complex, tortuous and heterogeneous structure (Nicholson and Hrabetova, 2017). Since it has been shown that the diffusion of immunoglobulins within the extracellular space greatly varies between brain areas (Pizzo et al., 2018), one may propose that limbic structures favour immunoglobulin retention due to extracellular space properties. In-depth investigations, at the single immunoglobulin level, are surely needed to tackle this question.

6. Conclusions and perspectives

The link between autoantibodies directed against neurotransmitter receptors and major brain disorders is emerging as a frontrunner in the field of neurology and psychiatry. The example of NMDAR-Abs is of particular interest as the pathogeny of the autoantibody has been well-established in anti-NMDAR encephalitis. The NMDAR-Abs impair NMDAR synaptic signalling, long-term synaptic plasticity, network activity, and associative memory. Yet, fundamental challenges remain in front of us. Defining the primary locus of action of the NMDAR-Abs, which would require us to identify whether synaptic or extrasynaptic NMDARs are the main disrupted pool, is still an open question that is often discarded. The number of B cell clones (expressing NMDAR-Abs) in each patient, the epitope(s) of the autoantibodies, their molecular pathogenicity, similarly remain exciting open questions. As most of the studies have employed the use of rodent biological substrate to unravel the mechanisms of human-generated NMDAR-Abs, exploring these questions using human-derived cells or neural tissue would also be of prime importance. Finally, when we consider the diverse array of mechanisms by which interneurons provide a local control of hippocampal network function, and the striking variety of functional roles for NMDAR populations expressed on these neurons, it is surprising that still relatively little is known about the possible contribution of different hippocampal cell types, such as interneurons, to anti-NMDAR antibody mediated pathologies. Particularly, given that it has long since been understood that interneuron dysfunction represents a significant contribution to both psychiatric and epileptic disorders with considerable symptom overlap with anti-NMDAR encephalitis.

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Synaptic and network dysfunctions in models of autoimmune encephalitis

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Abstract

Seizure activity and cognitive deficits represent a prominent phenotype associated with variable forms of autoimmune encephalitis, regardless of the epitope targeted by autoantibodies. The mechanistic underpinnings of these shared major neuropsychiatric symptoms remain however unclear. Here, we investigated the impacts of patient-derived monoclonal autoantibodies against the glutamatergic NMDA- (NMDAR-mAb) and inhibitory GABA_AR (GABA_AR-mAb) receptors in hippocampal network. Both NMDAR and GABA_AR-mAbs reduced excitatory and inhibitory synaptic receptor content and transmissions through altered receptor membrane diffusion. The cross-effect of NMDAR-mAb on inhibitory synapses and GABA_AR-mAb on excitatory synapses requires neuronal activity and protein kinase signalling. Functionally, both autoantibodies increase the excitation/inhibition balance of principal cell inputs. Furthermore, NMDAR- or GABA_AR-mAb leads to a convergent hyperactivation of hippocampal networks through distinct alterations of principal cell and interneuron properties. Thus, autoantibodies targeting excitatory or inhibitory receptor can produce similar network dysfunctions through a combination of shared and distinct mechanisms.

Converging synaptic and network dysfunctions in distinct autoimmune encephalitis

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SUMMARY

Seizure activity and cognitive deficits represent a prominent phenotype associated with variable forms of autoimmune encephalitis, regardless of the neurotransmitter receptor targeted by autoantibodies. The mechanistic underpinnings of these shared major neuropsychiatric symptoms remain however unclear. Here, we investigated the impacts of patient-derived monoclonal autoantibodies against the glutamatergic NMDA- (NMDAR-mAb) and inhibitory GABA_AR (GABA_AR-mAb) receptors in hippocampal network. Strikingly, NMDAR or GABA_AR-mAbs reduced both excitatory and inhibitory synaptic receptor content and transmissions through altered membrane diffusion. The cross-effect of NMDAR-mAb on inhibitory synapses and GABA_AR-mAb on excitatory synapses requires neuronal activity and involves protein kinase signalling. Functionally, both autoantibodies increase the excitation/inhibition balance of principal cell inputs. Furthermore, NMDAR- or GABA_AR-mAb leads to a hyperactivation of hippocampal networks through distinct alterations of principal cell and interneuron properties. Thus, autoantibodies targeting excitatory or inhibitory neurotransmitter receptor trigger convergent network dysfunctions through a combination of shared and distinct mechanisms.

INTRODUCTION

Recent years have seen the identification of an array of autoimmune neuropsychiatric disorders with patients expressing autoantibodies directed against membrane proteins. Two prominent diseases in this category are anti-NMDAR encephalitis and anti-GABA_AR encephalitis, in which patients develop antibodies directed against major excitatory or inhibitory ionotropic channels, respectively.¹⁻³ Most of the molecular investigations have delineated an acute pathway of action of these autoantibodies, focusing on the isolated impacts on the target antigens.⁴ Anti-NMDAR autoantibodies (NMDAR-Abs) bind to the extracellular N-terminal domain of the obligatory GluN1 subunit.^{5,6} Antibody binding causes a synaptic destabilisation of the NMDARs through altered interaction with the EphB2 receptor.^{7,8} This destabilisation causes an increase in the surface dynamics of synaptic NMDARs at acute timescales, promoting displacement to extrasynaptic sites.^{7,9} The overall amount of cell-surface NMDARs decrease, possibly through a promoted internalisation, resulting in an excitatory hypofunction on hippocampal neurons.^{6,7,9} For anti-GABA_AR autoantibodies (GABA_AR-Abs), some can elicit a direct antagonism of the inhibitory ion channel whereas others are without antagonistic action.¹⁰⁻¹² Direct antagonism of GABA_AR channels has been demonstrated to occur through competition by GABA_AR-Abs for GABA binding sites at the interface between α and β subunits, and also through negative allosteric modulation of the channel.¹¹ Such antagonism drives a hypofunction of the major inhibitory neurotransmission system. The effect of GABA_AR-Abs on the receptor surface organisation and diffusion has however not been examined. Thus, NMDAR-Abs and GABA_AR-Abs drive either excitatory or inhibitory hypofunction, respectively, in hippocampal networks.

Despite the obvious opposition in the function of these autoantibody targets, seizure phenotypes and cognitive deficits form a prominent facet of a shared symptomology in these two autoimmune diseases.^{3,10,13-17} In the case of GABA_AR-encephalitis, antagonism of inhibitory GABAergic transmission would intuitively lead to epileptogenesis, where the loss of inhibitory control results in over-excitation in brain regions that are dense in recurrent microcircuits such as the temporal lobe. It is not surprising then that approximately 80% of patients in this cohort present with severe seizure phenotypes, elevating this to the predominant disease feature of GABA_AR encephalitis.^{3,10,13-15} The mechanisms by which NMDAR-Abs induce excitatory hypofunction at the molecular scale result in a similar phenotype remains still enigmatic. Yet, seizures are observed in approximately 80% of cases, forming a considerable segment of clinical presentation along with psychiatric dysfunction (90%) and movement disorder (78%).¹³ It has been suggested that NMDAR-Abs may display a degree of selectivity in pathogenesis whereby inhibitory interneuron populations are primarily impaired.¹⁸ Some experimental evidence has begun to emerge in support of this hypothesis, suggesting a disinhibitory action of NMDAR-Abs in cortical networks that lead to hyperexcitation and network hypersynchrony based on disrupted excitatory-inhibitory balance and neuronal excitability.^{17,19,20} These findings are particularly intriguing considering the proposed role of interneuron-

specific hypofunction in other psychiatric conditions such as schizophrenia and bipolar disorder, which bare resemblance to early stages of NMDAR-encephalitis.²¹⁻²³ Seizure represents the clinical endpoint of significant perturbation to excitatory-inhibitory (E/I) balance and network homeostasis. The maintenance of physiological E/I balance in the healthy brain is supported by a complex interplay between many cell autonomous and network-level systems, including modulations of synaptic plasticity, scaling, synaptic density and intrinsic cellular excitability.^{24,25} Excitatory and inhibitory systems exert mutual influence over each other in order to reach a physiological equilibrium. This can be achieved through intracellular signalling cascades, for example the bidirectional linking of NMDAR-mediated calcium influx to local GABA_AR mobility and reorganisation.²⁶ Additionally, common interacting proteins such as type I and II dopamine receptors, which bind NMDA- and GABA_A receptors, respectively, can act as centralised orchestrators of excitatory and inhibitory signalling.²⁷⁻³¹

Given the array of homeostatic systems in place, each dependent on effective cross-talk between excitatory and inhibitory signalling, the mechanistic underpinnings of autoimmune encephalitis may reside in pathological co-regulation of excitatory and inhibitory receptors and transmissions. Yet, the characterisation of this cross-synaptic disruption remains to be determined. Here, we intended to tackle the issue on how autoantibodies that induce glutamatergic hypofunction or GABAergic hypofunction produce a similar set of symptoms in patients and behavioural deficits in rodents. Specifically, we investigated how patient-derived monoclonal autoantibodies against the excitatory NMDAR (NMDAR-mAb) and inhibitory GABA_AR (GABA_AR-mAb) (Figure 1A) impact excitatory and inhibitory drive and cell properties in the hippocampus, spanning from single molecule to neuronal network exploration.

RESULTS

Patient autoantibodies similarly alter NMDAR and GABA_AR membrane diffusion

Although NMDAR-Abs alter the membrane distribution and diffusion of NMDARs,^{7,32} their long-term impact on other neurotransmitter receptors are less clear. Regarding GABA_AR-Abs, their impact on GABA_ARs and other receptor surface dynamics has not been investigated. To shed light on this critical aspect, we performed single Quantum Dot (QD) tracking experiment to define the membrane diffusion of GluN1-containing NMDARs and $\gamma 2$ subunit-containing GABA_ARs in cultured hippocampal neurons. Synaptic areas were defined by expressing homer 1c-dsRed to locate glutamatergic synapses or gephyrin-mCherry to locate GABAergic synapses (Figure 1B-G). We then exposed neuronal networks to control, NMDAR- or GABA_AR-mAb (0.5 $\mu\text{g}/\text{ml}$) for 24 hours before tracking membrane NMDAR and GABA_AR. Noteworthy, NMDAR-mAb treatment did not alter the glial and microglia coverage in the hippocampus, as measured by the immunostaining of GFAP- and Iba1-positive cells (Figure S1). As expected, NMDAR-mAbs alter the membrane diffusion of synaptic NMDARs (Figure 1H) by inducing a decrease in the mean square displacement (MSD) curve, i.e. trajectories are more confined, and a significant decrease in the diffusion coefficient (Figure 1I-J). This indicates that a prolonged exposure to patient autoantibodies reduce the diffusion of the few remaining synaptic NMDARs. Surprisingly, analysis of GABA_AR surface diffusion also revealed that NMDAR-mAbs significantly alter GABA_AR surface trafficking at inhibitory synapses (Figure 1K). In this case, we observed an overall increase in the surface diffusion of GABA_ARs, reflected by a left shift in the MSD curve, i.e. trajectories are less confined, and a significant increase in the diffusion coefficient (Figure 1L-M). This indicates a cross-synaptic alteration in the trafficking of both NMDARs and GABA_ARs in the context of anti-NMDAR autoimmune encephalitis. Then, we investigated the putative effect of GABA_AR-mAbs on receptor membrane diffusion. After exposure of neuronal networks to GABA_AR-mAbs, surface GABA_AR were more diffusive within inhibitory synaptic compartments (Figure 1K). Specifically, GABA_AR-mAbs induced a left shift in the MSD curve, i.e. trajectories are less confined, and a significant increase in the diffusion coefficient (Figure 1L-M). In addition, synaptic NMDAR diffusion was also altered by GABA_AR mAbs (Figure 1H), as evidenced by inducing a right shift in the MSD curve, i.e. trajectories are more confined, and a significant decrease in the diffusion coefficient (Figure 1I-J). It is striking to note that, not only do both NMDAR- and GABA_AR-mAbs elicit cross-synaptic deficits, but the resulting perturbations in channel dynamics essentially mimic each other. Altogether, these data indicate that NMDAR- and GABA_AR-mAbs alter the membrane diffusion of their target as well as another key receptor, suggesting a major change in both excitatory and inhibitory synapses.

NMDAR- and GABA_AR-mAbs similarly reduce excitatory synaptic content

To characterise the impacts elicited by NMDAR- and GABAaR-mAbs at a macroscopic level, we examined the receptor content at glutamatergic synapses by quantifying the expression and localisation of two glutamatergic receptors: NMDAR and GluA1-containing AMPAR. To achieve staining of surface proteins neurons were transfected with receptor subunits tagged at their extracellular N-termini. Hippocampal neurons were exposed to control, NMDAR or GABAaR mAb for 24 hours prior to imaging at 14 days *in vitro* (DIV). Given the above mutual defect of NMDAR membrane diffusion induced by both pathogenic autoantibodies, one may predict a similar alteration of the receptor synaptic content. Confocal images of dendritic clusters of receptors were analysed to quantify the mean number of receptor clusters per micrometer of dendrite (cluster density), along with the size of identified clusters, expressed relative to Control mAb treated neurons. Exposure to both NMDAR- and GABAaR-mAbs elicited a significant reduction in NMDAR cluster density and size (Figure 2A-C), suggesting that chronic exposure to both patient autoantibodies induce a hypo-content of synaptic NMDARs. Similarly, NMDAR- and GABAaR-mAbs induce a moderate but significant reduction in the size of synaptic AMPAR clusters, although they did not impact the cluster density (Figure 2D-F). Given that NMDAR and AMPAR are stabilised at synapses, at least in part, by association with scaffolding proteins that are embedded within the postsynaptic density, we assessed the possibility that the postsynaptic density is impacted after 24 hours of autoantibody exposure. The cluster density or cluster size of Homer-1c remain unaffected in presence of autoantibodies (Figure 2I-H). Next, we tested whether these alterations in glutamatergic synaptic content are shaped by reduced function of NMDAR, independently of the aberrant surface diffusion induced by autoantibody exposure. To explore this, we exposed neuronal cultures to a partially-blocking concentration (1 μ M) of the NMDAR antagonist APV for 24 hours. This concentration was selected as to mimic the incomplete reduction of NMDAR signalling induced by NMDAR mAb exposure.^{6,7,17} This pharmacological manipulation failed to recapitulate any alteration in NMDAR or AMPAR localisation to glutamatergic synapses (Figure 2G), suggesting that the autoantibodies effects are primarily triggered by alteration in the surface dynamics of membrane proteins.

NMDAR- and GABAaR-mAbs similarly disrupt inhibitory synaptic content and scaffold proteins

Having uncovered substantial dysregulation at excitatory glutamatergic synapses by both encephalitis-derived autoantibodies, we further investigated the content and structuration of inhibitory synaptic compartments. Analysis of GABAaR content at inhibitory synapses demonstrated that both NMDAR- and GABAaR-mAbs decreased the synaptic content of GABAaRs. We report a significant reduction in synaptically-localised GABAaR cluster density, with no alteration in cluster size (Figure 3A-C). Given that synaptic GABAaR diffusion is regulated in part by scaffolding proteins,³³ we investigated the potential for autoantibody-mediated disturbance of the inhibitory synaptic scaffolding protein, gephyrin. In neurons exposed to either NMDAR or GABAaR mAbs for 24 hours, we observed a clear reduction in the density of gephyrin clusters

along dendritic segments (Figure 3D-E) without affecting gephyrin cluster size (Figure S2A). Since gephyrin phosphorylation can tune its synaptic clustering,^{34,35} we explored the phosphorylation status of gephyrin. Specifically, phosphorylation of a serine residue at position 270 (S270) has been demonstrated to induce a reduction in gephyrin cluster densities, through the enhanced recruitment of calpain, a protease that subsequently cleaves the scaffolding protein.³⁴ We exposed neurons to control-, NMDAR- or GABAaR-mAbs for 24 hours prior to immuno-labelling for total gephyrin expression or with a phosphorylation specific antibody, which binds selectively to gephyrin scaffolds that have been phosphorylated at S270 (Figure 3D).³⁶ As expected in control condition, the density of phosphorylated gephyrin clusters was significantly lower compared to total gephyrin (Figure 3F). However, phosphorylated (S270) gephyrin cluster density was similar to total gephyrin cluster levels after exposure to NMDAR-mAbs (93%) or GABAaR-mAbs (99%) (Figure 3F), indicating that both autoantibodies massively increased the phosphorylation of gephyrin. We further characterised the size of total and phosphorylated (S270) gephyrin clusters, reporting that the decrease in the cluster area of phosphorylated versus total gephyrin was also lost after exposure to NMDAR- and GABAaR-mAbs (Figure S2B). Since GABAaR mAbs, unlike their NMDAR-targeting counterparts, directly antagonize the ionotropic transmission of GABAaR channels,¹⁰ we investigated whether 24 h partial blockade with 5 μ M bicuculline – to mimic antibody-induced channel antagonism – was sufficient to elicit receptor and scaffold reorganisation at inhibitory synapses. The GABAaR synaptic content was unaltered (Figure 3G), suggesting that altered surface dynamics and redistribution of GABAaR is not dependent on its ionotropic modulation by autoantibodies. Furthermore, bicuculline failed to alter the density of gephyrin scaffolds (Figure 3G). Taken together, these results demonstrate that NMDAR or GABAaR autoantibodies converge to a similar macroscopic effect, likely instigated by the altered surface diffusion and not the lower ionotropic activity.

NMDAR- and GABAaR-mAbs reduce excitatory and inhibitory synaptic transmission

Based on this *in vitro* evidence that NMDAR- and GABAaR-mAbs reduce both glutamatergic and GABAergic receptor content at synapses, we next investigated the fast transmissions in preserved hippocampal networks. To do this, we used organotypic hippocampal slices to monitor synaptic activity. Both excitatory and inhibitory synaptic currents were revealed using whole-cell patch clamp electrophysiology in a voltage-clamp configuration. Hippocampal slices were exposed to NMDAR- or GABAaR-mAbs autoantibody for 24 h prior to recording. The voltage clamping at -70mV was performed for 10 minutes to uncover AMPAR-mediated spontaneous excitatory postsynaptic currents (sEPSC, Figure 4A-B). After which, cells were clamped at 0mV to reveal GABAaR-mediated spontaneous inhibitory postsynaptic currents (sIPSC, Figure 4E-F). The sEPSC amplitude was significantly reduced after exposure to NMDAR-mAb or GABAaR-mAbs (Figure 4C-D). There was no change in the frequency and either rise or decay kinetics of sEPSC of either pathogenic

antibody (Figure S3A-C). This suggests that the loss of AMPARs from excitatory synaptic compartments is not selective for particular subunit containing channels, instead reflecting an overall decrease in synaptic AMPARs. Quantification of sIPSC amplitudes revealed a similar impact on inhibitory signalling by NMDAR-mAb or GABAaR-mAbs. Compared to control, we observed a significant reduction after exposure to both NMDAR-mAbs and GABAaR-mAb (Figure 4G-H), with a 16% reduction in frequency after exposure to either mAb. However, this trend failed to reach significance and we found no impact on event kinetics (Figure S3E-F). The reduction in GABAergic signalling is expected by GABAaR-mAb exposure since the mechanisms of action of these antibodies is, in part, a direct antagonism of GABAaR channel function. However, the impact of NMDAR-mAbs on GABAergic fast transmission further indicate the effective cross-talk induced by autoantibodies. To compare the E/I balance of the synaptic inputs, we expressed the mean amplitudes of the AMPAR-mediated sEPSCs relative to the mean amplitudes of the GABAaR-mediated sIPSC for each recorded cell. This revealed a significant shift towards hyperexcitation in presence of NMDAR-mAbs (155%) and GABAaR-mAbs (157%) relative to the control-mAb condition (Figure 4I). This demonstrates that although there is a general reduction of synaptic inputs to CA1 pyramidal cells, the deficits in GABAergic transmission are consistently greater in magnitude compared to the impacts on glutamatergic transmission.

The ionotropic transmissions of both NMDA- and GABAaR are not confined solely to synaptic compartments, but also to tonic excitatory and inhibitory currents through extrasynaptically-recruited receptors. We thus measured tonic GABAergic current on CA1 pyramidal neurons after exposure to autoantibody samples. Whole cell patch-clamp recordings were collected in the presence of tetrodotoxin. After a stable baseline, current was recorded for 5 min, bicuculline was added into the extracellular solution to reveal the tonic GABAaR-mediated inhibitory current, apparent as a downward deflection in the trace baseline (Figure 4J). Analysis of baseline shift in hippocampal cells exposed to control antibody, revealed a tonic GABAergic current of approximately 18 pA in amplitude (Figure 4K-L). Exposure to NMDAR-mAbs significantly reduced this tonic inhibitory current amplitude, whereas exposure to GABAaR-mAbs virtually abolished the GABAergic tonic current (Figure 4K-L). Altogether, these recordings demonstrate that NMDAR- or GABAaR-mAbs induce comparable reduction in fast excitatory and inhibitory transmissions and shift the E/I balance toward excitation.

Excitatory and inhibitory synaptic crosstalk is activity dependent

Given the synaptic content of glutamatergic and GABAa receptors is modulated by hippocampal network activity,^{37,38} we investigated whether neuronal activity plays a role in the cross-talk effect of pathogenic autoantibodies. Hippocampal neurons were exposed 24 h with control, NMDAR- or GABAaR-mAb, either in standard cell culture medium or medium supplemented with 20nM TTX to block network activity (Figure 5A). NMDAR-mAbs decreased NMDAR cluster density in absence or presence of TTX (Figure 5B), indicating that

the membrane disorganisation of NMDARs by NMDAR-mAb is activity-independent. However, the GABA_AR cluster density was not anymore altered by NMDAR mAbs in presence of TTX (Figure 5C). While both NMDAR and GABA_AR decreased NMDAR cluster size regardless of the presence of TTX, no effect was observed on GABA_AR cluster size (Figure S2C-F). These data suggest that the cross-talk effects of NMDAR-mAbs on inhibitory synapses rely on neuronal activity. Remarkably, we observed the mirror effect of GABA_AR-mAbs on excitatory synapses. Indeed, GABA_AR-mAbs reduce GABA_AR cluster density regardless of TTX exposure (Figure 5D). Yet, the NMDAR cluster density was not anymore altered by GABA_AR-mAbs in presence of TTX (Figure 5E). Thus, the cross-talk effect of GABA_AR-mAbs on NMDAR is activity-dependent. These findings demonstrate a biphasic effect of autoantibodies: i) a direct and activity-independent effect of autoantibodies on their target synaptic organization, and ii) a cross-talk effect on another receptor type by alternative mechanisms that are dependent on neuronal activity.

Multiple protein kinase activity changes following exposure to autoantibodies

In order to identify the putative signalling pathway(s) underpinning the cross-talk effects of autoantibodies, we focused our attention to protein kinase activity in presence of NMDAR- and GABA_AR-mAbs. Indeed, synaptic activity is known to alter various protein kinase and phosphatase activities and our observation that gephyrin phosphorylation drastically changed following exposure to autoantibodies prompt us to measure protein kinase activity. To identify candidate protein kinases, we used a kinase screening assay based on fluorescent labelling of phosphorylated substrates developed with micro-array technology (PamGene, Figure 6A). Neuronal lysates, collected from cultured neurons exposed to control-, NMDAR- or GABA_AR-mAbs were screened for changes in activity of both tyrosine (PTK) and serine/threonine-specific (STK) protein kinases. We identified 1 (PTK) and 3 (STK) phosphosites that were significantly altered after exposure to NMDAR-mAbs, and 2 (PTK) phosphosites that were significantly altered after exposure to GABA_AR-mAbs (Figure 6B-C). These substrates identify then less than twenty protein kinases whose activities have been significantly altered by NMDAR- or GABA_AR-mAbs. Protein kinase whose activities were downregulated by both autoantibodies are: TEK Receptor Tyrosine Kinase (TEK), Focal adhesion kinase 2 (FAK2), Spleen tyrosine kinase (SYK), c-Jun N-terminal kinases 1 & 2 (JNK1&2) and p38 δ mitogen activated protein kinase (p38 δ) (Figure 6D). Conversely, upregulated protein kinases included: Fibroblast growth factor receptor 4 (FGFR4), Receptor tyrosine-protein kinase (RET), Protein kinase D1 (PKD1), Checkpoint kinase 2 (CHK2) and Glycogen synthase kinase-3 beta (GSK3 β) (Figure 6D). Intriguingly, of these identified kinases, GSK3 β is known to phosphorylate gephyrin at S270. As such, it is tempting to speculate that the upregulation of this kinase, induced by both NMDA- and GABA_AR-mAbs, may pose a likely candidate mechanism for the selective loss of inhibitory synapses. Furthermore, it is evident that both NMDAR and GABA_AR mAbs elicit substantial dysregulations of intracellular signalling cascades with 11 out of 18 protein kinase (~60%) whose activities

were similarly altered by both autoantibodies. Together, these data show a certain degree of convergence in intracellular signalling cascades between the autoantibodies, suggesting that intracellular kinase-mediated pathways likely form a complex substrate of altered signalling.

Dopamine-NMDA receptor interaction contributes to autoantibody-mediated cross-talk effect

Among the various intracellular cascades that can tune the membrane redistribution of NMDAR and GABA_AR following exposure to autoantibodies, the direct protein-protein interaction between transmembrane receptors is of prime interest.^{39,40} In this context, dopamine receptors are of particular interest since i) they directly interact with both NMDAR and GABA_AR, ii) their interaction is regulated phosphorylation, and iii) GSK3, which activity is upregulated by both autoantibodies, forms a complex with Casein kinase 1 (CK1), a known modulator of the NMDAR/D1R interaction (Figure 6E).^{29,39,41} We hypothesised that if D1R-GluN1 interaction plays a role in linking the neuronal-activity-modulated intracellular signalling cascades with surface organisation, then abolition of this interaction should in turn prevent the cross-organisational impacts of NMDAR and GABA_AR mAbs on GABA_AR and NMDAR, respectively. First, an inhibitor of the CK1, the CKI7 was applied together with the autoantibodies and observed that the effect of the GABA_AR mAb on NMDAR was lost when CKI7 was present (Figure 6F) To manipulate the D1R-GluN1 interaction, we employed a genetic strategy, in which neuronal cultures were transfected with either a WT D1R-expressing construct, or a D1R^{Δ12}-expressing construct, in which the GluN1 interacting peptide sequence is deleted from the intracellular tail. As a result, NMDAR and D1R^{Δ12} are unable to interact (Figure S4A). In these experiments, exposure to mAbs for 24 hours showed that NMDAR mAbs decrease dendritic NMDAR cluster density, irrespective of the interaction with D1R (Figure 6F-I). However, when the D1R-GluN1 interaction is lost, in cultures transfected with D1R^{Δ12}, NMDAR mAbs did not cause an effect on the cluster density of GABA_AR (Figure 6J). Thus, our observations suggest that D1R-GluN1 interaction may play a role in mediating the cross-effect of NMDAR mAbs on GABA_AR disorganisation. Similarly, while the effect of GABA_AR mAb on GABA_AR cluster density is preserved in neurons expressing WT D1R, the cross-organisational impact on NMDAR cluster density along the dendritic compartments is abolished (Figure 6K-L). Quantification of the expression of the exogenously expressed D1R or D1R^{Δ12} demonstrated that the observed changes were not due to a different cell surface expression but a displacement of the receptors (Figure S4B-D).

While we do not rule out the possibility that other surface proteins – and their interactions with NMDAR or GABA_AR – will also likely be modulated by the identified intracellular signalling dysregulations, it appears that D1R interaction with NMDAR may play a substantial role in orchestration of membrane-wide disorganisation of NMDAR and GABA_AR after exposure to GABA_AR and NMDAR mAbs, respectively. However, we note that the precise nature of this interaction, and indeed the modulation of this interaction between endogenous NMDAR and D1R, remains to be characterised in models of autoimmune encephalitis.

Yet, it is encouraging to observe that the global disorganisation of neuronal membrane channels may be manipulated – or attenuated – through alteration of subsets of protein-protein interactions, and further characterisation of these pathways may be warranted to identify potential therapeutic targets for the treatment of autoimmune encephalitis.

GABA_AR-mAbs increase principal cell excitability

Given membrane receptor disorganisation and synaptic transmission deficits, we tested whether autoantibodies also have the potency to alter intrinsic properties of hippocampal principal cells. We used a whole cell current clamp protocol, in which CA1 neurons were administered a series of hyper-to-depolarising current injections (from -150 to 400 pA in 50 pA increments) (Figure 7A-C). Input-output curves were generated, indicating an increased propensity for action potential firing after exposure to GABA_AR-mAbs, but not NMDAR-mAbs (Figure 7D). Further, exposure to NMDAR-mAbs had no impact on the resting membrane potential (-61 mV), whereas GABA_AR-mAbs elicited a depolarisation of the resting state potential (-48 mV; Figure 7E). Given this shift in the resting potential, we also characterised the threshold for action potential discharge and rheobase. None of autoantibodies altered the threshold for action potential (Figure S3G), whereas the rheobase was shifted in favour of hyperexcitation after exposure to GABA_AR-mAbs (41 pA) (Figure 7F), which is expected given the depolarised resting potential and unchanged threshold for action potential generation. Additionally, we observed a significant reduction in action potential amplitudes and an increase in their half-width (Figure S3J-L), which may be explained by an alteration in GABA_AR-mediated signalling at the axon initial segment. Taken together, these findings suggest that GABA_AR-mAbs, but not NMDAR-mAbs, alter the intrinsic excitability of principal cells in the hippocampus. The input resistance was also impacted by GABA_AR-mAbs (Figure 7G). Altogether, these data indicate that GABA_AR-mAbs alter the intrinsic properties of CA1 principal cells, favouring their excitability, whereas NMDAR-mAbs was without effect.

Both NMDAR- and GABA_AR-mAbs increase principal cell network activity

Because autoantibodies alter excitatory and inhibitory synaptic transmissions and, in part, cell intrinsic properties, an alteration of the principal cell network activity is possible. More specifically, one may predict that i) NMDAR-mAbs will increase principal cell firing and network activity since they increase E/I balance of inputs and decrease tonic GABAergic current, and ii) GABA_AR-mAbs will also increase principal cell firing and network activity since they increase E/I balance of inputs, decrease tonic GABAergic current, and increase principal cell excitability. To directly test the effect of NMDAR- and GABA_AR-mAbs on principal cell firing and network activity, we recorded cells in a cell-attached voltage-clamp mode, quantifying the spiking with a minimally invasive recording configuration. Both NMDAR- and GABA_AR-mAbs increased the firing of principal

cells (Figure 7H-I). To further address this question and explore the network activity, we employed a calcium imaging approach as a proxy for action potential discharges. CA1 principal cells of hippocampal slices were virally transfected with the calcium sensor, GCaMP6, to visualise somatic calcium influx and cell firing (Figure 7J). Consistently, the frequency of spontaneous calcium transients was significantly increased (Figure 7K), indicating that exposure to either NMDAR- or GABAaR-mAbs alone is sufficient to drive network hyperactivation. To further understand if this hyperactive state is driven by a generalised increase in principal cellular activity, or by a select subgroup of hyperactive neurons that in turn evoke network effects, we analysed the relative frequency distributions and maximum firing frequency of individual cells within each recorded slice. We found that the cumulative distributions of cellular activity are right-shifted by NMDAR- and GABAaR-mAbs, indicating an overall increase in cellular activity (Figure S5A). Additionally, we did not observe an alteration of the maximum firing frequency after exposure to either autoantibody (Figure S5B), suggesting that this increase is relatively homogeneous. Recent reports have indicated that NMDAR-mAbs induce a hypersynchronous network state between excitatory neurons in hippocampal circuits.²⁰ To confirm this observation and explore whether it also applies for GABAaR-mAbs, we calculated the mean correlation index⁴² from pairwise comparisons between all cells in the recorded networks. We uncovered a significant increase in the mean cell correlation index after exposure to NMDAR-mAbs, but only a trend for GABAaR-mAbs (Figure S5C). Analysis of simultaneously occurring cellular firing, here referring to calcium transients identified within the same imaging frame (300 ms), revealed that both NMDAR- and GABAaR-mAbs increase this rate (Figure S5D). Finally, we investigated the percentage of pairwise comparisons that yielded a positive correlation index, across all pairs of neurons. This analysis revealed a significant increase in the percentage of positively correlated spike trains after exposure to NMDAR-mAbs, and only a trend for the GABAaR-mAbs group (Figure 7L). Altogether, these data demonstrated that both NMDAR- and GABAaR-mAbs increase the firing rate of principal cells as well as CA1 principal cell network activity, which is further synchronized by NMDAR-mAbs.

NMDAR-mAbs decrease interneuron excitability and silence their firing

The activity of hippocampal neuronal network is highly tuned by interneurons.⁴³ To grasp a comprehensive view of the impact of autoantibodies on the hippocampal network, we thus recorded intrinsic properties and firing rate of CA1 *stratum radiatum* interneurons exposed to either NMDAR- or GABAaR-mAbs. Recording hyper- to depolarising current steps to interneurons revealed a significant decrease in the propensity for action potential generation in response to depolarising current injection in hippocampal slices exposed to NMDAR-mAbs, but not GABAaR-mAbs (Figure 8A-D). Moreover, we identified a modest but significant hyperpolarisation of the resting membrane potential in interneurons exposed to NMDAR-mAbs (Figure 8E). Since we did not observe any change in the threshold for action potential generation in these cells after

exposure to either NMDAR- or GABA_AR-mAbs (Figure S3H), we anticipate that this deflection in resting potential will serve to increase the requirement for excitatory input, before successful elicitation of neuronal firing in interneurons. This expectation was supported by the increased rheobase in interneurons exposed to NMDAR-mAbs (Figure 8F). Consistent with the above data, we identified a massive loss of interneuron firing after exposure to NMDAR-mAbs using a cell-attached voltage-clamp approach (Figure 8 G-H). This reduction in inhibitory interneuron activity was not observed after exposure to GABA_AR mAbs, suggesting that this silencing of GABAergic activity is a unique pathology related to NMDAR mAbs. Collectively, these data demonstrated that NMDAR-mAbs, but not GABA_AR-mAbs, down-regulate the interneuron excitability and massively decrease their firing within the hippocampal CA1 network. Taken in the context of our existing findings, we would postulate that this increase in excitatory output is primarily driven by a shift towards hyperexcitation of phasic synaptic inputs, that is compounded either by an increase in intrinsic excitability by GABA_AR mAbs; or by the ablated inhibitory networks in the case of NMDAR mAb exposure, which then fail to control excitatory connections between principal cells, resulting the hypersynchronous network phenotype we observed after exposure to NMDAR mAbs (Figure 8I).

DISCUSSION

How autoantibodies targeting neurotransmitter receptors mediating either excitation or inhibition lead to similar clinical symptoms and behavioural deficits recently emerged as a challenging question. Indeed, the precise molecular and cellular mechanisms underpinning brain dysfunctions in variable forms of autoimmune encephalitis have remained elusive. In this study, we demonstrate that autoantibodies from patients with anti-NMDAR or anti-GABA_AR encephalitis impact the membrane diffusion and distribution of glutamatergic and GABAergic receptors, resulting in deficits of both excitatory and inhibitory synaptic drive. Whereby both autoantibodies similarly reduce synaptic inputs onto pyramidal neurons, they induce a shift in E/I balance towards excitation. The effects of autoantibodies on other synaptic transmission types are dependent on the neuronal activity, and likely involve a group of protein kinases and protein-protein interactions between membrane receptors. Furthermore, NMDAR and GABA_AR autoantibodies impact the intrinsic excitability of CA1 inhibitory interneurons and pyramidal cells, respectively. NMDAR-mAbs act via a classical disinhibitory mechanism, whereas GABA_AR-mAbs drive hyperexcitation of principal cells. Taken together, we unveil that these compounding impacts across synaptic and cellular phenotypes converge to an overarching hyperexcitation of CA1 hippocampal networks. These data indicate that the pathological dysregulations initiated by autoantibodies in autoimmune encephalitis span beyond the direct antigen-mediated deficits on neuronal function and membrane organisation, with convergence at the network levels between different autoantibodies.

Both NMDAR- and GABA_AR-mAbs drive alterations in the surface diffusion of their target antigens, as expected from previous studies using patient-derived biological samples.^{7,32} Exposure to NMDAR-mAbs reduces the surface diffusion of NMDAR at excitatory synapses after 24h, suggesting a reduced membrane dynamics of remaining synaptic NMDARs after long-exposure to NMDAR-mAbs. On the other hand, GABA_AR-mAbs induce an increase in surface diffusion of GABA_AR at inhibitory synapses, likely dependent on the loss of the postsynaptic scaffold gephyrin in these synapses. Notably, a similar reduction in hippocampal gephyrin has recently been reported in a mouse model of NMDAR encephalitis (geis paper), suggesting that such destabilisation of inhibitory synapses also occurs *in vivo*. Moreover, we observed a striking recapitulation of mAb impacts on the synaptic depletion of NMDAR and GABA_AR, by their associated autoantibodies, as has been shown to occur after exposure to patient samples, including CSF and purified IgG.^{3,44} It has also been shown that the monoclonal autoantibodies used in these experiments are sufficient to elicit disease phenotypes, congruent to those observed in rodent models; including the development of cognitive dysfunction and spontaneous seizures.^{10,12,17} As such, it is compelling that exposure to single monoclonal samples are sufficient to elicit molecular and cellular cascades observed in models with patients

pooled IgGs of CSFs, indicating they constitute powerful tools to determine the major underlying pathogenic mechanisms.

Surprisingly, we uncovered that both NMDAR- and GABAaR-mAbs also provoke similar alterations in the surface diffusion of GABAaR and NMDAR, respectively. Autoantibody exposure, irrespective of the target antigen, impedes the correct localisation of receptors to synaptic compartments and induce a deficit in synaptic transmissions. Analysis of spontaneous AMPAR-mediated excitatory postsynaptic currents revealed that both NMDAR and GABAaR mAbs reduce excitatory input onto CA1 hippocampal pyramidal neurons. Remarkably, despite the relatively moderate macroscale disorganisation of AMPAR at excitatory synapses, the larger functional reduction in excitatory current amplitudes would suggest that autoantibodies elicit a substantial disruption of nanoscale synaptic architecture,^{9,32} possibly through a destabilisation of nanocolumns, in which AMPAR clusters are closely aligned with presynaptic glutamate release sites.^{45,46} It would therefore be interesting to characterise the subsynaptic nanoscale organisation of the remaining synaptic AMPAR population after exposure to autoantibodies using super-resolution approaches. Moreover, GABAaR-mediated inhibitory signalling was substantially reduced by both NMDAR- and GABAaR-mAbs. Consistently, the magnitude of reduction in inhibitory transmission was greater than that observed in excitatory transmission, yielding a clear shift in the excitation-inhibition balance of synaptic inputs in favour of hyperexcitation. While such a shift may have been anticipated in the case of GABAaR-mAb action, with direct antagonism of GABAaR, it is compelling to observe a congruent impact by NMDAR-mAbs. These homogenous impacts, by both NMDAR- and GABAaR-mAbs, suggest convergent routes towards pathogenesis across distinct autoimmune encephalitides. Consistent with the fact that neuronal activity modulates the surface expression, trafficking and organisation of many synaptic receptors, we observed that blockade of neuronal activity with TTX application during mAb exposure maintained the direct antigen effects of both pathogenic mAbs but this manipulation abrogated their cross-system effects. mAbs alter the activity of an array of protein kinases, with a high degree of convergence between autoantibodies. Given that a high degree of kinase regulatory processes are intricately linked with neuronal activity, it is tempting to speculate that convergent network hyperactivity – induced by both NMDAR- and GABAaR-mAbs – lies upstream of kinase pathway dysfunction. As such, blockade of network and neuronal activity by TTX may abrogate the cross-synaptic effects by impeding pathological alterations in kinase activity. Among the putative mechanisms that could be affected, we show that the protein-protein interaction between NMDAR and dopamine receptors contribute to the cross-effects likely through the regulation of the interaction by protein kinases.

The major alterations of synaptic inputs are compounded by shifts in intrinsic cellular excitability of pyramidal neurons exposed to GABA_AR-mAbs. While this shift towards hyperexcitability was not observed in principal cells exposed to NMDAR-mAbs, an opposing reduction in excitability of interneurons was observed. One may propose that this bidirectional action on opposing neuronal systems yields the same endpoint of increased hippocampal activity at the network scale. A common overarching hyperactivation of hippocampal pyramidal cells, culminating from the dysregulation across the synaptic and cellular functions. Noteworthy, subtle disparities in the network phenotypes, i.e. NMDAR-mAbs drive hippocampal networks to a hypersynchronous state that is not observed following GABA_AR-mAb exposure, were observed. Interestingly, network hypersynchrony induced by NMDAR-mAbs has recently also been described in hippocampal *ex vivo* recordings in a passive-transfer mouse model of NMDAR encephalitis and in further recent studies.^{19,20} Here, we found severely reduced excitability in hippocampal interneurons by NMDAR-mAbs which might serve as a potential driver of network hyperexcitability.^{10,12,17,19} Although the molecular mechanisms underpinning these cell intrinsic property changes remain to be defined, one may envision that autoantibodies alter the membrane organization and trafficking of ion channels controlling the cell intrinsic properties. Antibody-based cross-linking of NMDAR, which could resemble to some aspect the impact of NMDAR-Abs, alter the firing pattern of dopamine neurons from the ventral tegmental area.⁴⁷ Although a dysfunctional cross-talk between NMDAR and SK potassium channels is suspected in this specific phenotype, the functional interplay between NMDAR and various ion channels and transporters, e.g. Kv4.2,⁴⁸ fuel the hypothesis that autoantibodies against neurotransmitter receptors can indirectly alter ion channel membrane content and distribution, and consequently impair cell intrinsic properties.

Synaptic deficits induced by NMDAR- and GABA_AR-mAbs revealed that surprisingly both pathogenic autoantibodies drive a homogenous dysregulation in the surface dynamics, synaptic content and excitatory and inhibitory synapses. These direct- and cross-effects clearly suggest that individual autoantibodies induce a wide-reaching disorganisation at the plasma membrane. Future studies should aim to explore this possibility, including investigations into diffusive properties of other surface proteins. This indicated that autoimmune disruption of neuronal surface receptors extends beyond the single identified antigen, with substantial trafficking dysregulation in presumably well-segregated membrane proteins. One may propose that pathogenic mechanisms involved in autoimmune encephalitic syndromes should not be considered as single channelopathies, whereby autoantibodies solely impacting their isolated antigens, but instead as disease processes with wider and sometime convergent routes to pathogenesis. Considering the array of research that has focussed on single protein mediated defects, for instance in genetic models of epilepsy or neurodevelopmental conditions, we would suggest that the high degree of orchestrated co-regulation between many neuronal proteins, can itself become a substrate for pathogenesis. Ultimately, dysregulation

of individual neuronal proteins can in a very short time window result in large-scale modifications of other membrane receptors, ion channels, and transporters. Defining the putative “dominoes effect” on membrane proteins that is triggered by patients’ autoantibodies will thus shed new light on the interplay between major systems in neurons and open new avenues of intervention in the blooming field of autoimmune neuropsychiatric disorders.

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Author contributions

DH, MP-P, NB performed and analyzed experiments. MC, JK, HP, CG generated resources and provided tools. DH, MP-P, LG conceived the project and designed the experiments. DH, MP-P, LG wrote the manuscript.

Declaration of interests

The authors declare that they have no conflict of interest.

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Figure Legends**Figure 1. Patient autoantibodies mutually disrupt the surface dynamics of NMDA receptors and GABA_A receptors**

(A) Schematic representation of experimental protocols for the derivation and production of human monoclonal autoantibodies.

(B) Representative image of cultured hippocampal neuron expressing GluN1-SEP (green) and Homer1c-dsRed (red). Scale bar = 20 μm .

(C) Schematic representation of quantum dot (QD) tracking method of NMDAR.

(D) Illustrative schematic to show the tracking of NMDAR (solid black line) in the membrane of the neuronal dendrite (dashed line). Scale bar = 1 μm .

(E) Representative image of cultured hippocampal neuron expressing γ 2-SEP (green) and gephyrin-mVenus (red). Scale bar = 20 μm .

(F) Schematic representation of quantum dot (QD) tracking method of GABA_AR.

(G) Illustrative schematic to show the tracking of GABA_AR (solid black line) in the membrane of the neuronal dendrite (dashed line). Scale bar = 1 μm .

(H) Representative single molecule tracks of NMDAR in synaptic compartments, after 24h exposure to Control-mAb (grey), NMDAR-mAb (green) or GABA_AR-mAb (magenta).

(I) Mean square displacement and (J) diffusion coefficients of synaptic NMDAR trajectories (Control-mAb n = 66 trajectories from 6 cells; NMDAR-mAb n = 80 trajectories from 6 cells; GABA_AR-mAb n = 109 trajectories from 5 cells; Kruskal-Wallis test).

(K) Representative single molecule tracks of GABA_AR in synaptic compartments, after 24h exposure to Control-mAb (grey), NMDAR-mAb (green) or GABA_AR-mAb (magenta).

(L) Mean square displacement and (M) diffusion coefficients of synaptic GABA_AR trajectories (Control-mAb n = 66 trajectories from 4 cells; NMDAR-mAb n = 61 trajectories from 6 cells; GABA_AR-mAb n = 89 trajectories from 5 cells; Kruskal-Wallis test).

Figure 2. NMDAR and GABA_AR autoantibodies mutually disrupt excitatory synaptic content

(A) Representative immunostainings of hippocampal neuronal dendrites expressing GluN1-Flag (green) and Homer1c-dsRed (magenta). Binary representations of thresholded colocalisation can be seen as white puncta on the far right. Scale bar = 10 μm .

(B-C) Mean synaptic NMDAR cluster density and (C) size, normalised to the Control-mAb condition (Control-mAb n = 46 cells; NMDAR-mAb n = 55 cells; GABA_AR-mAb n = 45 cells; one-way ANOVA).

(D) Representative immunostainings of hippocampal neuronal dendrites expressing GluA1-SEP (green) and Homer1c-dsRed (magenta). Merge of the two signals is shown, in which colocalisation of AMPAR and Homer1c can be seen as white puncta, and the regions of colocalisation are shown on the far right as binary images. Scale bar = 10 μ m.

(E-F) Quantified mean per cell of synaptic AMPA cluster density and (F) size, normalised to the Control-mAb condition (Control-mAb n = 46 cells; NMDAR-mAb n = 57 cells; GABAaR-mAb n = 31 cells; one-way ANOVA).

(G) From left to right: Quantified means per cell of NMDAR (Control n = 15 cells; APV n = 15 cells; Student's t-test), AMPAR (Control n = 28 cells; APV n = 28 cells; Student's t-test), and homer1c cluster densities (Control n = 28 cells; APV n = 28 cells; Student's t-test), of hippocampal cell dendrites, from cultures exposed to 5 μ M of the NMDAR antagonist, APV; or untreated controls.

(H) Quantified mean per cell of homer1c cluster density and (I) size, normalised to the Control-mAb condition (Control-mAb n = 46 cells; NMDAR-mAb n = 55 cells; GABAaR-mAb n = 46 cells; one-way ANOVA). Error bars represent the standard error of the mean.

Figure 3. NMDAR and GABAaR autoantibodies mutually disrupt inhibitory synaptic content and scaffolding proteins

(A) Representative immunostainings of hippocampal neuronal dendrites expressing γ 2-SEP (green) and gephyrin-mVenus (magenta). Binary representations of thresholded colocalisation can be seen as white puncta on the far right. Scale bar = 10 μ m.

(B-C) Mean synaptic GABAaR cluster density and (C) size, normalised to the Control-mAb condition (Control-mAb n = 40 cells; NMDAR-mAb n = 43 cells; GABAaR-mAb n = 41 cells; one-way ANOVA).

(D) Mean gephyrin cluster density, normalised to the Control-mAb condition (Control-mAb N = 40 cells; NMDAR-mAb n = 43 cells; GABAaR-mAb n = 41 cells; one-way ANOVA).

(E) Representative immunostainings of total gephyrin puncta and gephyrin phosphorylated at S270. Scale bar = 20 μ m.

(F) Mean total versus phosphorylated gephyrin puncta density, normalised to the level of total gephyrin staining (Control-mAb Total Gephyrin n = 20 cells; Control-mAb Phospho-Gephyrin n = 20 cells; NMDAR-mAb Total Gephyrin n = 20 cells; NMDAR-mAb Phospho-Gephyrin n = 26 cells; GABAaR-mAb Total Gephyrin N = 24 cells; GABAaR-mAb Phospho-Gephyrin n = 24 cells; multiple t tests with Benjamini and Hochberg correction for false discovery rate).

(G) Quantified means per cell of synaptic GABAaR (Control n = 81 cells; BCC n = 49 cells; APV n = 28 cells; one-way ANOVA) and (H) gephyrin cluster densities (Control n = 81 cells; BCC n = 49 cells; APV n = 28 cells; one-way ANOVA), of hippocampal cell dendrites, from cultures exposed to 1 μ M of the GABAaR antagonist,

bicuculline (BCC); 5 μ M of the NMDAR antagonist, APV; or untreated controls. All error bars represent the standard error of the mean.

Figure 4. Autoantibodies mutually dysregulate spontaneous excitatory and inhibitory neurotransmission

(A-B) Representative trace recordings from CA1 principal cells in organotypic hippocampal slices and (B) mean event traces of AMPAR-mediated spontaneous excitatory postsynaptic currents, AMPAR-sEPSC.

(C-D) Cumulative frequency distributions of AMPAR-sEPSC amplitudes (Control-mAb n = 1020 events; NMDAR-mAb n = 296 events; GABAaR-mAb n = 449 events) and (D) mean amplitude (Control-mAb n = 25 cells; NMDAR-mAb n = 9 cells; GABAaR-mAb n = 15 cells; one-way ANOVA).

(E-F) Representative trace recordings from CA1 principal cells in organotypic hippocampal slices and (F) mean event traces of GABAaR-mediated spontaneous inhibitory postsynaptic currents, GABAaR-sIPSC.

(G-H) Cumulative frequency distributions (Control-mAb n = 1952 events; NMDAR-mAb n = 538 events; GABAaR-mAb n = 873 events) of GABAaR-sIPSC amplitudes and (H) mean amplitude (Control-mAb n = 25 cells; NMDAR-mAb n = 9 cells; GABAaR-mAb n = 15 cells; one-way ANOVA).

(I) Excitatory-inhibitory balance quantifications resulting from mean cellular AMPAR-sEPSC amplitude divided by mean cellular GABAaR-sIPSC amplitudes (Control-mAb n = 24 cells; NMDAR-mAb n = 9 cells; GABAaR-mAb n = 15 cells; one-way ANOVA).

(J) Representative trace recordings of GABAaR-sIPSC before and after wash in of 20 μ M of GABAaR antagonist, bicuculline (BCC).

(K) Frequency distributions of the normalised point count, generated from event-free baselines before and after BCC application, and (L) mean cellular amplitude of the recorded tonic GABAergic current (Control-mAb n = 10 cells; NMDAR-mAb n = 10 cells; GABAaR-mAb n = 12 cells; one-way ANOVA). All error bars represent the standard error of the mean.

Figure 5. Excitatory and inhibitory synaptic crosstalk is activity dependent

(A) Cell quantification of synaptic NMDAR cluster density after 24h exposure to Control-mAb or NMDAR-mAb, in the presence or absence of TTX (Control-mAb n = 46 cells; NMDAR-mAb n = 55 cells; Control-mAb +TTX n = 18 cells; NMDAR-mAb +TTX n = 29 cells; one-way ANOVA).

(B) Cell quantification of synaptic NMDAR cluster density after 24h exposure to Control-mAb or GABAaR-mAb, in the presence or absence of TTX (Control-mAb n = 46 cells; GABAaR-mAb n = 46 cells; Control-mAb +TTX n = 18 cells; GABAaR-mAb +TTX N = 26 cells; one-way ANOVA).

(C) Cell quantification of synaptic GABA_AR cluster density after 24h exposure to Control-mAb or NMDAR-mAb, in the presence or absence of TTX (Control-mAb n = 40 cells; NMDAR-mAb n = 43 cells; Control-mAb +TTX n = 35 cells; NMDAR-mAb +TTX n = 43 cells; one-way ANOVA).

(D) Cell quantification of synaptic GABA_AR cluster density after 24h exposure to Control-mAb or GABA_AR-mAb, in the presence or absence of TTX (Control-mAb n = 40 cells; GABA_AR-mAb n = 41 cells; Control-mAb +TTX n = 35 cells; GABA_AR-mAb +TTX n = 37 cells; one-way ANOVA). All error bars represent the standard error of the mean.

Figure 6. Intracellular signalling cascades and surface protein interactions are dysregulated by NMDAR and GABA_AR autoantibody exposure

(A) Schematic representation of experimental protocols employed in the microarray kinase assay.

(B-C) Volcano plots of the quantified fluorescence change of individual phosphosites after processing of cell lysates from hippocampal cultures exposed to NMDAR-mAb (green) or GABA_AR-mAb (pink) relative to Control-mAb treated cultures using STK or PTK array. Arrowheads indicate phosphosites that show significant difference compared to control ($p < 0.05$). Results are expressed by plotting the effect size (x-axis, LFC or delta) versus significance (y-axis, $-\log_{10}(p \text{ value})$) of the test.

(D) Upstream Kinase Analysis (UKA) to predict differential kinase activity in the test condition compared to the control based on the phosphorylation of a set of phosphosites, “#” represents cases in which significant alterations were identified after exposure to both NMDAR-mAb (green) and GABA_AR-mAb (pink) compared to a control mAb.

(E) Schematic illustration showing the loss of interaction between NMDAR and D1R after introduction of a deletion on the D1R C-terminal domain, D1R Δ t2.

(F) Cell mean quantification of synaptic NMDAR cluster density after 24h exposure to Control-mAb, NMDAR-mAb or GABA_AR-mAb, in the presence or absence of CK1 antagonist, CKI (No CKI: Control-mAb n = 22 cells; NMDAR-mAb n = 28 cells; GABA_AR-mAb n = 24 cells; Plus CKI: Control-mAb n = 29 cells; NMDAR-mAb n = 23 cells; GABA_AR-mAb n = 37 cells; one-way ANOVA).

(G-H) Representative immunostainings of hippocampal neurons expressing GluN1 (magenta) and γ 2 (cyan), with wildtype D1R or (H) D1R Δ t2. Scale bar = 10 μ m.

(I-J) Mean dendritic NMDAR and (J) GABA_AR cluster density after exposure to Control-mAb and NMDAR-mAb, with coexpression of endogenous-only (-), wildtype D1R or D1R Δ t2 (untransfected: Control-mAb n = 20 cells; NMDAR-mAb n = 20 cells; D1R-WT: Control-mAb n = 26 cells; NMDAR-mAb n = 29 cells; D1R Δ t2: Control-mAb n = 29 cells; NMDAR-mAb n = 28 cells).

(K) Quantified cell means of dendritic GABA_AR and (L) NMDAR cluster density after exposure to Control-mAb and GABA_AR-mAb, with coexpression of endogenous-only (-), wildtype D1R or D1R Δ t2 (Untransfected: Control-mAb n = 20 cells; GABA_AR-mAb n = 20 cells; D1R-WT: Control-mAb n = 26 cells; GABA_AR-mAb n = 28 cells; D1R ^{Δ t2}: Control-mAb n = 29 cells; GABA_AR-mAb n = 27 cells). All error bars represent the standard error of the mean.

Figure 7. GABA_AR-mAbs increase principal cell excitability & Both NMDAR- and GABA_AR-mAbs increase principal cell network activity

(A-C) Representative current-clamp recordings from CA1 principal cells in organotypic hippocampal slices exposed to Control-mAb, (B) NMDAR-mAb and (C) GABA_AR-mAb.

(D) Input-output quantification of mean number of action potential discharges elicited in response to depolarising current injections (Control-mAb n = 9 cells; NMDAR-mAb n = 7 cells; GABA_AR-mAb n = 7 cells; one-way ANOVA).

(E-F) Mean resting membrane potential and (F) rheobase of CA1 pyramidal neurons (Control-mAb n = 9 cells; NMDAR-mAb n = 7 cells; GABA_AR-mAb n = 7 cells; one-way ANOVA).

(G) Mean input resistance of CA1 pyramidal neurons (Control-mAb n = 15 cells; NMDAR-mAb n = 9 cells; GABA_AR-mAb n = 14 cells; one-way ANOVA).

(H-I) Representative trace recordings of CA1 pyramidal neurons in cell-attached voltage-clamp configuration, after exposure to Control-, NMDAR- or GABA_AR-mAbs and (I) the mean spontaneous spike frequency (Control-mAb n = 29 cells; NMDAR-mAb n = 11 cells; GABA_AR-mAb n = 13 cells; one-way ANOVA).

(J) Representative frames taken from postprocessed calcium imaging acquisitions, and the mean cellular correlation indices.

(K) Mean network frequency from recorded CA1 hippocampal networks and (L) the mean percentage of thresholded correlation indices (Control-mAb n = 12 slices; NMDAR-mAb n = 12 slices; GABA_AR-mAb n = 12 slices; one-way ANOVA). All error bars represent the standard error of the mean.

Figure 8. NMDAR-mAbs decrease interneuron excitability and silence their firing

(A-C) Representative current-clamp recordings from CA1 inhibitory interneurons in organotypic hippocampal slices exposed to Control-mAb, (B) NMDAR-mAb and (C) GABA_AR-mAb.

(D) Input-output quantification of mean number of action potential discharges elicited in response to depolarising current injections (Control-mAb n = 14 cells; NMDAR-mAb n = 8 cells; GABA_AR-mAb n = 11 cells; one-way ANOVA).

(E-F) Mean resting membrane potential (Control-mAb N = 11 cells; NMDAR-mAb N = 8 cells; GABAaR-mAb N = 17 cells; One-way ANOVA) and (F) rheobase of CA1 pyramidal neurons (Control-mAb n = 19 cells; NMDAR-mAb n = 10 cells; GABAaR-mAb n = 26 cells; one-way ANOVA).

(G-H) Representative trace recordings of CA1 inhibitory interneurons in cell-attached voltage-clamp configuration, after exposure to Control-, NMDAR- or GABAaR-mAbs and (H) the mean spontaneous spike frequency (Control-mAb n = 10 cells; NMDAR-mAb n = 11 cells; GABAaR-mAb n = 6 cells; one-way ANOVA).

(I) Left: Schematic representation of synaptic alterations in the context of NMDAR- and GABAaR-mAb exposure, specifically demonstrating the depletion of AMPAR, NMDARs and GABAaRs from synaptic compartments, altered mobility of remaining NMDARs and GABAaRs, resulting in disrupted EI balance of synaptic inputs. Right: Schematic representation of antibody selective alterations of principal (triangular) and inhibitory neuron (circular) intrinsic excitability, and resulting overall network hyperactivity. All error bars represent the standard error of the mean.

FIGURE 1

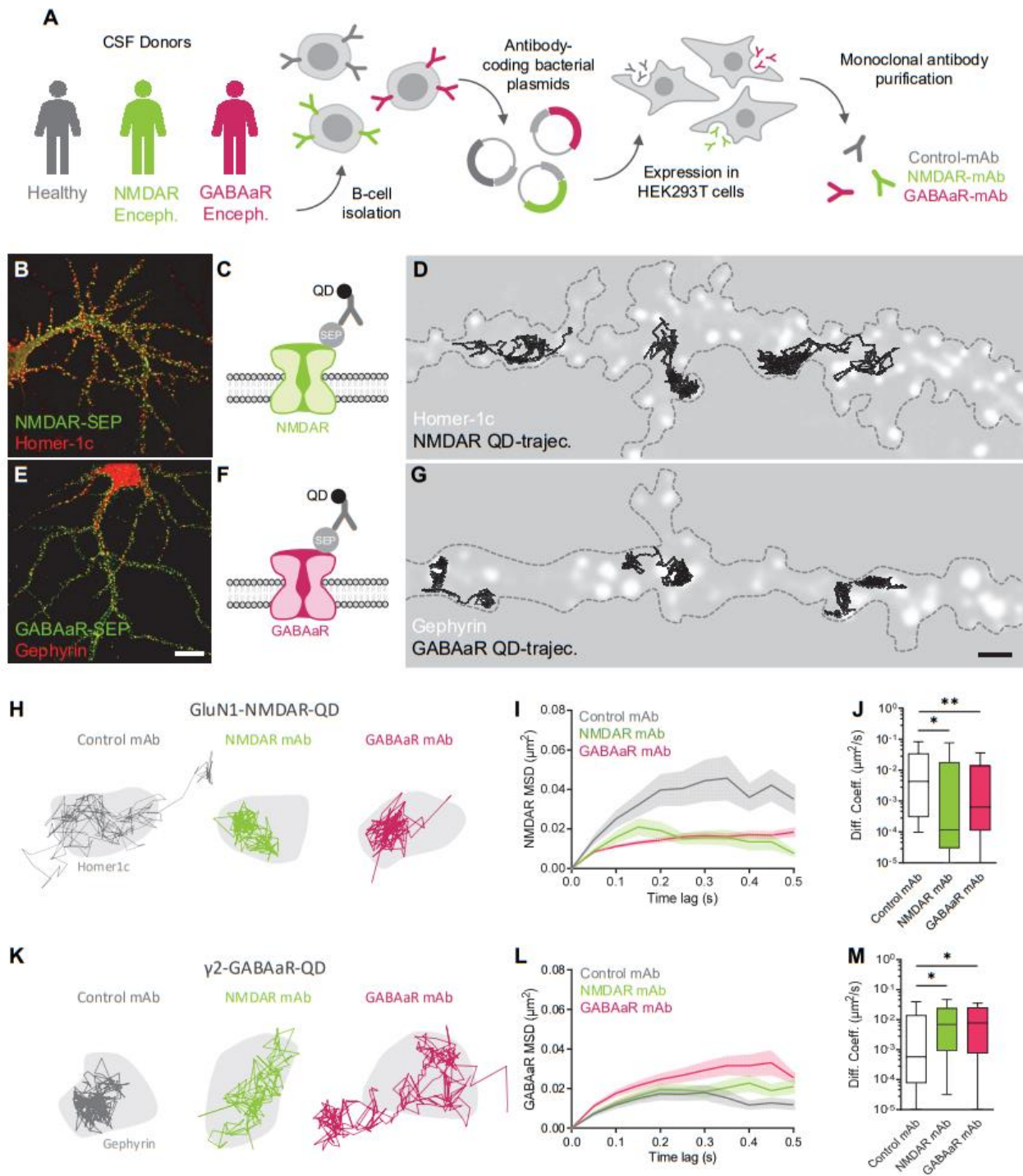


FIGURE 2

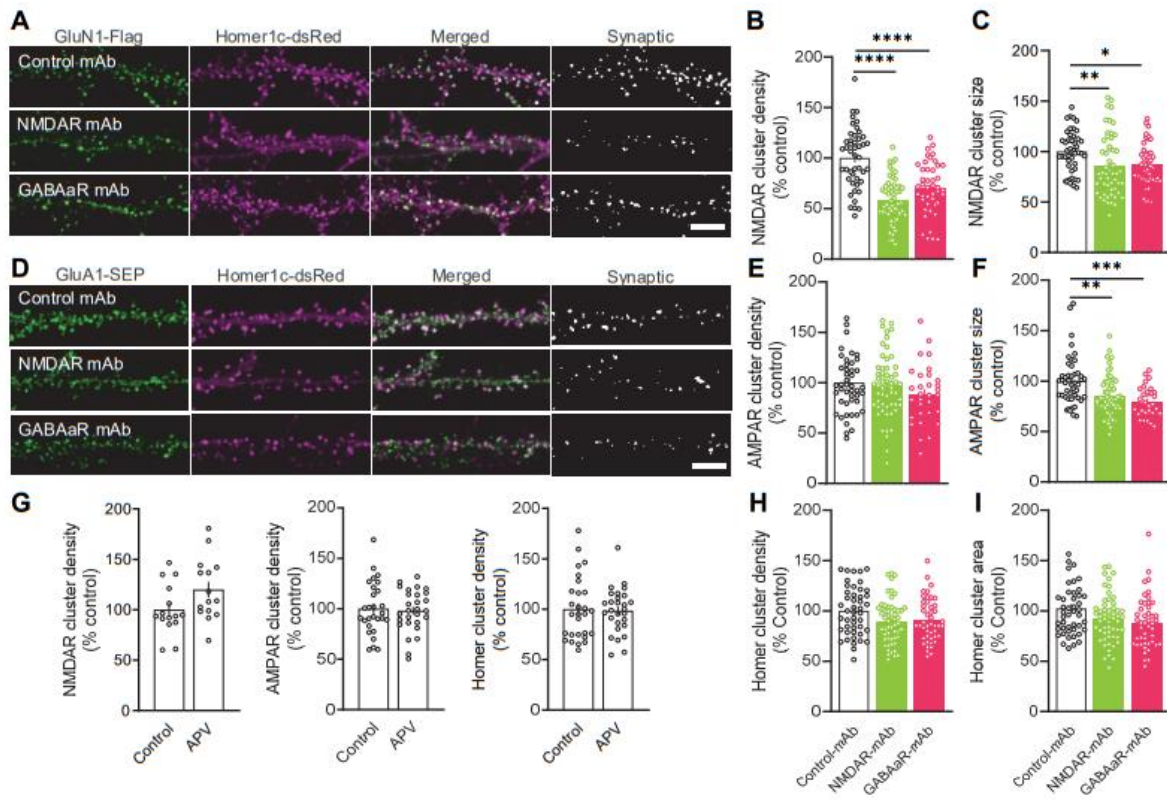


FIGURE 3

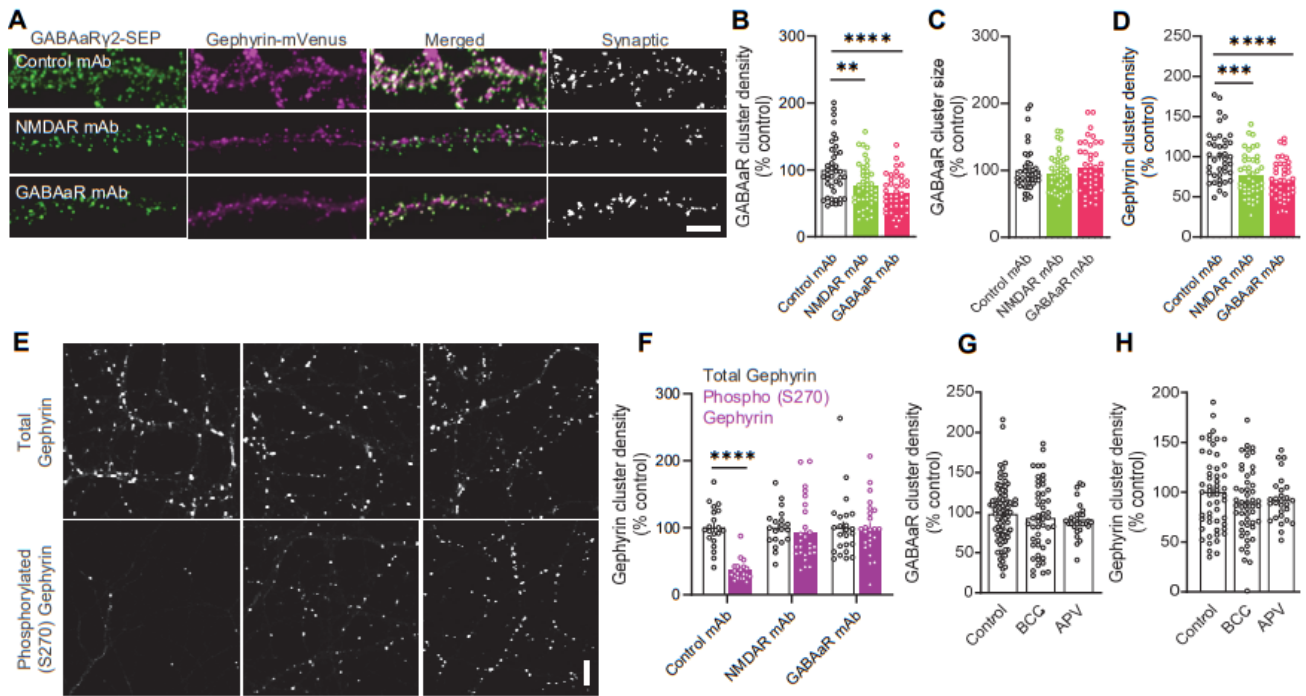


FIGURE 4

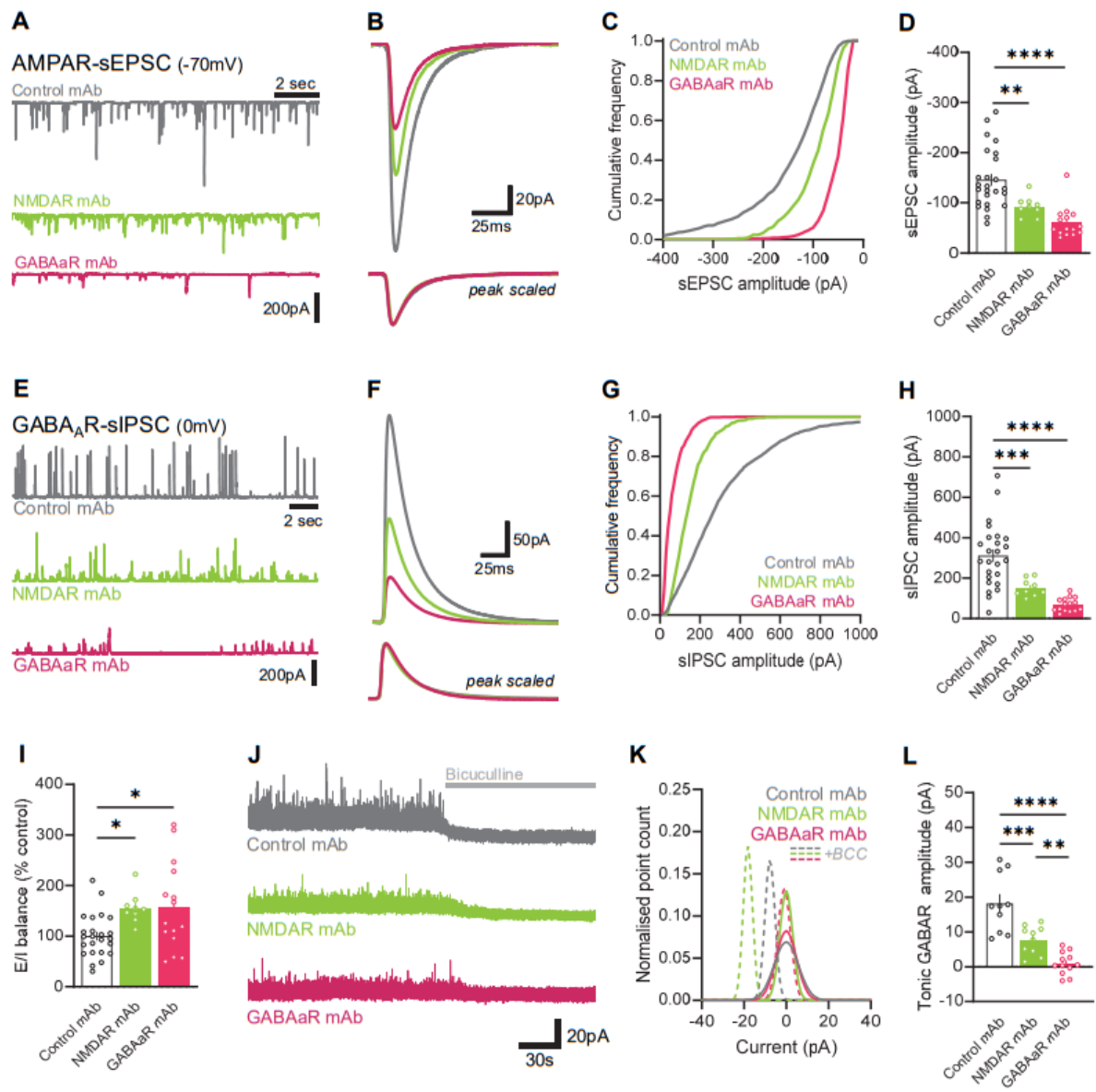


FIGURE 5

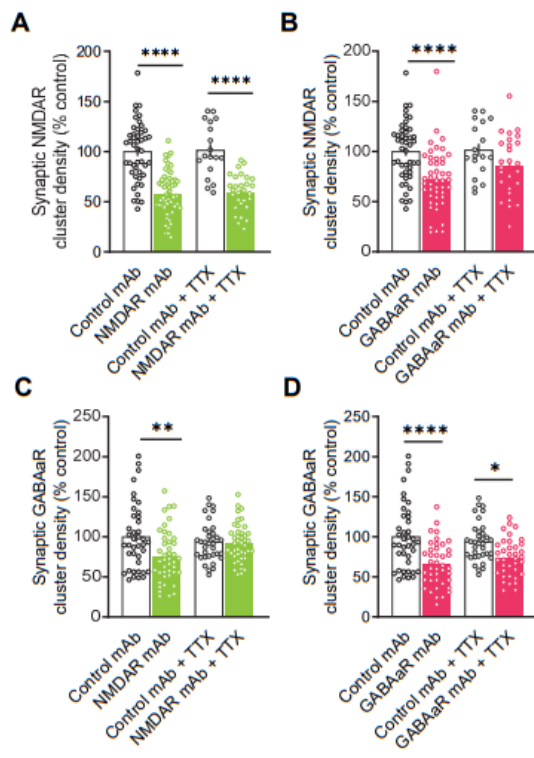


FIGURE 6

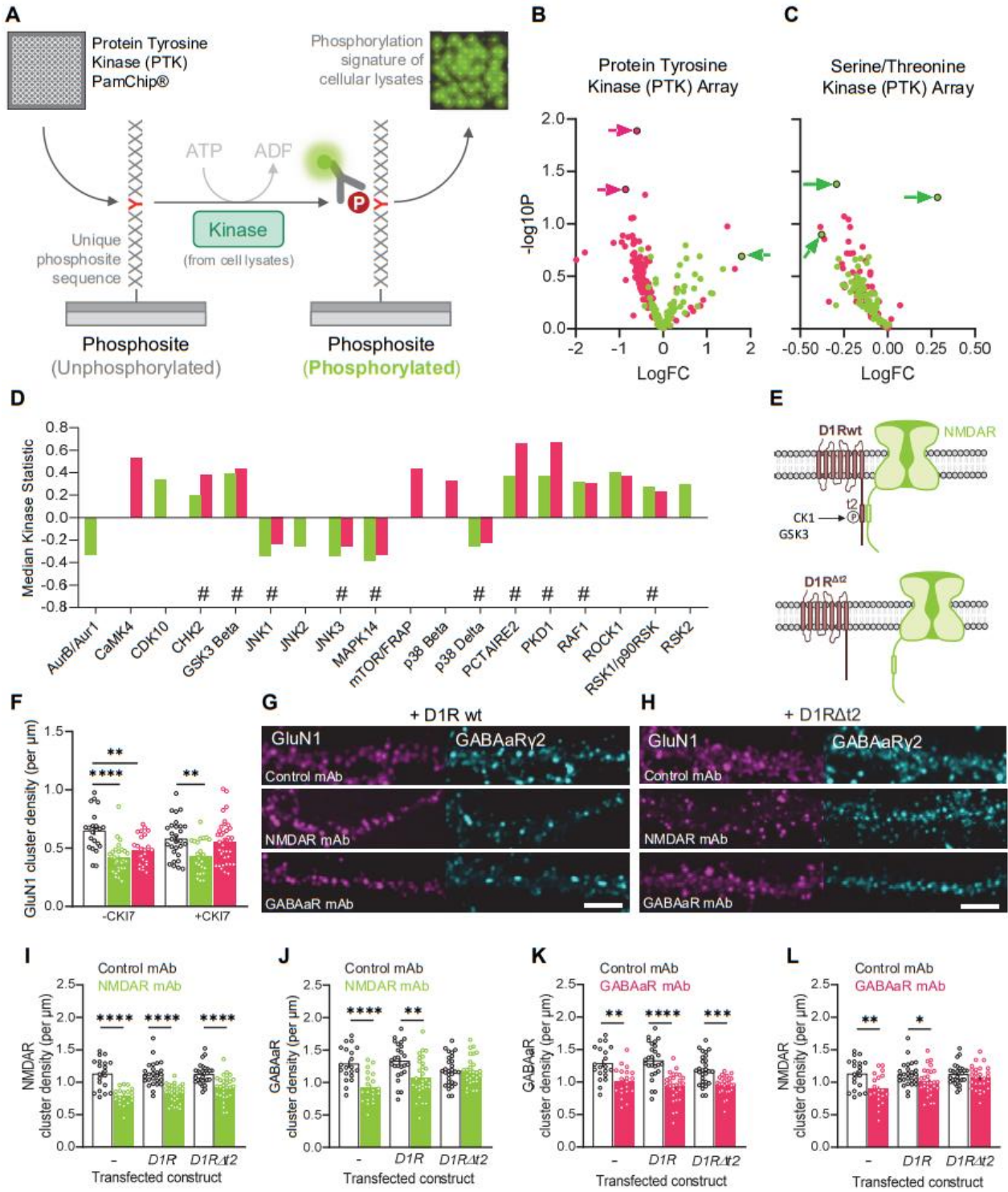


FIGURE 7

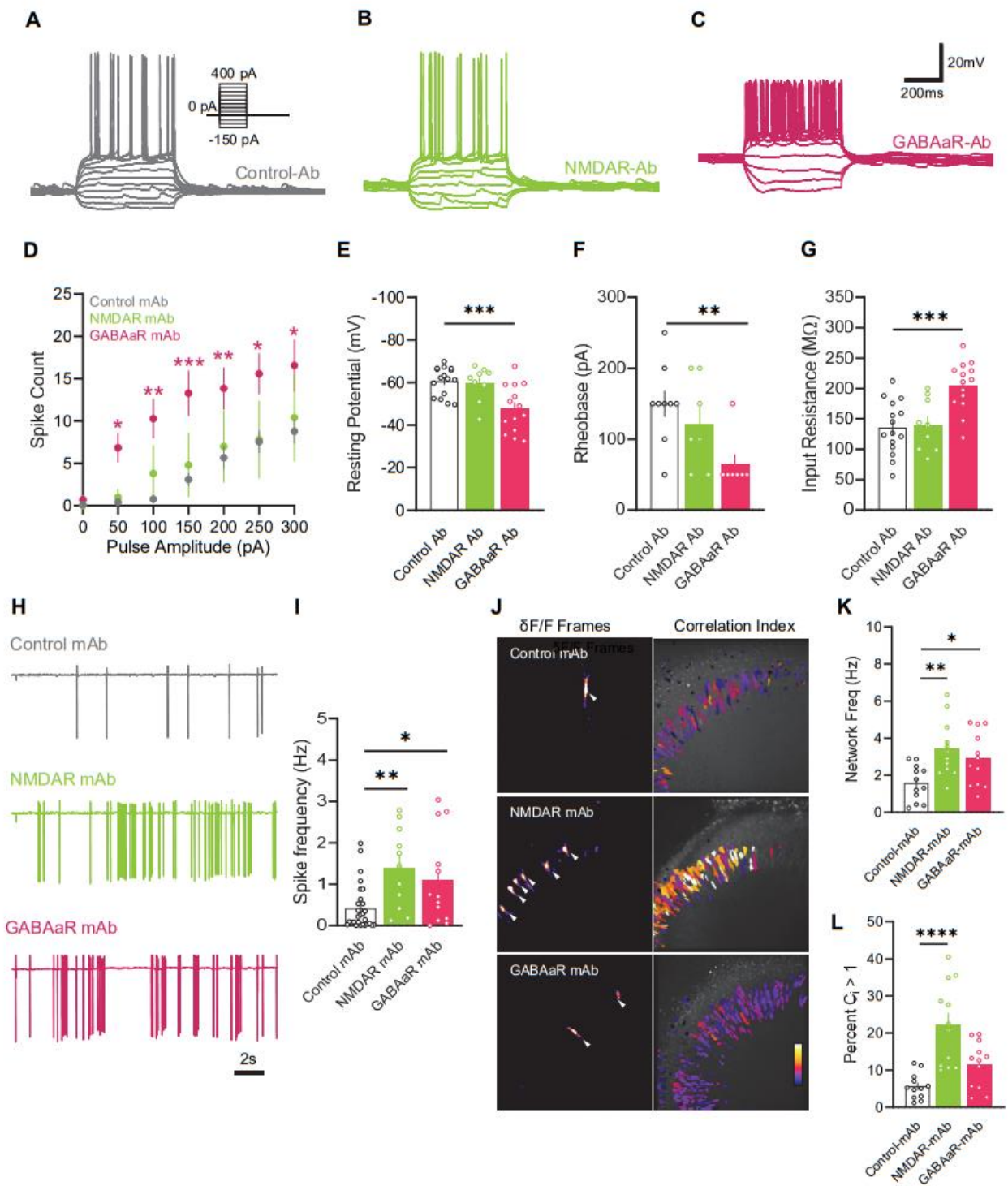
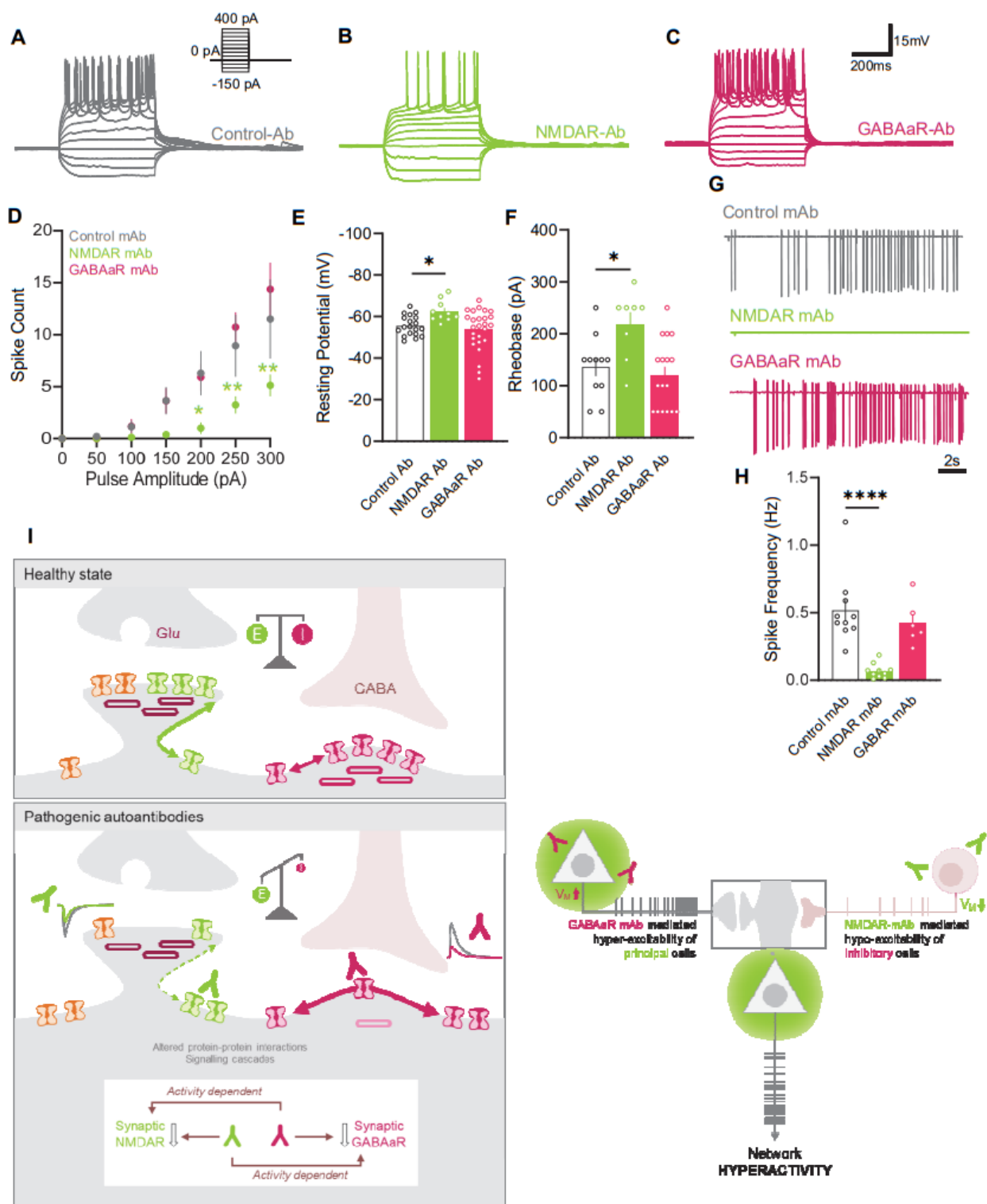


FIGURE 8



Additional Publications:

Article published in Nano Letters, Volume 17, September 2022.

Near-Infrared Carbon Nanotube Tracking Reveals the Nanoscale Extracellular Space around Synapses

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Abstract

We provide evidence of a local synaptic nanoenvironment in the brain extracellular space (ECS) lying within 500 nm of postsynaptic densities. To reveal this brain compartment, we developed a correlative imaging approach dedicated to thick brain tissue based on single-particle tracking of individual fluorescent single wall carbon nanotubes (SWCNTs) in living samples and on speckle-based HiLo microscopy of synaptic labels. We show that the extracellular space around synapses bears specific properties in terms of morphology at the nanoscale and inner diffusivity. We finally show that the ECS juxta-synaptic region changes its diffusion parameters in response to neuronal activity, indicating that this nanoenvironment might play a role in the regulation of brain activity

Article uploaded to BioRxiv, March 2022.

Human NMDAR autoantibodies disrupt excitatory-inhibitory balance leading to hippocampal network hypersynchrony

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Abstract

Specific autoantibodies against the NMDA-receptor (NMDAR) GluN1 subunit cause severe and debilitating NMDAR-encephalitis. Autoantibodies induce prototypic disease symptoms resembling schizophrenia, including psychosis and cognitive dysfunction. Using a mouse passive transfer model applying human monoclonal anti-GluN1-autoantibodies, we observed CA1 pyramidal neuron hypoexcitability, reduced AMPA-receptor (AMPA) signaling, and faster synaptic inhibition resulting in disrupted excitatory-inhibitory balance. Functional alterations were supported by widespread remodeling of the hippocampal proteome, including changes in glutamatergic and GABAergic neurotransmission. At the network level, anti-GluN1-autoantibodies amplified gamma oscillations and disrupted theta-gamma coupling. A data-informed network model revealed that lower AMPAR strength and faster GABAA-receptor current kinetics chiefly account for these abnormal oscillations. As predicted by our model and evidenced experimentally, positive allosteric modulation of AMPARs alleviated aberrant gamma activity and thus reinforced the causative effects of the excitatory-inhibitory imbalance. Collectively, NMDAR-hypofunction-induced aberrant synaptic, cellular, and network dynamics provide new mechanistic insights into disease symptoms in NMDAR-encephalitis and schizophrenia.

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In the writing of these acknowledgements, I wish to emphasise that this thesis is not just my accomplishment, but a collective accomplishment made possible, only by the perpetual love and support of my truly remarkable family and friends. Thank you to all of you for making this possible.

With love and gratitude,

Daniel

A handwritten signature in black ink, appearing to read 'Daniel', with a long horizontal flourish extending to the left and right.