FROM RECEPTOR TO BRAIN:

SYNAPTIC, CELLULAR AND BEHAVIORAL PATHOPHYSIOLOGY IN ANTI-GABAB RECEPTOR AND ANTI-NMDA RECEPTOR ENCEPHALITIDES

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DEDICATION

To my wife, Julia, for her tireless love and unflinching support. Thank you for being my most enthusiastic cheerleader and my toughest critic.

To my son, Eli, for reminding me every day what it all is really for. Thank you for teaching me how to truly love.

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ABSTRACT

SYNAPTIC, CELLULAR AND BEHAVIORAL PATHOPHYSIOLOGY IN ANTI-GABAB RECEPTOR AND ANTI-NMDA RECEPTOR ENCEPHALITIDES

Ankit Jain

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A new class of severe but treatable autoimmune encephalitides is associated with serum and CSF autoantibodies to cell surface receptors that are thought to cause disease by disrupting the normal function of their target protein. Consistent with disruption of the major neurotransmission pathways and thus circuit malfunction in the central nervous system, symptoms of these newly characterized diseases are severe and include psychosis, memory loss, confusion, seizures, and autonomic instability normal function. The majority of symptoms resolve with aggressive immunosuppresive therapy.

The best characterized of these encephalitides is associated with antibodies to ionotropic glutamate receptors, N-methyl-D-aspartate receptor (NMDAR) or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR). NMDAR and AMPAR autoantibodies cross-link the cognate synaptic receptors on the surface of cultured neurons and result in a selective internalization of the target without disruption of any other synaptic component or injury to neurons. In a passive transfer animal model, chronic perfusion of NMDAR antibodies into the cerebrospinal fluid (CSF) of mice also decreases NMDA receptors after 14 days, which results in anhedonia and diminished

spatial memory. Consistent with the reversible course of disease, the effects of NMDAR and AMPAR autoantibodies can be reversed in both *in vivo* and *in vitro* model systems. These mechanisms may underlie the neurological and psychiatric manifestations of these forms of autoimmune encephalitis, and account for recovery of many patients with immune therapy aimed at lowering antibody titer.

Patients with antibodies to the metabotropic gamma-aminobutyric acid receptor type B ($GABA_B$ receptor) develop severe intractable seizures, but little is known about the underlying pathophysiological mechanisms. Deletion mapping and expression in heterologous cells showed that patient anti-$GABA_B$ antibodies bind to alternatively spliced Sushi domains present in the presynaptically localized $GABA_{B1a}$ isoform. In contrast to patient anti-NMDAR antibodies, $GABA_B$ autoantibodies did not result in a decrease in surface $GABA_B$ receptors or their internalization. Treating neurons with GABABR autoantibodies for two hours blocked the activation of $GABA_B$ receptors by baclofen in a titer-dependent manner. Autoantibody-bound GABABRs are still signaling competent, because baclofen block was circumvented by directly activating $GABA_{B2}$ with a selective agonist, CGP7930.

Patient $GABA_B$ receptor autoantibodies are selective $GABA_{B1}$ antagonists that may contribute to seizures by interfering with GABA-mediated inhibition. Brain penetrant $GABA_{B2}$ agonists may be useful to treat intractable seizures in anti-$GABA_B$ receptor encephalitis patients.

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PREFACE

Recently, several novel, potentially lethal and treatment-responsive syndromes that affect hippocampal and cortical function have been shown to be associated with auto-antibodies against synaptic antigens, notably glutamate or $GABA_B$ receptors. Patients with these autoantibodies (sometimes associated with teratomas and other neoplasms) present with psychiatric symptoms, seizures, memory deficits and decreased levels of consciousness. These symptoms often improve dramatically after immunotherapy or tumor resection. In this work I review and extend several studies of the cellular and synaptic effects of these antibodies in hippocampal neurons *in vitro* and preliminary work in rodent models. Our work suggests that patient antibodies lead to rapid and reversible removal of neurotransmitter receptors from synapses, leading to changes in synaptic and circuit function that, in turn, are likely to lead to behavioral deficits. I also discuss several of the many questions raised by these and related disorders. Determining the mechanisms underlying these novel anti-neurotransmitter receptor encephalitides will provide insights into the cellular and synaptic bases of the memory and cognitive deficits that are hallmarks of these disorders, and potentially suggest avenues for therapeutic intervention.

In my thesis, I have focused on the pathophysiological mechanisms of two autoimmune encephalitides, one associated with anti-NMDA receptor antibodies, and the other associated with anti-GABAB receptor antibodies. The investigation of these diseases ranges from patient-oriented research to mechanistic experiments in cell culture and in animal models. The rich tapestry of clinical and mechanistic insights into this new class of diseases caused by neuronal cell surface autoantibodies is

introduced in Chapter 1. Rapid progress has been made in our understanding of these diseases since the discovery of anti-NMDA receptor encephalitis as the founding member of this class of autoimmune disease in 2003. Since then, many previously known as well as newly identified syndromes have been found to be caused by autoantibodies to a diverse group of surface proteins such as neurotransmitter receptors, synaptic scaffolding proteins, and also proteins whose function has not yet been discovered.

In Chapter 2, I explore the mechanistic underpinnings of pathogenesis by $GABA_B$ autoantibodies in a neuronal cell culture model. The autoantibodies in anti-$GABA_B$ receptor encephalitis affect $GABA_B$ receptor function by a mechanism distinct from what has been described thus far for anti-NMDA receptor, anti-AMPA receptor and anti-$GABA_A$ receptor encephalitis. Rather than decreasing the level of GABAB receptors at the surface of neurons, $GABA_B$ autoantibodies directly antagonize the activation of $GABA_B$ receptors.

In Chapter 3, I present a multi-center collaborative effort to generate and describe a passive immunization model of anti-NMDA receptor encephalitis. My role in this work was to generate the animal model, and discover the conditions and tools that resulted in a successful infusion of anti-NMDA receptor encephalitis patient CSF into mouse cerebral ventricles. Like in vitro, exposure of mouse brains to patient CSF reduces NMDA receptor levels and results in defects in spatial and object memory. Remarkably, both the reduction in NMDA receptor levels and the cognitive decline recovers after the cessation of patient CSF infusion. This animal model will be vital to test potential therapeutic interventions that may reduce receptor loss and cognitive decline or hasten recovery.

In Chapter 4, I describe a small case series of the first known pregnant

women with anti-NMDA receptor encephalitis. The two term infants potentially exposed to anti-NMDA receptor antibodies in utero did not have any signs of encephalitis.

The importance of these disorders is that they offer human models of brain–immune interactions in which the target antigens have critical roles in neuronal synaptic transmission and plasticity. Therefore, their study will improve our understanding of the effects of the antibodies at the cellular, synaptic and circuit levels, eventually impacting the clinical management of the patients.

CHAPTER 1. Introduction

Cellular, synaptic and circuit effects of antibodies in synaptic autoimmune encephalitides

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Establishing a pathogenic role for anti-synaptic antibodies

Multiple lines of evidence strongly suggest that anti-synaptic antibodies are not simply markers of disease but rather are directly pathogenic. When live, unpermeabilized dissociated rodent brain cells are treated with patient CSF, immunoglobulins (IgG) isolated from patient CSF or serum, unique patterns of punctate surface staining are observed (Fig. 2A; Ances et al., 2005). This shows that patient derived antibodies bind distinct neuronal cell surface proteins. This is in contrast to classically described paraneoplastic autoimmune encephalitides, in which the isolated antibodies target intracellular antigens, resulting in a cytotoxic T cell response, neuronal cell death and a result, poor prognosis (Dalmau and Rosenfeld, 2008). In the case of synaptic autoimmune encephalitides rodent brain sections immunostained with patient CSF also show distinct patterns of immunoreactivity in the neuropil of hippocampus, cortex and other brain regions, while there is no immunoreactivity to neuronal cell bodies (Vitaliani et al., 2005). Brain biopsies from a small number of patients demonstrate minimal pathologic changes or cell loss but demonstrate rare T cell and B cell infiltrates, microgliosis and astrocytosis (Fig. 4; Martinez-Hernandez et al., 2011). These results are consistent with a direct pathogenic role for patient antibodies in synaptic autoimmunities rather than a cell mediated immune response as seen in paraneoplastic encephalitides associated with intracellular antigens.

Mass spectrometry has been particularly useful for identifying specific binding targets of anti-synaptic antibodies from immunoprecipitates of rat brain lysates with patient antibdodies (Fig. 2C; Lai et al., 2009; Lancaster et al., 2011). Immunostaining of heterologous cells that overexpress an antigenic candidate protein identified by mass spectrometry with patient CSF has been used to confirm binding of patient antibodies to a particular antigenic protein (Fig. 2D-F). These in vitro approaches,

together with other analyses, have been used to establish that each distinct syndrome corresponds to a distinct cell surface antigen targeted by patient CSF antibodies (Fig. 1).

The function of a surface protein antigen and the particular neuropsychiatric encephalitis syndrome associated with that antigen are, not surprisingly, strongly correlated. Moreover, the titer of a specific antibody is correlated to disease severity. Upon aggressive immunomodulation therapy, these patient often improve in concert with a decrease in antibody titer of the CSF (Dalmau et al., 2008; Florance et al., 2009), although many patients can have positive antibody titers several years after their recovery. Work from several labs has shown that associated antibodies likely cause the neuronal and circuit abnormalities that manifest as neuropsychiatric symptoms, using several complementary approaches.

In assays of antibody-antigen binding that rely on immunoprecipitation or radioligands, it is not possible to distinguish between binding of antibody directly to the protein of interest or to a non-antigenic protein that is bound to the antigenic protein in a multiunit complex. To show that the putative target antigen is indeed sufficient to bind patient antibodies, it is important to demonstrate that patient antibodies can immunostain heterologous cells exogenously expressing the putative target antigen, and do not bind to heterologous cells not expressing the exogenous protein. The CSF of autoimmune encephalitis patients may contain antibodies against several distinct antigens, and the results from immunoprecipitation, neuronal staining and heterologous cell staining would be consistent with the possibility of antibodies to multiple antigens. To show that the putative target antigen is specific and necessary to bind to immunoglobulins in a patient's CSF, a demonstration that patient antibodies do not bind to neuronal tissue from rodents that lack the target antigen is required (Fig. 1).

Another confounding factor is that any effect of CSF on neurons in vitro or in vivo may be the result of some factor(s) other than self-reactive immunoglobulins that does not specifically bind to the putative target, for example elevated cytokines and other sequelae of inflammation. To show that the immunoglobulin fraction of the CSF is necessary and sufficient to disrupt normal functioning of the target antigen, one needs to demonstrate that the effect of CSF is reproduced by IgG purified from CSF, but not by the CSF fraction depleted of IgG. To confirm that antibodies cause neuronal dysfunction and hence are the most likely candidate CSF factor to cause disease, the specific antibodies are purified from CSF by incubation with immobilized antigen. Treatment with this immunodepleted CSF results in no adverse effects on neurons, while the specific antibodies purified from patient CSF recapitulate the effect of whole patient CSF. The specific binding of antibodies along with an isolated change in the properties of the cognate antigen provides a plausible biological mechanism for the causal link between patient antibodies and the neuropsychiatric manifestation of the associated disease.

Cellular and synaptic mechanisms mediated by antibodies

Antibodies against ionotropic glutamate receptors

The best characterized autoimmune encephalitides in patients as well as in the laboratory are ones associated with antibodies to ionotropic glutamate receptors: NMDA receptors (NMDAR) and AMPA receptors (AMPAR). Anti-NMDAR encephalitis patients classically present with severe aggression, paranoia, hallucinations and delusions characteristic of acute psychosis along with personality changes and memory loss (Dalmau et al., 2007; Sansing et al., 2007). Within days or weeks of the appearance of psychiatric symptoms, patients begin to deteriorate neurologically; they have debilitating seizures, decreased level of consciousness, abnormal movements, autonomic instability,

and hypoventilation. In contrast to the varied symptoms in anti-NMDAR encephalitis, anti-AMPAR encephalitis most often presents with isolated memory loss and seizures (Lai et al., 2009).

Both NMDARs and AMPARs transmit glutamatergic excitation, but serve distinct functions at the cellular and circuit level. AMPA receptors mediate most of the fast excitatory synaptic transmission in the brain (Shepherd and Huganir, 2007), while NMDARs act as molecular coincidence directors. Open NMDARs allow calcium to enter the postsynaptic cell where the high local concentration of calcium acts as a second messenger. Together, they are essential for synaptic plasticity and memory.

NMDA receptors are usually formed from heteromers of two NR1 and two NR2 subunits (Kendrick et al., 1996; Laube and Kiderlen, 1997). There are four NR2 subunits (NR2A-D), which have 50-70% sequence identity in the extracellular domain; NR1 is ubiquitously distributed in the brain (Monyer et al., 1994; Standaert et al., 1994; Waxman and Lynch, 2005). AMPA receptors are predominantly heterotetramers composed of GluR1, 2, 3 or 4 subunits, and distinct combinations of subunits are expressed in a region-specific manner (Palmer et al., 2005). GluR1/2 and GluR2/3 levels are high in hippocampus and other limbic regions (Sprengel, 2006), similar to the distribution of immunostaining with patients' antibodies.

There are several possible molecular mechanisms by which antibody binding to synaptic proteins may result in a disruption in synaptic transmission that in turn alters circuit properties pathologically. Antibody binding may block the agonist binding site or inhibit conformational changes that promote the activation of receptors. In a classically known autoimmune disease of the neuromuscular synapse, myasthenia gravis, application of the pathogenic antibodies against nicotinic acetylcholine receptor

(nAChRs) from patients to outside-out patches of mouse myotubes irreversibly blocks AChR currents (Jahn et al., 2000). In addition to agonist binding sites, both AMPAR and NMDAR have additional N-terminal binding sites for channel modulators such as zinc and polyamines that may be obscured by patients' antibodies (Rassendren et al., 1990; Herin and Aizenman, 2004; Paoletti and Neyton, 2007).

On the opposite end of the spectrum, the antibodies may promote the active conformation of the antigenic receptor and thus result in a constitutive or increased activity of the receptor. NMDA receptor NR2 subunit specific antibodies from patients with systemic lupus erythematosus (SLE) cause neuronal death when injected into mouse brain (DeGiorgio et al., 2001). This effect is attenuated by treatment with the NMDAR blocker, MK-801, suggesting the antibodies mediate cell death by enhancing channel activation (DeGiorgio et al., 2001). Synaptic transmission is finely tuned over many episodes of concurring electrical and biochemical activity in the cell. A change in the gain of synaptic transmission by an exogenous agent can result in the loss of information encoded by the synapse or even result in cellular injury from excitotoxicity or increased intracellular calcium levels. Thus, either activation or inhibition of the cognate receptor by antibodies may disrupt circuit dynamics by changing the finely tuned synaptic gain or by causing cellular injury.

If antibody binding does not change the properties of the cognate antigen at the molecular level, it can result in clustering of target receptors and their consequent displacement from their multiprotein complexes at the synapse. This can change target antigen localization in synaptic domains, deplete the target antigen by antibody mediated capping and internalization, or even alter the turnover or intracellular trafficking of the target antigen (Fig. 3A-C). Any of these possibilities would result in a loss of the role of the target receptor at synapses and hence adversely affect synaptic

transmission. In addition to an acute blockade of nAChR by pathogenic antibodies, nAChR antibodies from patients with myasthenia gravis have also been observed to cause a loss of surface nAChRs by cross-linking and internalization (Drachman et al., 1978). Similarly, cross-linking and internalization of voltage gated calcium channels by autoantibodies has also been shown to occur in patients with Lambert-Eaton syndrome (Nagel et al., 1988; Peers et al., 1993).

In the anti-glutamate receptor encephalitides, incubation of cultured neurons with patient CSF for 15-30 minutes does not change synaptic transmission mediated by NMDARs or AMPARs (Moscato et al., 2014, Peng et al., 2014). However, both anti-NMDAR1 and anti-AMPAR antibodies decrease the frequency and amplitude of mini EPSCs (Hughes et al., 2010; Moscato et al., 2014; Peng et al., 2014) within 12 hours of CSF treatment. This loss of glutamatergic signaling at the synapse after several hours of exposure of neurons to patient CSF is due to a reversible loss of receptors from synapses. For both diseases, incubation of neurons grown 14 to 21 days in vitro and treated with patient CSF for as little as 12 hours results in a titer-dependent decrease in density of synaptic receptors monitored by immunostaining (Fig 3D-E). There is also a decrease in the amount of surface receptors as seen by surface biotinylation followed by western blotting, and a consistent decrease in receptor clusters that colocalize with other synaptic proteins as assayed by immunostaining (Hughes et al., 2010; Moscato et al., 2014). The receptors lost from the surface are internalized because a pool of antibody bound receptors can be stained only upon permeabalization of the cell (Fig. 3B-C; Moscato et al., 2014).

The molecular and subcellular effects of patient CSF on NMDA and AMPARs are specific to the antigenic target of autoantibodies. The receptors are lost from the synapse without affecting neuron morphology, synapse density or the

surface localization or amount of other synaptic proteins. In anti-NMDAR encephalitis, despite a loss of NMDARs from synapses, there is no evidence for neuronal death, synaptic pruning, or a loss of AMPARs or GABARs at either glutamatergic or gabaergic synapses (Hughes et al., 2010). Similarly, anti-AMPAR antibodies exclusively affect synaptic AMPARs, without a decrease in PSD-95, vesicular glutamate transporter, NMDARs, or the AMPAR scaffolding protein, stargazin (Peng et al., 2014). This precise molecular excision of the target antigen from synapses reflects the specificity of anti-target antibodies.

Remarkably, the synaptic loss of NMDARs or AMPARs is reversible. After incubation for 24 hours, CSF can be washed out and replaced with culture medium. In both cases, the cluster density of NMDARs or AMPARs returned to baseline within several days. This reversibility of loss of synaptic glutamate receptors, together with the fact that there is little or no neuron death after treatment with patient antibodies, may explain the remarkable recovery of patients after CSF antibody titers are lowered by immunotherapy. It is unclear, however, whether the prolonged time of recovery (usually several months) represents persistence of the immune response in the brain or slow recovery of circuit dysfunction caused by the decrease of synaptic proteins like NMDARs and AMPARs that are critical for synaptic function and plasticity.

More is known about the mechanism of synaptic loss of NMDARs and AMPARs than other antigens. NMDARs that are lost from the surface are internalized into recycling endosomes and to some degree in degradation lysosomes (Moscato et al., 2014). This internalization effect of patient antibodies can be lost if the antibodies are converted into Fab fragments and lose their divalency (Fig. 2H). The divalency seems to be more important than the presence of the Fc domain as clustering the NMDAR bound Fab fragment with a second divalent anti-Fab antibody restores the ability of

the pathogenic antibodies to internalize NMDAR (Hughes *et al.*, 2010). This requirement for divalency of the antibody-antigen complex results in the sequestering of receptors in clusters and this signals internalization of the receptor clusters. In vitro immunostaining of neurons shows that treatment with patient CSF not only reduces the density of receptor clusters, but the remaining clusters are much larger than in control treated cultures. So, patient antibodies in anti-NMDAR encephalitis disrupt NMDAR contribution to glutamatergic synapses by their clustering effect, and may not need to agonize or antagonize NMDARs directly to cause the circuit dysfunction from abnormal glutamatergic signaling in anti-NMDAR encephalitis. One suggested mechanism for how antibody clustering of NMDAR results in their internalization is that upon antibody binding, NMDARs have a weaker interaction with the EphB2 receptor (Mikasova et al., 2012), which stabilize and retain NMDARs at synapse (Dalva et al., 2000, 2007). This effect is strengthened several fold when EphB2Rs are activated by the ligand ephrin-B2. In fact, exposing neurons to ephrin-B2 along with the pathogenic antibodies partially rescues the antibody-dependent internalization and loss of NMDARs both in vivo and in vitro (Mikasova et al., 2012). Measuring the diffusion of single particles using quantum dot imaging showed that when the NMDAR-EPHB2R complex is disrupted, NMDAR lateral mobility in the membrane is increased and NMDARs redistribute from being synaptically clustered to uniformly and diffusely distributed between the synaptic and extrasynaptic domains. Consistent with the observation that treatment with patient CSF reduces NMDAR density without affecting other synaptic components (Hughes et al., 2010), treatment with NMDAR antibodies has no effect on surface diffusion of GluA1, metabotropic dopamine receptor D1, alpha2 $GABA_AR$ subunits, or Kv1.3 (Mikasova et al., 2012).

Another mechanism for cellular and tissue damage seen in other

autoimmune diseases is opsonization of cells with autoantibodies followed by complement mediated inflammatory cytotoxicity. Muscle biopsies from patients with myasthenia gravis have revealed extensive deposits of components of the complement cascade (Engel et al., 1977; Sahashi et al., 1980). Autopsy and *in vitro* studies have also linked complement activation with Rasmussen's encephalitis and neuromyelitis optica, the latter characterized by antibodies to aquaporin-4 (Whitney et al., 1999; Lucchinetti et al., 2002; Waters et al., 2008). Although antibodies from patients with synaptic autoimmune encephalitides can fix complement in vitro, biopsies from patient brains do not contain complement deposits (Martinez-Hernandez et al., 2011). Also, given the minimal gross tissue damage and the near complete recovery of most patients that are diagnosed and treated promptly, it is unlikely that complement mediated cell death plays a major role in clinically relevant pathogenesis.

Antibodies against voltage gated K^+ channel associated proteins

Two overlapping syndromes are associated with proteins that are part of the voltage gated K channel complex (Klein et al., 2013). The CNS dominant disease called Morvan syndrome is caused by antibodies against the leucine rich glioma activated 1 protein (LGI1; Lai et al., 2010). It is characterized by limbic encephalitis and autonomic dysfunction, but with neuromyotonia less frequently (Liguori et al., 2001). The PNS dominant disease, Isaac's syndrome, is caused by antibodies against contactin-associated protein-like 2 (CASPR2; Shillito et al., 1995; Lancaster et al., 2011), although around half of anti-CASPR2 encephalitis patients also exhibit encephalitis.

LGI-1 is a secreted protein that has been linked to a pediatric epilepsy disorder known as autosomal dominant lateral temporal lobe epilepsy (ADLTE; Nobile et al., 2009). Animal experiments support the role of normal LGI-1 activity in preventing seizure activity; mice with LGI-1 knocked out in pyramidal neurons during embryonic

stages exhibited early-onset seizures that were commonly lethal (Boillot et al., 2014). LGI-1 when knocked out in rats, mice or zebrafish causes hyperexcitability and seizures (Fukata et al., 2010; Teng et al., 2010; Yu et al., 2010).

There are several known functions of LGI-1, any or all of which could be disrupted by the pathogenic anti-LGI-1 antibodies in patients. LGI-1 It integrates into a complex with presynaptic voltage-gated potassium channel Kv1.1 and prevents its inactivation by blocking the cytoplasmic regulatory protein Kvbeta1 (Schulte et al., 2006). It has also been shown to complex with synaptic scaffolding proteins ADAM22 and ADAM23, which stabilize and retain AMPARs at the synapse. LGI-1 knockout rats had decreased AMPA mEPSP amplitude in the hippocampus of acute brain slices suggesting that LGI-1 potentiates AMPAR-mediated synaptic transmission (Ohkawa et al., 2013).

In anti-LGI-1 encephalitis, antibodies result in a loss of AMPARs from glutamatergic synapses. The antibodies disrupt LGI-1 and ADAM22/23 interaction that may destabilize the machinery that sequesters AMPARs to synaptic membranes. Soluble extracellular ADAM22 mimicked the effect of anti-LGI-1 antibodies on AMPAR trafficking out of the synaptic domain. This is similar to the disruption of the interaction between NMDARs and EphB2Rs by anti-NMDAR antibodies (Mikasova et al., 2012). It would be interesting to measure the diffusion coefficient of quantum dot labeled AMPARs after exposing the neuron to anti-LGI-1 antibodies. The role of LGI-1 in maintaining AMPAR levels has been confirmed in vivo. LGI-1 null mice have reduced AMPARs at synapses. The ADAM22 complex binds to ADAM23 presynaptically and this is thought to regulate levels of synaptic AMPAR levels. Changes in AMPAR levels underlie plasticity, global brain excitability, and homeostatic scaling that maintains the average synaptic gain while preserving the information encoded in the relative strengths of synaptic inputs onto a

neuron (Shepherd and Huganir, 2007; Lu et al., 2009; Ohkawa et al., 2013)

Antibodies against GABA receptors

Patients with antibodies against either the ionotropic $GABA_{A\beta3}$ subunit or metabotropic $GABA_B$ receptor B1a subunit typically present with seizures that are refractory to antiepileptic treatment. Patients with either disease have a higher risk of mortality typically due to unmitigated status epilepticus (Höftberger et al., 2013; Petit-Pedrol et al., 2014). The patient presentations are consistent with the known functions of both ionotropic and metabotropic GABA receptors. Human mutations in either $GABA_A$ or $GABA_B$ have been associated with genetic epilepsy syndromes. Mice that are genetic nulls for either $GABA_A$ subunits or $GABA_B$ receptors have a lower seizure threshold (Prosser et al., 2001). Important antiepileptic and anxiolytic drugs such as benzodiazepines and barbiturates modulate $GABA_A$ activity. Similarly, baclofen, a GABAB antagonist, is used as an anti-spasmodic.

Despite the similarity in presentation and clinical course, the mechanism of antibody-mediated disruption of the cognate receptor is different in the anti-GABAA and anti-$GABA_B$ encephalitis. Similar to the encephalitides with antibodies against glutamatergic ionotropic receptors, patient anti-GABAA antibodies reduce both synaptic GABAA receptors as seen by immunostaining and picrotoxin sensitive IPSCs in cultured hippocampal neurons. The effects of these antibodies are specific for the cognate receptors; anti-GABAA antibodies do not affect NMDARs, glutamatergic synaptic transmission, or the localization or amount of the GABAA synaptic anchoring protein, gephryn.

In contrast to anti-GABAA receptor antibodies, it is striking that anti-$GABA_{B1}$ receptor antibodies do not result in a loss of surface $GABA_B$ receptors. How then do anti-

$GABA_B$ antibodies result in a change in inhibitory tone via $GABA_B$ activation that is likely to underlie the refractory status epilecticus in patients? In live cultured neurons, application of a $GABA_B$ agonist such as baclofen results in a reduction in global synaptic activity. Anti-$GABA_B$ antibodies block this effect. If $GABA_B1$ antibodies do not reduce the density of $GABA_B$ receptors, then they must inactivate or interfere with the target receptors. One possibility is that the antibodies act as an orthosteric or an allosteric blocker of $GABA_B$ receptors. This would predict that patient antibodies can disrupt $GABA_B$ signaling with an acute application, and the antibody blocking effect should be dose dependent. Additionally, an allosteric agonist of $GABA_{B1}$ or an activator of $GABA_{B2}$ should circumvent the $GABA_B$ signaling block by patient antibodies. Another possibility is that the patient antibodies activate the $GABA_B$ receptors and a prolonged incubation with patient antibodies results in a tonic constitutive $GABA_B$ signaling, which then results in deactivation of receptors. These questions are currently being addressed by studying the pharmacological properties of anti-$GABA_B$ antibodies in both cultured neurons and heterologous cultured cells that have been transfected to express $GABA_B$ receptors exogenously.

Homeostatic plasticity in response to antibody-mediated decrease of receptor levels

Homeostatic plasticity is important for maintaining the stability of neuronal network activity in the face of potentially destabilizing changes in the strengths of individual synapses. Compensatory mechanisms at the cellular and synaptic level have been shown to occur in autoimmune disorders of the nervous system in humans and in experimental model systems. Studies from mouse models of myasthenia gravis and patients' tissue have shown an enhanced rate of synthesis of AChRs and increased expression levels of AChR α, β, δ, and ε subunits, as well as increased

ACh release upon stimulation (Wilson et al., 1983; Guyon et al., 1994, 1998; Plomp et al., 1995). Similarly, in the CNS, Purkinje cells treated with IgG from patients with Lambert-Eaton syndrome show a loss of P/Q-type voltage gated calcium channel currents and a concomitant increase in R-type currents (Pinto et al., 1998). The NMDAR hypofunction in anti-NMDAR encephalitis led us to the question of whether patient antibody treatment can induce homeostatic changes in cultured neurons.

It is well known that after pharmacological blockade of glutamate receptors, cultured neurons compensate by changing both excitatory and inhibitory synaptic strength. Several studies have shown that, after 48 hours of NMDAR or AMPAR blockade, mEPSC amplitude is enhanced (Turrigiano et al., 1998; Sutton et al., 2006). Treatment of cultured neurons with antibodies from patients with anti-NMDAR encephalitis results in a decrease in glutamatergic transmission via NMDA receptors. Therefore it is plausible that neurons compensate for a loss of functional NMDA receptors by reducing inhibitory synaptic transmission or by increasing NMDAR and AMPAR expression. Treatment of cultured neurons does not increase expression of NMDARs or AMPARs, but result in a decrease in inhibitory synapse density visualized as colocalizing GABAA and VGAT clusters (Moscato et al., 2014).

Similarly, in anti-AMPAR encephalitis, antibody treated neurons compensate for the loss of AMPAR conductance by decreasing GABAAR mIPSC amplitude. In addition to changes at the synapse, treatment with anti-AMPAR antibodies increases intrinsic neuronal excitability (Peng et al., 2014). While the average action potential firing frequency was unaffected, antibody treatment changed the pattern of firing as evidenced by a decrease in inter-spike interval. These compensatory changes are consistent with numerous observations that pyramidal neurons tend to homeostatically maintain firing rate in response to chronic inactivity (Turrigiano et al., 1998; Burrone et al.,

2002). This may explain why despite reducing excitatory synaptic transmission via glutamate receptors, patient antibodies are capable of inducing synaptic and neuronal changes that contribute to the short-term memory loss and seizures observed in patients with anti-AMPAR encephalitis.

In vivo effects of anti-receptor antibodies on circuits and behavior

Patient antibodies disrupt their cognate antigens at synapses in cell culture. This synaptic dysfunction and the accompanying homeostatic changes are likely to underlie the circuit malfunction and psychologic and neurologic deficits in patients. To test whether anti-NMDAR antibodies from patients can disrupt NMDAR-dependent circuit properties in vivo, Zhang et al. (2012) tested the effect of a short treatment with patient antibodies on LTP induction at Schaffer collateral–CA1 synapses in rodent acute hippocampal slices. Genetic and pharmacological block of NMDAR blocks induction of LTP at these synapses although their activity is not necessary for maintenance of LTP. In slices treated with anti-NMDAR antibodies for 15 minutes, the induction of LTP by theta burst stimulation was suppressed (Fig. 4C-D; Zhang et al., 2012). This suppression was not present when slices were treated with patient CSF that had antibodies adsorbed on HEK cells overexpressing NMDAR. LTP in hippocampal synapses is a leading candidate for the circuit property that encodes place memory and loss of LTP induction in the hippocampus of rodents after exposure to patient antibodies may disrupt memory in awake behaving animals.

In a large rodent study, Planaguma et al. (2014) generated a passive immune transfer model of anti-NMDAR encephalitis to test the effects of patient antibody infusion on animal behavior. Using osmotic pumps, they infused a dialyzed mixture of CSF from several patients or controls into the cerebral ventricles of mice for 14 days. The pumps

were removed after 14 days to enable studying recovery from a chronic exposure to pathogenic antibodies from patients. A dramatic loss of novel object memory and anhedonia in the mice with chronic infusion of patient antibodies was observed over 14 days of infusion. However, by day 18 (day 4 after cessation of infusion), both the memory deficits and anhedonia had returned to control levels (Fig. 4E). The behavioral deficits correlated with a NMDAR levels in the brain, which also decreased during the 14-day infusion and recovered completely by day 21 (Planagumà et al., 2014). This study demonstrates the pathogenic potential of antibodies present in patients with anti-NMDAR encephalitis. It also establishes the relationship between the effects of pathogenic antibody on synapse and circuit function, and the changes in behavior, memory and cognition that are hallmarks anti-NMDAR encephalitis.

Recent translational studies have begun to shed light on whole brain activity during anti-NMDAR or anti-AMPAR encephalitis. Diffusion tensor MRI in anti-NMDAR patients shows decreased functional connectivity between the two hippocampi and extensive white matter changes that correlates with disease severity (Finke et al., 2013). Imaging studies in anti-AMPAR encephalitis patients have identified hypermetabolism in the limbic area on 18-F-FDG PET, which correlates with severity of clinical course (Spatola et al., 2014). EEG studies identified a characteristic electrographic pattern in anti-NMDAR encephalitis known as “extreme delta brush”, characterized by rhythmic delta activity at 1-3 Hz with superimposed 20-30 Hz bursts superimposed on each delta wave (Schmitt et al., 2012; Armangue et al., 2013). The clinical significance of the extreme delta brush pattern is unclear, but reflects widespread cortical and subcortical network dysfunction.

Mechanisms of autoimmunity

In the majority of autoimmune encephalitides, patients are much more likely than normal to have an associated neoplasm. These encephalitides were initially classified as paraneoplastic syndromes. The associated tumors are often dedifferentiated tissues that express many neuronal proteins. Among patients with teratomas, the ones with a comorbid NMDAR encephalitis were more likely than neurologically intact patients to have dysplastic CNS neurons in teratomas that express NMDARs (Day et al., 2014). The tumor microenvironment along with the inappropriate expression of neuronal proteins in non-neuronal contexts may contribute to the breakdown of immune tolerance (Maueröder et al., 2014). Another possibility is that mutant genes in tumors produce proteins are structurally different enough from the wild type protein to escape immune tolerance and induce an immune response that is cross-reactive to both the mutant and wild type gene product (Engelhorn et al., 2006). A well-studied example is scleroderma, an autoimmune connective tissue disease, in which a subset of patients that have autoantibodies to RNA polymerase III are more likely to have cancer (Shah et al., 2010). The cancers in these scleroderma patients harbor missense mutations in the gene encoding for RNA polymerase III, *POLR3A*, and the T cells in these patient that were reactive to mutant RNA polymerase cross-reacted with the wild type form of the protein (Joseph et al., 2014).

Not all patients with autoimmune encephalitis have a co-morbid neoplasm. It is possible, but unproved, that an immune response elicited by the tumor and the subsequent antibody synthesis decreases the size or eliminates the tumor via antibody binding and complement mediated cytotoxicity. Thus, at the time of diagnosis, antibodies are present, but no tumor is detected.

A genetic predisposition to autoimmunity may contribute to sensitizing the immune system against essential synaptic proteins. Another evidence for the

association between tumor and synaptic autoimmunity is that higher titers correlated with poor outcome and presence of teratoma. In the case of anti-NMDAR encephalitis, the presence of a tumor is correlated with more severe disease and higher titers. Conversely, in patients with tumors, excision of the tumor was one of the most effective treatment modality (Gresa-Arribas et al., 2014).

Identification of the epitope of pathogenic antibodies in anti-NMDA, anti-AMPAR, anti-CASPR2 and anti-$GABA_{B1}$ encephalitides raise an intriguing possibility that regions of these synaptic proteins may be inherently immunogenic. In all these diseases, the identified epitope is nearly identical regardless of the age, sex, tumor status, or disease severity of patients (Gleichman et al., 2012). Moreover, in anti-NMDAR encephalitis, the epitope remained constant over disease progression (Gresa-Arribas et al., 2014). Domain swapping and point mutation experiments identified the N368/G369 region of the LIVBP domain of NMDAR1 to be the minimal epitope necessary for antibody binding (Gleichman et al., 2012).

Molecular mimicry or the cross reactivity of antibodies against infectious epitopes with normal host proteins can occur if the epitopes are sufficiently similar. Guillain-Barré syndrome (GBS), an immune mediated peripheral neuropathy affecting axons and myelin sheaths is a classic example of an autoimmune neurologic disease that is known to arise from molecular mimicry (Hahn, 1998; Hughes and Cornblath, 2005). The disorder is frequently preceded by an infection, often by *Campylobacter jejuni* (Rees et al., 1995; McCarthy and Giesecke, 2001). Patients' serum antibodies react with peripheral nerve gangliosides as well as lipooligosaccharide from *Campylobacter jejuni* (Oomes et al., 1995; Yuki et al., 2004). Similarly, a syndrome known as Sydenham's chorea is characterized by abnormal movements, hypotonia, and neuropsychiatric symptoms, and characteristically occurs after an

infection by group A streptococci (Marques-Dias et al., 1997; Kirvan et al., 2006). Like in GBS, antibodies from patients with Sydenham's chorea react with gangliosides expressed in the basal ganglia and cross-react with group A streptococcal N-acetyl-glucosamine (Bronze and Dale, 1993; Kirvan et al., 2003). Patients with SLE, a multisystem autoimmune disease, can harbor antibodies to double stranded DNA that also cross react with a single epitope present in the extracellular region of NR2A and NR2B of the NMDAR (DeGiorgio et al., 2001; Kowal et al., 2006). Cross reacting anti-double stranded DNA antibodies are more prevalent in SLE patients with neuropsychiatric symptoms, but can also be seen in neurologically normal patients.

The source and brain access of autoantibodies

Two different, but not mutually exclusive, sources of pathogenic antibodies can synthesis of antibodies within the intrathecal space by plasma cells derived from anti-neuronal targeted B cells that translated across the blood brain barrier and matured within the intrathecal space or the antibodies are synthesized peripherally and cross the blood brain barrier that has been pathologically disrupted.

Antibodies that are synthesized in peripheral lymph nodes and serum can cross the blood brain barrier passively and down their concentration gradient if the barrier becomes more permeable in disease. There are several methods for experimentally increasing BBB permeability to test the effect of peripherally generated antibodies on CNS functions including focal ultrasound (Kinoshita et al., 2006), hypertonic solute (Neuwelt et al., 1988) and lipopolysaccharide (Xiao and Jan, 2009). More physiologically relevant models of BBB disruption include peripheral inflammation (Rabchevsky et al., 1999; Huber et al., 2001), acute stress (Esposito et al., 2002), and epinephrine (Huerta et al., 2006). At least in rodent models, crossing of serum protein including antibodies

has been shown to increase after inducing a “leaky” blood brain barrier experimentally. Iodinated antibodies injected into rats were detected in the brain following osmotic opening of the BBB (Neuwelt et al., 1988). Kinoshita et al. showed that focal sonication caused BBB disruption allowing intravenously injected dopamine receptor antibodies to enter the brain and bind to antigen at sites of barrier breakdown (Kinoshita et al., 2006). A clinical observation that is consistent with the possibility of peripheral antibody synthesis with transudation across a disrupted blood brain barrier is that many patients with autoimmune encephalitis report a history of flu-like prodromal symptoms or demonstrated viral infection prior to presenting with neuropsychiatric syndrome of autoimmune encephalitis (Dalmau et al., 2004). It is possible that a systemic inflammation induced by a bacterial or viral infection transiently disrupts the BBB, and allows antibodies that were already present in the serum to enter the intrathecal space.

Memory B-cells that are able to cross a normal BBB will undergo re-stimulation, antigen-driven affinity maturation, clonal expansion, and differentiation into NMDAR antibody-secreting plasma cells. This mechanism, which occurs in other autoimmune diseases such as multiple sclerosis (Hauser et al., 2008), would explain the detection of intrathecal synthesis of antibodies in most patients with anti-NMDAR encephalitis. Clinical pathology investigations of CSF from autoimmune encephalitis patients support intrathecal synthesis of pathogenic antibodies. These patients often have an increased ratio of CSF IgG to serum IgG concentration. This could result from the intrathecal space being the source of antibodies and the peripheral space being the sink or anti-neuronal antibodies present in serum when given access to neuronal tissue get sequestered close to the neuronal tissue. Additionally, protein electrophoretic analyses of the CSF from autoimmune encephalitis patients demonstrates multiple distinct bands of IgG that are absent in serum (oligoclonal bands), supporting the presence of plasma cell

clones within the thecal space that that are absent in serum and thus may have matured after their progenitor B cells extravasated intrathecally (Dalmau et al., 2008).

The relative lack of efficacy of strategies for treating autoimmune encephalitis by depleting serum IgG (IVIg, plasma exchange) is another piece of evidence to support the presence of the source of pathogenic antibodies intrathecally. IVIg and plasmapharesis alone are no longer considered standard of care or appropriate first line treatment for autoimmune encephalitis (Martinez-Hernandez et al., 2011). Patients that do not respond to IVIg or plasma exchange often improve with immunomodulatory drugs that affect immune cells such cyclophosphamide and rituximab. Also, a retrospective study of titers and disease progression showed that higher titers of antibodies in CSF but not in serum correlated with poor neurologic outcome (Gresa-Arribas et al., 2014). Additionally, relapses of neuropsychiatric decline correlated more with CSF titer change than serum titer.

There is also direct evidence for the presence of CD138+ plasma cells in perivascular, interstitial and Virchow-Robin spaces in brain biopsies from patients (Fig. 5). Biopsies in 14 cases and autopsy of three patients showed microgliosis, occasional inflammatory B-cell and plasma cell infiltrates, and very rare T-cell infiltrates (Martinez-Hernandez et al., 2011), in contrast to other paraneoplastic syndromes in which only cytotoxic T-cell infiltrates are prominent (Stein-Wexler *et al.*, 2005). These lymphocytes may be recruited by chemokines secreted by monocytes and microglia. Patients have a higher level of B-cell–attracting C-X-C motif chemokine 13 (CXCL13) in their CSF compared to controls but there was no difference in serum (Leypoldt et al., 2014). Moreover, anti-NMDAR patients from whom brain biopsy samples are available showed infiltration of monocytes and microglia that express CXCL13 (Fig. 5C). This suggests that despite being an immune privileged organ, the intrathecal space

of autoimmune encephalitis patients displays all the hallmarks of an organ with an adaptive immune system response: antigen presenting cells secreting chemokines, extravasation of immature lymphocytes, and mature plasma cells producing anti-neuronal antibodies.

Effects of synaptic receptor antibodies related to neurologic symptoms

Glutamate binding to NMDARs and AMPARs is crucial for synaptic transmission and plasticity. Pharmacological blockade or genetic reduction of NMDARs or AMPARs has been shown to alter measures of learning and memory and other behaviors in animal models (Mohn et al., 1999; Kapur and Seeman, 2002; Schmitt et al., 2007; Labrie et al., 2008). The balance between excitatory and inhibitory synaptic inputs is also altered, and this has been shown to affect circuit function and behavior (Prange et al., 2004; Epsztein et al., 2006; Kehrer et al., 2008). In addition, NMDA (Olney et al., 1999; Coyle and Tsai, 2003; Stahl, 2007) and/or AMPA (Wiedholz et al., 2008; Zavitsanou et al., 2008) receptor hypofunction has been proposed to be part of the pathophysiological mechanisms underlying schizophrenia.

It is interesting to consider why patients with anti-NMDAR antibodies develop a complex syndrome that includes psychosis, learning and memory dysfunction, abnormal movements, autonomic instability and frequent hypoventilation, while those with AMPAR antibodies preferentially develop psychiatric and amnestic symptoms. Studies using genetic deletion of NMDAR or AMPAR subunits in mouse models provide some insight into this issue. While NR1 knockout mice die shortly after birth due hypoventilation (Li et al., 1994), mice with spatially restricted NR1 deletion can survive into adulthood (Nakazawa et al., 2004). CA1-specific NR1 knockouts have impaired spatial and temporal memory and a loss of CA1 LTP (Tsien et al., 1996). Mice with an inducible,

reversible knockout of NR1 in forebrain show impairment in the maintenance of long-term memory if NR1 expression is turned off during the memory storage phase.

In addition to memory deficits, targeted manipulation of NR1 expression can result in schizophrenia-like symptoms. Hypomorphic expression of NR1 leads to increased stereotypic behavior and decreased sociability, while early postnatal loss of NR1 in a subset of cortical and hippocampal interneurons results in decreased pre-pulse inhibition and increased social isolation-induced anxiety (Mohn et al., 1999; Belforte et al., 2010). Moreover, subanesthetic doses of NMDAR blockers such as phencyclidine and ketamine are psychotomimetic, and they recapitulate many of the positive and negative signs of schizophrenia in humans and rodents as well as repetitive orofacial movements, autonomic instability and seizures (Luby et al., 1962; Krystal et al., 1994; Lahti, 2001; Krystal, 2002).The remarkable similarity between these phenotypes, the effect of patients' antibodies resulting in a dramatic decrease of synaptic NMDAR clusters and function, and the reduced levels of NMDARs in autopsied patients, support an antibody-mediated pathogenesis of anti-NMDAR encephalitis, and strengthen the NMDAR hypofunction hypothesis of schizophrenia (Belforte et al., 2010).

The consequences of loss of AMPAR expression have also been studied in mouse models. Spatial learning and memory are largely unaffected in GluR1 knockout mice despite the fact that LTP is reduced in CA1 and CA3 (Zamanillo, 1999) and working memory is diminished (Reisel et al., 2002; Sanderson et al., 2007). GluR2 knockout mice show reduced exploration and impaired motor coordination. In these animals, AMPAR mediated synaptic transmission is reduced, but LTP is enhanced (Jia et al., 1996; Gerlai et al., 1998). GluR2 knockout mice also have increased cell death (Feldmeyer et al., 1999; Oguro et al., 1999), possibly due to excitotoxicity related to increased, compensatory insertion of GluR1 homomeric AMPARs. While

AMPAR subunit knockout mice have not provided a satisfying explanation for the role of AMPARs in synaptic plasticity related to learning and memory, the fact that patients with AMPAR antibodies have short-term learning and memory deficits argues that further studies at the circuit and behavioral levels are warranted.

$GABA_{B1}$ receptor knockout mice display a variety of neurologic and behavioral abnormalities, including spontaneous seizures, enhanced anxiety, hyperactivity, hyperalgesia, and impaired memory (Prosser et al., 2001) suggesting dysfunction of the limbic system. Consistent with these experimental data, patients with anti-$GABA_{B1}$ receptor antibodies present with an encephalitis that associates with early and prominent seizures, confusion, agitation, behavioral problems and severe short-term memory deficit along with MRI abnormalities predominantly involving the hippocampi. Interestingly, both $GABA_{B1}$ receptor knock out mice and mice treated with a $GABA_{B1}$ receptor antagonist, CGP56423A, exhibit antidepressant-like behavior in a forced swim test and a learned helplessness paradigm (Nakagawa et al., 1999; Mombereau et al., 2004), suggesting that GABA signaling may have disparate effects on different aspects of mood such as depression and anxiety. Combined with animal studies, these patients can provide rich insight into the role of $GABA_{B1}$ receptor signaling in memory, behavior, and cognition.

Conclusion

Each of the clinical syndromes under the umbrella of autoimmune encephalitis is an opportunity to not only understand the mechanism by which the human body fails to distinguish self from non-self but also how autoantibodies cause CNS disease. The basic science and translational work on the unique aspects on each of the autoimmune encephalitides underscores the fact that despite a failure in immunotolerance, cellular

and humoral factors can disrupt their target antigen in distinct ways.

Figures

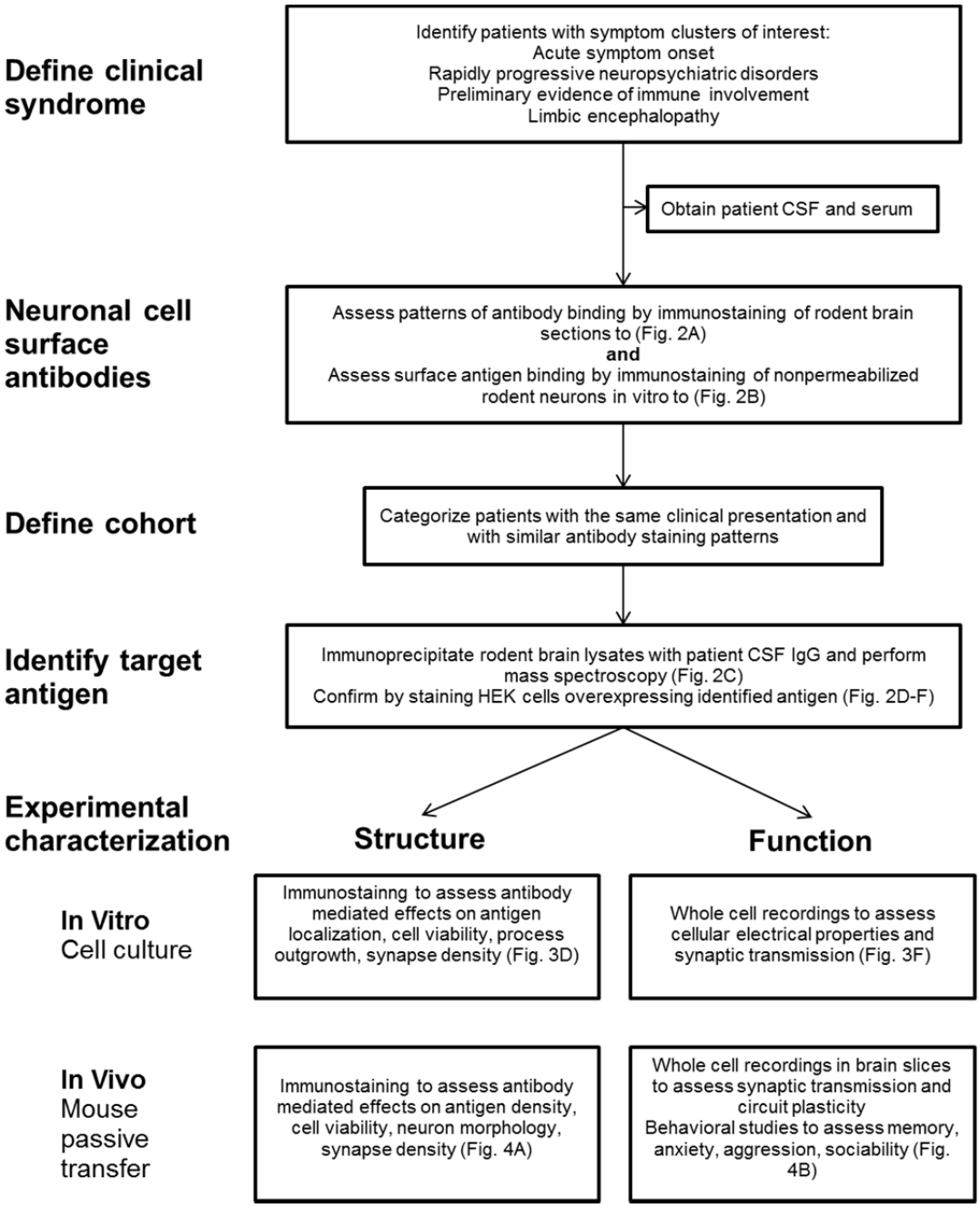

Fig 1. Flowchart of identifying a cluster of autoimmune encephalitis patients, identifying the target antigen, and laboratory characterization.

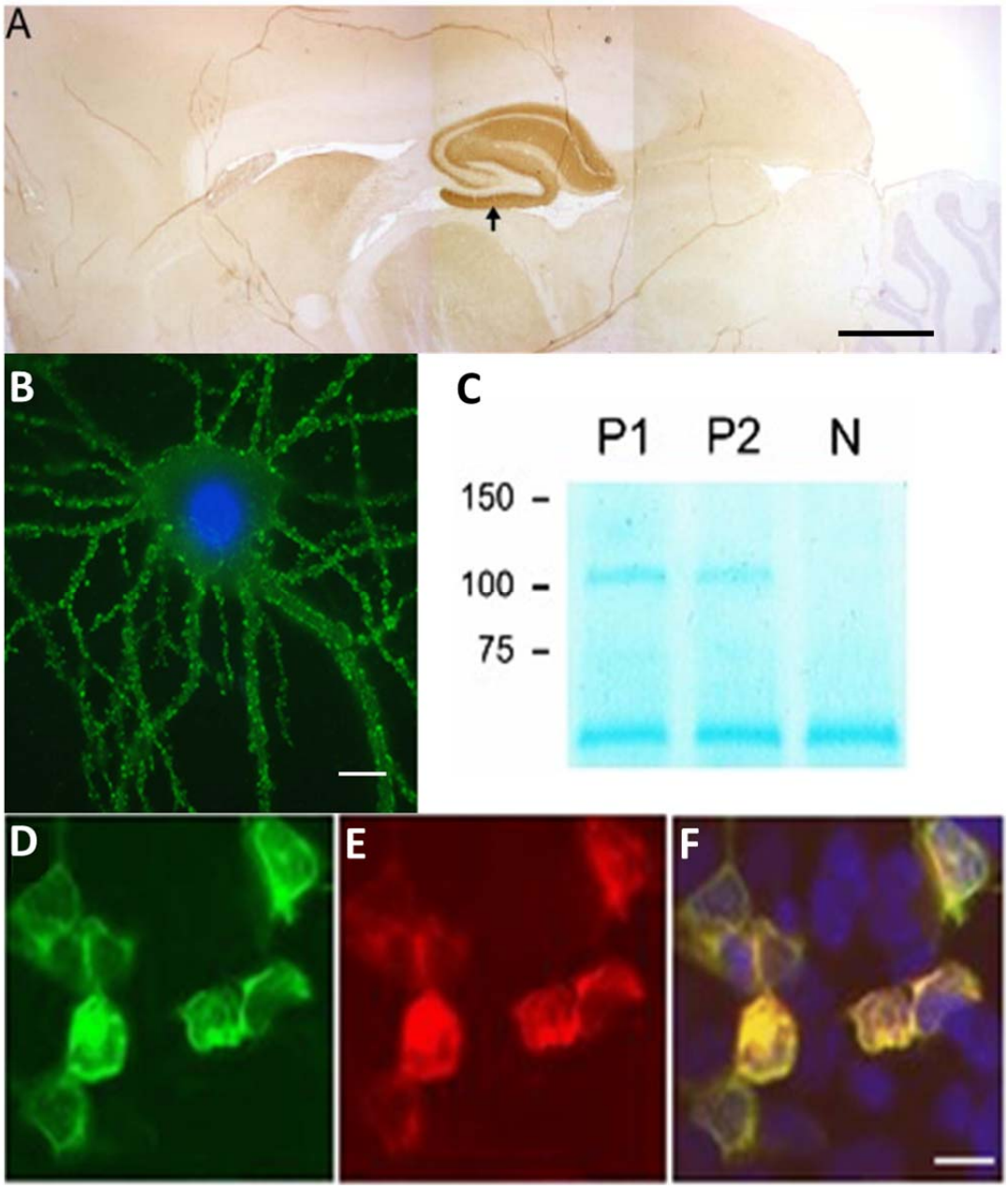
A
B
C
P1
P2
N
150
100
75
D
E
F

Figure 2. Process of characterization, antigen immunoprecipitation, and development of diagnostic tools. (A) Immunohistochemistry of rat brain section stained with patient CSF (anti-NMDA receptor encephalitis; Dalmau et al., 2007). Scale bar = 1mm. (B) Dissociated rat hippocampal neurons maintained *in vitro* and incubated (live, nonpermeabilized) with patient CSF. Note the binding of of patient's antibodies with clusters of cell surface antigens; scale bar = 10 μm. (C) Precipitation of these antigens using two patients' CSF antibodies is shown in a gel in which proteins are visualized with EZBlue. Note that patients' antibodies (P1, P2) precipitated a single band at ~100 kDa; this band is not seen in the precipitate generated with CSF from a control individual (N). Analysis of the 100 kDa band by mass spectrometry demonstrated sequences derived from GluR1/GluR2 subunits of the AMPA receptor. The ~50 kDa band across all samples corresponds to IgG. Transfer of the proteins to nitro-cellulose and Western blot with GluR1 and GluR2 antibodies confirmed that the 100 kDa band contained both GluR1 and GluR2 subunits (not shown). (D-F) Further validation of the antigen was done in heterologous cells expressing GluR1/2, showing reactivity with patient's antibodies (D, green), a commercial monoclonal GluR1 antibody (E, red), and merged (F, yellow). Scale bar = 10 um. Figure panels from Lai et al., 2009.

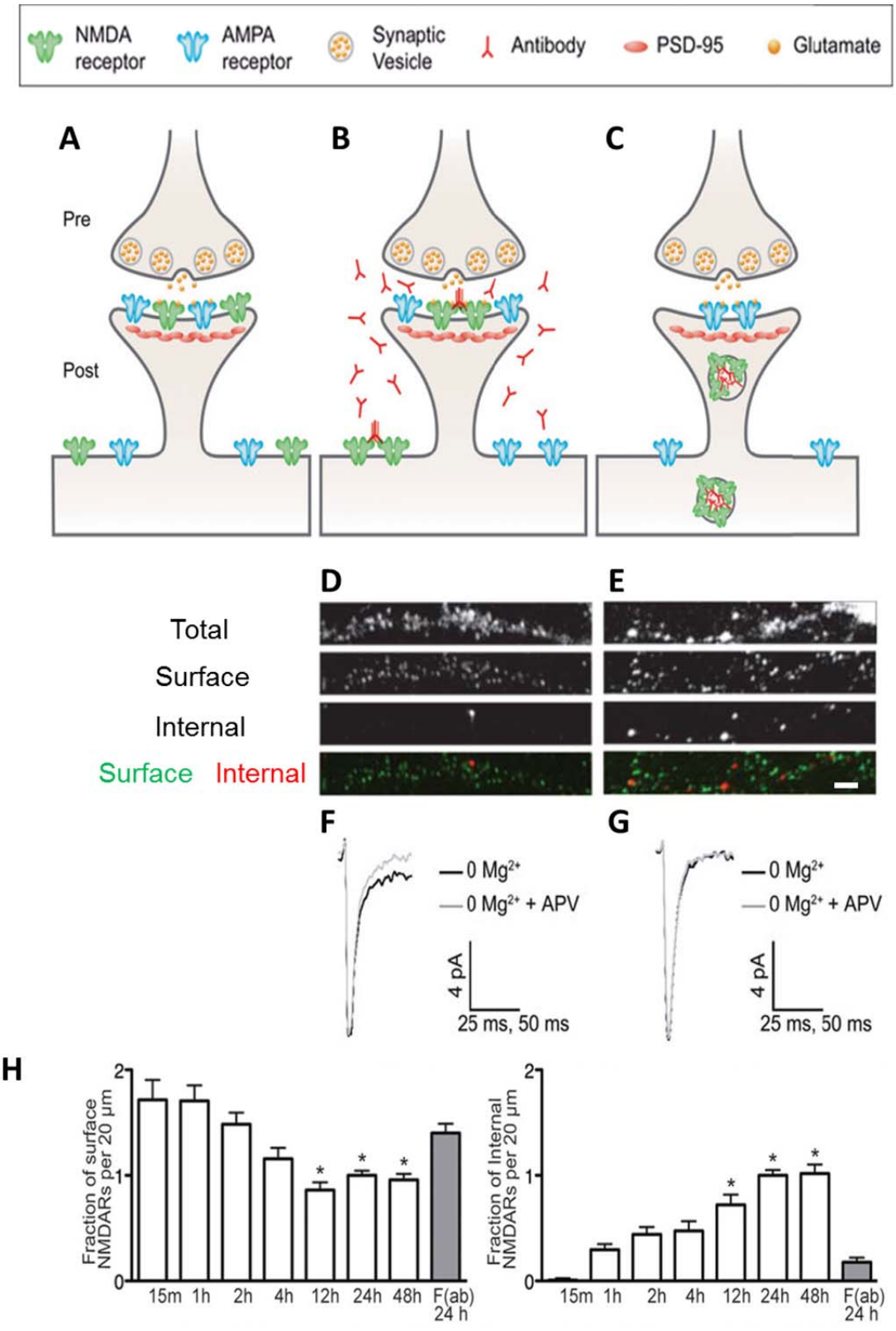
NMDA receptor
AMPA receptor
Synaptic Vesicle
Antibody
PSD-95
Glutamate
A
B
C
Pre
Post
D
E
Total
Surface
Internal
Surface Internal
F
G
0 Mg2+
0 Mg2+ + APV
4 pA
25 ms, 50 ms
H
Fraction of surface NMDARs per 20 µm
Fraction of Internal NMDARs per 20 µm
15m 1h 2h 4h 12h 24h 48h F(ab) 24 h

Figure 3. Synaptic effects of antibodies from patients with anti-NMDAR encephalitis. (A–C) AMPA and NMDA receptors are localized in the postsynaptic membrane and are clustered at the postsynaptic density. (A) Patient antibodies bind selectively to synaptic and extrasynaptic NMDA receptors, and this binding leads to receptor cross-linking (B). Cross-linked and clustered NMDA receptors are internalized, resulting in a decrease of surface NMDA receptors while other synaptic components, such as postsynaptic AMPA receptor clusters and PSD-95, as well as presynaptic terminals, dendrite branches, dendritic spines and cell viability, are unaffected (C). Panels A-C adapted from Moscato et al 2010. (D–E) Rodent cultured neurons treated within patient's CSF results in internalization of antibody bound NMDA receptors. After treatment with patient CSF for 1h (D) or 12h (E), surface NMDA receptors were stained using an anti-human IgG secondary antibody conjugated to Alexa 488 on live cultured neurons. After fixing and permeabalization, an anti-human IgG secondary antibody conjugated to Alexa 594 is used to stain the internalized antibody bound fraction of NMDA receptors. Counterstaining with a commercial antibody labeled the total pool of NMDA receptors. Scale bar = 1 um. (F–G) Whole-cell patch recordings of miniature excitatory postsynaptic currents (mEPSCs) that consist of a fast AMPA receptor–mediated component and a slower later component mediated by NMDA receptors that is APV sensitive. Compared with neurons without patient antibody mediated internalization, those treated with CSF from a patient with NMDA receptor antibodies show a loss of the APV-sensitive NMDA receptor component G (Hughes et al., 2010). (H) Quantification of surface and internalized NMDARs following treatment. Surface NMDAR density was significantly decreased after 12 hours of patient CSF treatment compared with patient antibody-derived F(ab) fragments after which surface levels reached a plateau. This was paralleled by an increase over time in the density of internalized NMDARs.

Panels D-H adapted from Moscato et al., 2014.

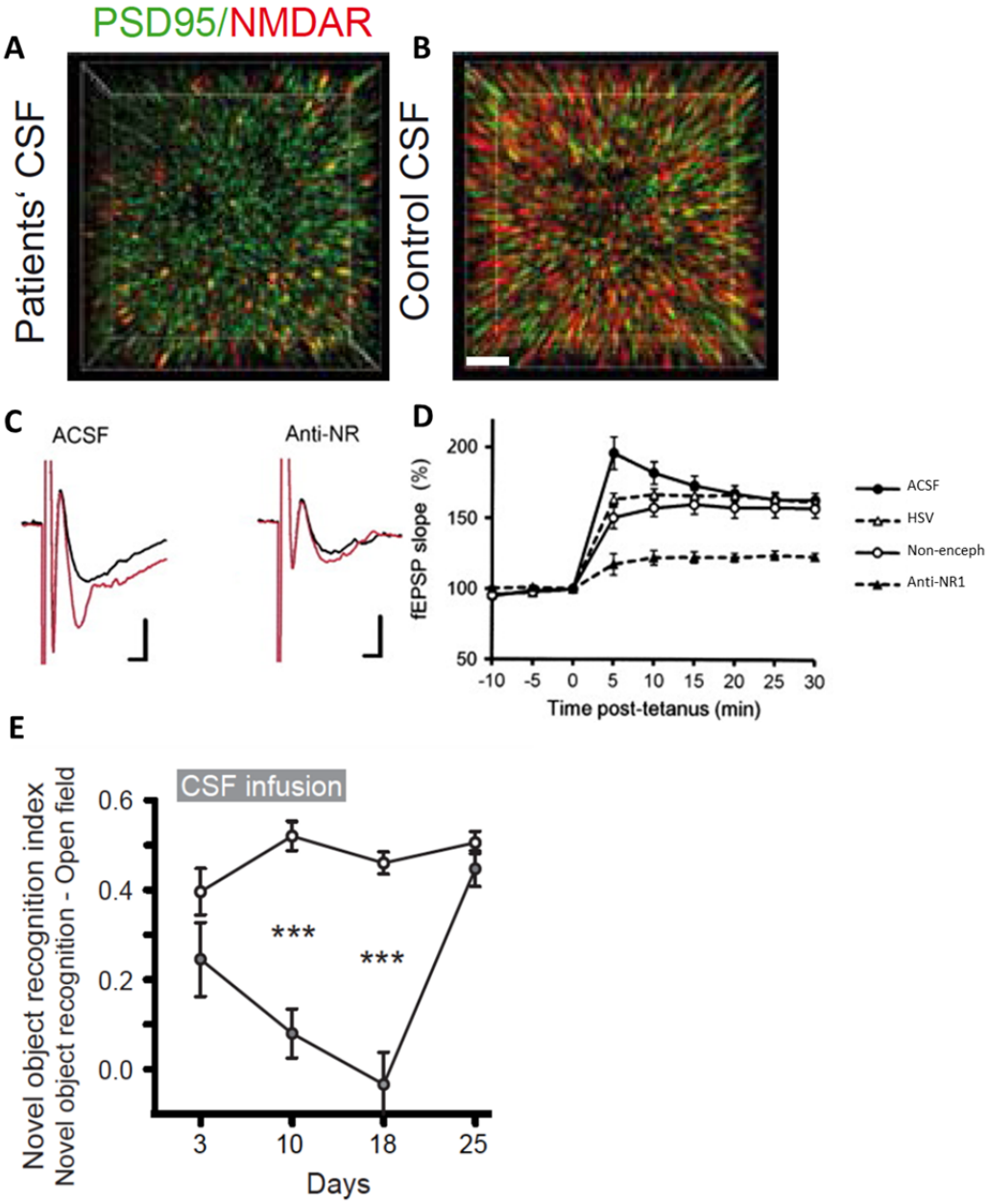
PSD95/NMDAR
A
Patients' CSF
B
Control CSF
C
ACSF
Anti-NR
D
fEPSP slope (%)
200
150
100
50
-10
-5
0
5
10
15
20
25
30
Time post-tetanus (min)
ACSF
HSV
Non-enceph
Anti-NR1
E
CSF infusion
Novel object recognition index
Novel object recognition - Open field
0.6
0.4
0.2
0.0
***
***
3
10
18
25
Days

Figure 4. Infusion of CSF from patients with NMDAR antibodies into rodent brains reduces synaptic NMDA receptors and results in deficits circuit plasticity, memory and behavior (A-B) Three-dimensional projection and analysis of the density synaptic clusters of NMDA receptors (defined as NMDAR clusters colocalized with PSD95) in CA3 region of hippocampus. (C-D) Suppression of LTP by CSF obtained from an anti-NMDAR encephalitis patient (Zhang et al., 2011). (C) Superimposed field EPSPs (fEPSPs) recorded at Schaffer colateral-CA1 synapses immediately before (black) and 30 min after TBS (red). For assessment of the magnitude of LTP, the initial slope of post-tetanic fEPSP (red) was expressed as percent of the pre-tetanic slope (black). The slope was measured as indicated in the middle panel (dotted lines). Scale bars: 2 ms and 0.5 mV. (D) Averaged LTP time course. The slope of fEPSPs was expressed as percent of those recorded immediately before TBS. The lines represent data from the artificial CSF control (solid line with filled circles), the non-encephalitis control (solid line with open circles), the herpes simplex virus encephalitis control (HSV; chain line with open triangles) and the NMDAR antibody positive case (chain line with filled triangles). (E) Infusion of CSF from patients with NMDAR antibodies causes deficits in memory, anhedonia and depressive-like behavior. Novel object recognition index in open field is reduced in animals treated with patients' CSF (grey circles) but not control CSF (white circles) after 14 days of chronic infusion. The patient CSF infused animals recover their novel object memory 11 days after the cessation of infusion (day 25 post surgery). A higher novel object Index indicates better object recognition memory. Figure adapted from Planaguma et al., 2014 and Zhang et al., 2011.

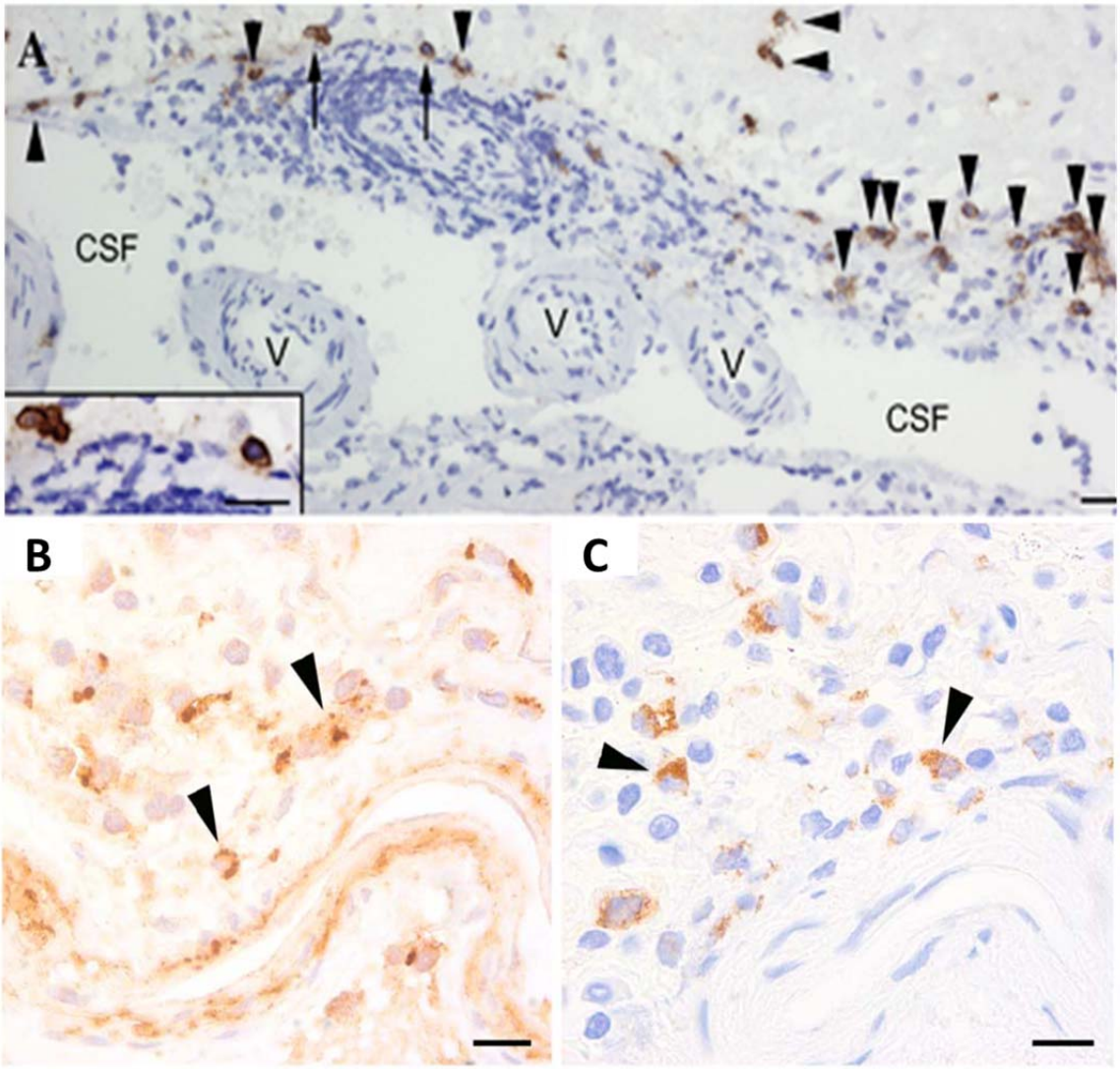
A
CSF
V
V
V
CSF
B
C

Figure 5. Brain biopsy of a patient with anti-NMDAR encephalitis showing perivascular lymphocytic and monocytic Infiltrates. (A) In Virchow-Robin spaces, $CD138^{+}$ plasma cells are in perivascular regions (arrows) and along the tissue surface (arrowheads) that delineates spaces containing CSF and small vessels (v). The plasma cells/plasmablasts indicated with arrows are amplified in the inset in K. Scale bars = 20 m. (B-C) Monocytic infiltrates expressing CXCL13, a B-cell attracting chemokine (B, arrow heads). Infiltrates were mainly composed of monocytes and macrophages (B, arrow heads indicate CD68 expressing monocytes and macrophages). Scale bar = 14 μm. Figure adapted from Stein-Wexler et al., 2005 and Leypoldt et al., 2014.

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CHAPTER 2. GABAB autoantibodies are antagonists

Antibodies from anti-GABAB receptor encephalitis patients are selective GABAB1 antagonists

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Abstract

Patients with antibodies to the metabotropic gamma-aminobutyric acid receptor type B ($GABA_B$) develop severe intractable seizures, but little is known about the underlying pathophysiological mechanisms. Using deletion mapping and expression in heterologous cells, we show that patient anti-$GABA_B$ receptor antibodies bind to alternatively spliced Sushi domains that is present in the presynaptically localized $GABA_{B1a}$ isoform. In contrast to anti-NMDAR antibodies from patients, $GABA_B$ receptors autoantibodies did not decrease in surface $GABA_B$Rs or their internalization. Treating neurons with $GABA_B$ receptors autoantibodies for as little as 30 minutes blocked the activation of $GABA_B$ receptors by baclofen in an autoantibody titer-dependent manner. Autoantibody-bound $GABA_B$Rs are still signaling competent, however, because baclofen block was circumvented by directly activating $GABA_{B2}$ with a selective agonist, CGP7930. Thus, patient $GABA_B$ receptor autoantibodies are selective $GABA_{B1}$ antagonists that may contribute to seizures by interfering with GABA-mediated inhibition. Brain penetrant $GABA_{B2}$ agonists may be useful to treat intractable seizures in anti-GABABR encephalitis patients.

Introduction

A growing family of autoimmune neurological diseases defined by pathogenic antibodies to neuronal cell surface proteins has been recognized over the last decade (Leypoldt et al., 2014). These diseases typically cause profound neuropsychiatric disability, and may be fatal (Vitaliani et al., 2005; Dalmau et al., 2008; Finke et al., 2012; Petit-Pedrol et al., 2014), but most patients improve markedly with immunosuppression, and many patients recover (Iadisernia et al., 2012; Gresa-Arribas

et al., 2014). Some of these disorders target important synaptic proteins including the ionotropic glutamate receptors, N-Methyl D-Aspartate receptor (NMDAR, Sansing et al., 2007; Dalmau et al., 2008) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR, Lai et al., 2009), the glycine receptor, the ionotropic gamma-aminobutyric acid receptor type A ($GABA_AR$, Petit-Pedrol et al., 2014) and the metabotropic gamma-aminobutyric acid receptor type B receptor ($GABA_BR$, Lancaster et al., 2010). The neuropsychiatric manifestations of these autoimmune encephalitides are similar to those caused by the genetic or pharmacologic disruption of the corresponding receptors in animal models and in human genetic diseases (Prosser et al., 2001; Brown et al., 2003). The specificity of the autoantibodies for receptor subunits, and often dominant epitopes of these proteins, is exquisite (Gleichman et al., 2012, 2014). While studies of the effects of patient antibodies on neurons, synapses, circuits, and behavior have begun to provide important insights into the pathophysiologic etiology of patient symptoms, many questions remain.

The cellular effects mediated by NMDAR and AMPAR antibodies have been studied in detail (Lai et al., 2009; Hughes et al., 2010; Moscato et al., 2014; Peng et al., 2014). These antibodies do not directly inhibit receptor function. Rather, NMDAR and AMPAR autoantibodies cross-link the cognate synaptic receptors on the surface of cultured neurons and result in a selective internalization of the target without disrupting other synaptic components or injurying neurons (Hughes et al., 2010; Moscato et al., 2010; Peng et al., 2014). Chronic perfusion of NMDAR antibodies into the cerebrospinal fluid (CSF) of rodents also decreases NMDA receptors after 14 days (Planagumà et al., 2014). This passive transfer of autoimmunity in mice results in anhedonia and diminished spatial memory. These effects are reversible in both *in vivo* and *in vitro* model systems (Moscato et al., 2010; Planagumà et al., 2014). Thus, these

mechanisms may underlie the neurological and psychiatric manifestations of these forms of autoimmune encephalitis, and account for recovery of many patients with immune therapy aimed at lowering antibody titer.

Antibodies to the extracellular domain of B1 subunit of the metabotropic $GABA_B$ receptor ($GABA_{B1}$) have been have been identified in a subset of patients with autoimmune limbic encephalitis (15, 10, and 20 patients in Lancaster et al., 2010; Höftberger et al., 2013; Dogan Onugoren et al., 2014, respectively). These patients typically present with subacute seizures that are resistant to treatment, along with confusion, memory loss and behavioral problems. Nearly half the patients have a co-morbid small cell lung cancer, and many patients improve with immunosuppression. Other reports have confirmed this association of antibodies to $GABA_{B1}$ with encephalitis, severe seizures, and small cell lung cancers (Lancaster et al., 2010). $GABA_{B1}$ may be the most common antigen in patients with small cell lung cancer and autoimmune encephalitis.

$GABA_B$ receptors are G-protein coupled receptors (GPCRs) for the inhibitory neurotransmitter GABA (Kaupmann et al., 1997). They are obligate heterodimers of $GABA_{B1}$, which binds GABA, and $GABA_{B2}$, which activates intracellular G-protein mediated signaling (Robbins et al., 2001; Schwenk et al., 2010). The $GABA_{B1}$ subunit has two distinct isoforms with different localization and function (Vigot et al., 2006). GABAB1a is found in presynaptic receptors, which can decrease in synaptic vesicle release by inhibition of presynaptic calcium influx (Harrison, 1990). The GABAB1b subunit is found in post-synaptic receptors (Hannan et al., 2012), which can reduce excitability by the opening of G-protein coupled inwardly rectifying K^+ channels (GIRK, Lüscher et al., 1997). Hypoactivity of $GABA_B$ signaling is implicated in epilepsy, spasticity, anxiety, addiction, autism and pain. $GABA_{B1}$ null mice have

decreased sensorimotor gating, spontaneous seizures, hyperlocomotion and memory impairment (Prosser et al., 2001). Baclofen, a selective $GABA_B$ agonist, is used routinely to treat spasticity in patients.

Here, we describe the target epitope of human $GABA_{B1}$ autoantibodies and demonstrate that these antibodies preferentially target the GABAB1a isoform of the receptor. The cellular effects of anti-GABAB1a antibodies on cultured hippocampal neurons are strikingly different from those of antibodies to ionotropic glutamate receptors. $GABA_{B1}$ antibodies do not result in internalization of surface $GABA_B$ receptors. Rather, patient $GABA_{B1}$ antibodies prevent activation of the $GABA_{B1}$ subunit by the agonist baclofen, without affecting the ability of the $GABA_{B2}$ subunit to induce G-protein mediated signaling. $GABA_{B1}$ receptor antagonism may account for the severe and intractable seizures that are prominent in patients with CSF $GABA_{B1}$ antibodies.

Materials and Methods

Patients and human samples

Cerebrospinal fluid and serum were obtained from patients with well-characterized clinical manifestations of anti-$GABA_B$ receptor encephalitis. Control samples were obtained from patients undergoing CSF screening for various disorders not associated with antibodies against known neuronal antigens. Control CSF used in this study was confirmed to not have any anti-neuronal antibodies using the methods described below. The clinical characteristics of patients with $GABA_B$ antibodies whose CSF was used in this study have been described previously (Lancaster et al., 2010; Höftberger et al., 2013).

Primary neuron cell culture and treatment

Hippocampi from embryonic day 18 rat pups were subjected to proteolytic digestion and mechanical disruption as previously described (Hughes et al., 2010). Briefly, dissociated cells were plated onto poly-L-lysine coated 15 mm coverslips at a density of 25,000 cells/cm^2 and incubated in Neurobasal medium. Neurons were treated on days in vitro (div) 17 – 21 with CSF from patients or controls at dilutions specified in the figure legends.

Antibodies

The following antibodies were used at the indicated dilutions for Western blots (WB), HEK293 cells (H) or dissociated neurons (N): GluN1 (rabbit, WB 1:1000, Millipore AB9864R), GluA1 (rabbit, WB 1:200, Millipore AB1504), GABAB1Rs (guinea pig, WB 1:200 N 1:500 H 1:2000, Millipore AB2256), GABAARβ2/3 (mouse, WB 1:200, Millipore 05-474), and β-actin (chicken, WB 1:1000, Abcam ab13822), myc tag (mouse, 1:1000, Sigma 9E10). Omission of primary antibodies was used as a control for each of the secondary antibodies, which were raised in goat and conjugated to

various Alexa Fluor or Dylight dyes (Jackson Immunoresearch).

Immunostaining and microscopy

Hippocampal neurons on coverslips were fixed and stained as previously described (Hughes et al., 2010). Briefly, neurons were fixed with freshly prepared 4% paraformaldehyde/4% sucrose for 10 min, blocked in 5% goat normal serum/ 0.1% Triton X-100 for 1 hour and washed in phosphate buffered saline (PBS) three times for 5 min each, incubated with primary antibodies diluted in block overnight at 4^{o}C and washed, then incubated in secondary antibodies for 1-2 hours at room temperature and washed. Coverslips were mounted, sealed and imaged on a confocal microscope (TCS SP5, Leica, Wetzlar, Germany).

To selectively label internalized human IgG, neurons were incubated with patient CSF (1:50), followed by incubation with goat anti-human secondary antibody conjugated to Alexa 488 (1:100). After 1-2 hours, neurons were fixed, permeabalized, and stained with goat anti-human secondary antibody conjugated to Alexa 594 to selectively label human IgG bound internalized receptors.

Images acquired on the confocal microscope were analyzed using custom-written ImageJ macros as previously described (Hughes et al., 2010). Briefly, images were background corrected by subtracting a Gaussian blur of the image, followed by adaptive thresholding and cluster detection to produce a binary mask of clusters (Bergsman et al., 2006).

Immunohistochemical staining of brain sections was carried out as described previously (Gable et al., 2009). Briefly, mouse brain hemisections were immersion fixed in freshly prepared 4% paraformaldehyde for 2 hours and then cryoprotected in 30% sucrose until equilibrated. Snap frozen brains were saggitally sectioned at 10 um, and

mounted on glass slides. Sections were immersed in 0.3% hydrogen peroxide for 10 min and then washed, blocked in 5% goat normal serum, incubated with patient CSF (1:50) overnight at 4^{o}C and washed, then incubated with biotinylated goat anti-human IgG secondary antibody (1:2000) and washed, incubated with avidin-biotin-peroxidase complex (ABC kit, Vector Labs) for 1 hour and washed. The sections were incubated with diaminobenzidine (DAB Eqv, Vector Labs) for 5 - 15 mins; the reaction was stopped when a section not incubated with patient CSF begins developing staining. The sections were washed, counterstained with hematoxylin, dehydrated in ethanol, cleared in Citrasolv (Fisher) and mounted with Permount (Sigma).

Biotinylation of surface proteins and analysis by Western blot

Five coverslips of dissociated hippocampal neurons were treated with patient or control CSF (1:25) for 24 hours and prepared for protein extraction as previously described (Hughes et al., 2010). Briefly, neurons were washed in PBS, incubated with 1mg/ml sulfo-NHS-biotin (Thermo Fisher Scientific) for 30 min at 4^{o}C, lysed in RIPA buffer (150 mM NaCl, 1 mM EDTA, 100 mM Tris-HCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH 7.4, supplemented with 1:500 protease inhibitor mixture III (Calbiochem) for 1 h at 4°C, and centrifuged at 4000g for 20 minutes. An aliquot of the supernatant was assayed for total protein. The biotinylated fraction of proteins in the remaining supernatant was adsorbed onto avidin-linked agarose beads (Immobilized Monomeric Avidin; Thermo Fisher Scientific) overnight at 4°C, beads precipitated, and the supernatant was saved to assay proteins in the intracellular fraction. Protein bound to beads was eluted to assay proteins in the surface fraction. Protein samples were denatured and reduced by boiling in Laemmli buffer, separated on a 4-15% gradient SDS-PAGE gel and transferred to nitrocellulose membranes. The surface, intracellular and total fractions were probed with primary antibodies. Membranes were

incubated with alkaline phosphatase (AP)-conjugated goat secondary antibodies (1:3000, Cell Signaling), and signals were visualized using Western-Star chemiluminescent detection system (Applied Biosystems).

Heterologous cell transfections and cDNA constructs

GABAB1a and $GABA_{B2}$ cDNAs were the gift of Dr. Steve Moss (Calver et al., 2000). mCD8 cDNA was a generous gift from Dr. Steve Scherer. All deletion constructs were generated with the Quickchange kit following a modification of the manufacturer's protocol.

HEK293 cells (ATCC) were grown on Poly-D-lysine coated coverslips in 24 well plates in DMEM supplemented with 5% fetal bovine serum, 5% horse serum, glutamine and penicillin/streptomycin cocktail. At log phase of growth (60-80% confluency), the media was exchanged for 400 µL of serum-free media (OptiMEM, Invitrogen). For each well, transfection was carried out with a total of 1 µg of plasmid DNA premixed and preincubated with 3 µL of Lipofectamine LTX (Invitrogen) in 100 µL of OptiMEM. Twenty four hours after transfection, HEK 293 cells were fixed in 4% paraformaldehyde for 10 minutes, fixed in 5% goat normal serum for one hour and either used immediately for immunocytochemistry or frozen at -80^{o}C for later use.

Electrophysiological assays

Whole cell current clamp and voltage clamp measurements were recorded from div 17-21 neurons that had been treated with patient or control CSF for the duration specified. Extracellular solution was perfused at 1 ml/min and was composed of (in mM): 140 NaCl, 3 KCl, 2.5 $CaCl_2$, 0.6 $MgCl_2$, 30 glucose, 10 HEPES, pH titrated to 7.4 with 10N NaOH. Patch pipette electrodes were prepared from borosilicate glass with resistance within 4-8 MΩ. The composition of intracellular solution was (in mM): 140 Kgluconate, 2 $MgCl_2$, 10 HEPES, 11 EGTA, 7 glucose, 2 ATP, 0.3 GTP, pH

7.3. The pharmacological agents used were racemic baclofen (1 mM in water, pH 7.4 with NaOH, Tocris) and CGP7930 (10 mM in DMSO, Tocris) at the indicated concentrations. Before a gigaOhm seal was formed on neurons, voltage offset was compensated. Membrane resistance, series resistance, and membrane capacitance were determined from current transients elicited by a 5 mV depolarizing step from a holding potential of -80 mV before switching to current clamp mode with a command current of 0 mA. Membrane potential was amplified and low pass filtered with a 5 KHz cutoff Bessel filter before being sampled and digitized at 10 KHz. Firing rate was determined offline using StimFit (Schlögl et al., 2013). Action potential recordings were acquired in current clamp mode. Action potentials were detected by counting threshold crossing of the first time derivative of the membrane potential trace.

Statistical Analysis

Data that passed tests for normality by the D'Agostino and Pearson test were analyzed with parametric tests, specifically t test for one factor with two levels, one-way ANOVA for multi-level experiments and two-way ANOVA for two factor experiments. Post-hoc testing for group differences in parametric tests was one using the Dunn test. In experiments where the sample statistic had a theoretical maximum or a minimum and deviated from normality were tested using non-parametric Kruskal-Wallis test with the Holm-Sidak post-hoc test for group differences.

Analysis of antagonism by patient CSF was done using Gaddum/Schild EC_{50} shift analysis by parametric nonlinear fitting in GraphPad Prism. The firing rate in the presence of $GABA_B$ ligands was converted to efficacy in percent. Firing rate that was identical to baseline was set as 0% efficacy while a firing rate of 0Hz i.e. maximal inhibition in the presence of drug was set as 100% efficacy. pA2, B and Schild slope were shared parameters between CSF concentration or duration of CSF

incubation but, the Hill slope parameter was unconstrained.

Results

Patient CSF contains antibodies that bind to $GABA_{B1}$ receptors

CSF samples from 10 patients with $GABA_B$ antibodies whose clinical characteristics were described previously (Lancaster et al., 2010; Höftberger et al., 2013) were used in these studies. We determined that all patients whose CSF was used in this study had anti-$GABA_B$ immunoglobulins by immunostaining unfixed, unpermeabilized cultured rat hippocampal neurons. Confocal microscopy confirmed that 10/10 patient CSF samples labeled clusters on the surface of cell bodies and processes (N = 3 replicates, 2-3 coverslips each, 4 neurons/coverslip). Whether these clusters contained $GABA_{B1}$ receptors was determined by analyzing images from neurons that were subsequently fixed, permeabalized and labeled with a commercial antibody to an intracellular $GABA_{B1}$ receptor epitope. Nearly all (85 $\pm$ 7%; N = 1 replicate, 3-8 coverslips each, 4 neurons/coverslip) human IgG-bound surface clusters were also labeled with the $GABA_{B1}$ antibody (Fig. 1B) overlapped with a subset of clusters (41 $\pm$ 15%) recognized by a commercial antibody to an intracellular epitope on $GABA_{B1}$ receptor (Fig. 1B). This confirms that the immunoglobulins in patient CSF recognize the $GABA_{B1}$ receptor subunit (Lancaster et al., 2010).

To verify that patient CSF autoantibodies bind directly to $GABA_{B1}$ receptors and not to other proteins that colocalize with $GABA_{B1}$, HEK293 cells were transfected with cDNA constructs encoding GABAB1a and $GABA_{B2}$ receptors and immunostained with patient CSF and/or commercial $GABA_{B1}$ receptor antibodies. Cells that expressed $GABA_{B1}$ were identified after immunostaining with commercial $GABA_{B1}$ antibodies (N = 2-10 replicates, 2-5 coverslips each). All cells that expressed $GABA_{B1}$ also bound human IgG from the CSF of the 10 patients used (Fig. 1C).

While CSF from patients contains antibodies to $GABA_{B1}$

receptors, it is possible that some of the neuronal reactivity with CSF could be explained by antibodies to other neuronal proteins. To evaluate this possibility, we immunostained sagittal brain sections from *Gabbr1*-null mice and wild-type littermates with CSF from patients and controls (Fig. 1D). All three patient CSFs tested, but not three control CSFs, strongly stained hippocampus, thalamus and striatum in wild-type brain sections in a pattern similar to their staining of wild-type rat brains (Lancaster et al., 2010). In contrast, neither patient CSF nor control CSF stained *Gabbr1*-null brain sections (Fig. 1D). These results suggest that the patient CSFs tested contain antibodies only to $GABA_{B1}$ receptor subunit.

Taken together, these results confirm that patients with anti-$GABA_{B1}$ receptor encephalitis have immunoglobulins in their CSF that bind to $GABA_{B1}$ receptors and that this binding accounts for the reactivity of their CSF with brain tissues.

Patient antibodies bind to GABAB1a receptor Sushi domains

Antibodies in patient CSF bind to an extracellular region of the $GABA_{B1}$ receptor subunit, as evidenced by surface staining of unfixed, unpermeabilized neurons (Fig. 1; Lancaster et al., 2010). $GABA_{B1}$ is comprised of a large N-terminal extracellular domain (NTD) that is linked to the seven transmembrane domain typical for the GPCR superfamily. The only extracellular epitopes of $GABA_{B1}$ are the NTD and the linkers between transmembrane helices 2-3, 4-5, and 6-7. To determine the minimal $GABA_{B1}$ epitope required for patient antibody binding, cDNAs encoding $GABA_{B1}$ receptors with different deletions (Fig. 2A) were expressed in HEK cells, followed by immunostaining with patient CSF.

When the NTD was deleted, 10/10 patient CSFs tested (N = 4 replicates, 6-10 coverslips, 100-200 cells) no longer bound to HEK293 cells expressing $GABA_{B1}$□NTD

(Fig. 2B). These results demonstrate that the NTD is necessary for autoantibody binding.

The NTD in GABAB1a has two subdomains, the ligand-binding Venus Flytrap Domain (VFT) and the alternatively spliced doublet of Sushi domains (SD) at the amino-terminus (Fig. 2A). All (7/7) patient CSFs tested bound to HEK cells transfected with the construct lacking the VFT domain (ΔVFT) (Fig. 2C; N = 3 replicates, 3 coverslips, 100-200 cells). This result shows that the VFT is not the immunodominant domain of $GABA_{B1}$.

Since the majority of the residues in the NTD make up the VFT, we next assessed the smaller SDs and short inter-domain loops as potential immunodominant domains. When SDs were deleted from GABAB1a, 3/3 patient CSFs tested no longer bound to transfected HEK cells (Fig. 2D; N = 2 replicates, 1 coverslip, 100 cells). This result indicates that the SDs is necessary for autoantibody binding.

It is possible that deleting $GABA_{B1}$ domains interfered with the correct folding of the remaining tertiary structure. Even if a misfolded protein remnant contained the epitope, it might fail to bind antibodies. To further confirm that the immunodominant epitope is within the SD, and the loss of autoantibody staining in the ΔSD construct was not due to a misfolded or otherwise blocked epitope on the remaining protein, the SD was fused to a membrane carrier derived from mouse CD8 receptors (NTD::CD8). HEK cells transfected with the NTD::CD8 fusion construct bound human autoantibodies from 3/3 patient CSFs (Fig 2E; N = 2 replicates, 2 coverslips; 100 cells).

Taken together, these data show that the alternatively spliced SDs in GABAB1a are necessary and sufficient for antibody binding.

Patient CSF antibodies do not reduce surface receptor cluster density

Previous work has shown that divalent antibodies in CSF from patients with anti-

NMDAR or anti-AMPAR encephalitis induces surface receptor loss, due to receptor crosslinking and internalization into recycling endosomes and lysosomes (Lai et al., 2009; Hughes et al., 2010; Moscato et. al., 2014; Peng et al., 2015). To determine whether $GABA_{B1}$ autoantibodies similarly crosslinked and internalized surface $GABA_B$ receptors, cultured neurons were treated with patient CSF for 24 hours followed by immunostaining and image analysis obtained by confocal microscopy.

As previously reported (Hughes et al., 2010; Moscato et al., 2014), a decrease in surface NMDA receptor clusters was observed after 24 h treatment with patient compared to control CSF (Fig. 3A-B, right patient CSF 9.6 $\pm$ 1.4 clusters/20 μm; control CSF 5.5 $\pm$ 1.4 clusters/20 μm; significantly different, $p < 0.05$; N = 2 patient and 2 control CSFs, 3 coverslips/patient, 4 neurons/coverslip). To assess antibody bound clusters that were internalized, neurons were treated with patient CSF for 24 hours, fixed, permeabilized and incubated with a secondary antibody to human IgG conjugated to Alexa 594. A concomitant increase in internalized receptor clusters was observed after 24 h treatment with anti-NMDA receptor encephalitis patient compared to control CSF, as previously reported (Hughes et al., 2010; Moscato et al., 2014) (Fig. 3C, right; anti-NMDA receptor encephalitis patient CSF 4.0 $\pm$ 0.2 clusters/20 μm; control CSF 0.1 $\pm$ 0.2 clusters/20 μm; significantly different, $p < 0.001$). Surprisingly, no change in the density of surface $GABA_B$ receptor clusters was observed after 24 h treatment with patient compared to control CSF. Treatment with 5 patient and 5 control CSFs followed by analyses of receptor cluster density as well as area and intensity were used to confirm the lack of receptor internalization by patient $GABA_{B1}$ antibodies (N = 3 experiments, 8-15 coverslips/CSF, 4 neurons/coverslip). Treatment of cultured hippocampal neurons for 24 h with patient or control CSF resulted in no change in cluster density (patient CSF

12.3 ± 1.22 clusters/20 μm; control CSF14.1 ±1.11 clusters/20 μm; not significantly different, p = 0.29), area (patient CSF 0.17 ± 0.008 μm^2; control CSF 0.18 ± 0.008 μm^2, not significantly different, p = 0.50) or intensity (patient CSF 80.1 ± 6.7; control CSF 78.1 ± 5.3 arbitrary values; not significant, p = 0.81) (Fig. 3D-F). Taken together, these data indicate that total surface $GABA_{B1}$ receptors are unchanged by patient antibodies after 24 h of treatment. No change in internalized receptor clusters was observed after 24 h treatment with patient compared to control CSF (Fig. 3C, left; patient CSF 0.1 ± 0.1 clusters/20 μm; control CSF 0.1 ± 0.1 clusters/20 μm; not significantly different, p = 0.20).

To determine whether longer antibody exposure led to loss of surface $GABA_{B1}$ receptor clusters and/or other structural changes, neurons were treated for 1, 2, 3 and 7 days with patient and control CSF followed by immunostaining with patient CSF to detect surface $GABA_B$ receptors, a commercial antibody to an intracellular $GABA_B$ receptor epitope to detect both intracellular and surface $GABA_B$ receptors, and Bassoon antibodies to stain presynaptic terminals. No change in surface $GABA_B$ receptor clusters was observed after 1 to 7 days of treatment (Fig. 3D; 1 day patient 16.2 ± 4.0 clusters/20 μm, control 15.6 ± 4.0, p = 0.99; 2 days patient 13.7 ± 4.9 clusters/20 μm, control 13.9 ± 5.0, p = 0.99; 3 days patient 11.5 ± 3.7 clusters/20 μm, control 19.0 ± 3.6, p = 0.17; 7 days patient 14.7 ± 5.8 clusters/20 μm, control 11.6 ± 6.0, p = 0.97; not significantly different, 2-way ANOVA, CSF treatment F(1,37) = 0.17, p = 0.67 followed by Holm-Sidak multiple comparisons). In addition, no reduction was observed in $GABA_{B1}$ receptor clusters recognized by the commercial $GABA_B$ antibody (Fig. 3E; 2-way ANOVA, CSF treatment F(1,37) = 1.46, p = 0.233). Treatment with patient or control CSF for 1 to 7 days did not result in a change in presynaptic terminal density visualized by staining for

Bassoon (Fig. 3F; 2-way ANOVA, CSF treatment $F(1,39) = 0.02$, $p = 0.88$).

We used an alternate method to measure the quantity of surface $GABA_B$ receptors. Surface biotinylation followed by Western blot analysis of $GABA_{B1}$ showed no difference between surface $GABA_{B1}$ protein after either 24 h or 72 h treatment in patient or control CSF (Fig. 3G). Similarly, no difference was observed in total $GABA_B$ receptor protein, surface GABAA or GluR1 receptor protein after 24 or 72 h treatment with patient or control CSF.

Taken together, these data show that, contrary to autoantibodies from anti-NMDA receptor, anti-AMPA receptor or anti-GABAA receptor encephalitis patients, $GABA_{B1}$ autoantibodies do not change surface $GABA_B$ receptor cluster density or protein level, total receptor protein expression, internalized clusters, other synaptically localized receptors (GABAA, GluR1 receptors) or presynaptic terminals.

Patient antibodies block $GABA_B$ receptor activation

We next investigated whether patient $GABA_B$ receptor antibodies have functional effects on $GABA_B$ receptor mediated signaling. We used the inhibition of action potential firing rate by a $GABA_B$ agonist as a functional readout of $GABA_B$ activation in cultured hippocampal neurons. Cultured hippocampal neurons form networks in vitro characterized by spontaneous action potential firing ranging from 0.5 to 5 Hz. Baclofen, a $GABA_B$ receptor specific agonist (Bowery, 1993) that inhibits action potential firing was used as a positive control. Treatment with 1-100 μM baclofen for 0.5-5 min eliminates action potential firing (Misgeld et al., 1995), likely due to inhibition of presynaptic terminals via GABAB1a-containing receptors (Howe et al., 1987; Harrison, 1990; Scanziani et al., 1992).

Consistent with previous work, whole cell recording in current clamp mode with no

current injection showed that cultured hippocampal neurons fired in the range of 1-10 Hz (mean 3.53 ± 0.91 Hz, N = 15 coverslips, 2 neurons/coverslip). After treatment with 100 μM baclofen, the action potential firing rate was dramatically reduced to at most 0.05 Hz (Fig. 4A-B; mean 0.031 +/- 0.008 Hz, N = 20 coverslips, 1 neuron/coverslip). However, cultured hippocampal neurons do not have a high baseline $GABA_B$ tone, because a high concentration (1 μM) of a potent $GABA_B$ antagonist CGP55845 (Kd = 30 nM; Brugger et al., 1993) did not change the firing rate (data not shown). Consistent with this, baseline firing rate is similar in neurons treated with either patient or control CSF (control CSF 3.54 ± 0.92 Hz, N = 2 CSFs, , 9 coverslips, 1 neuron/coverslip; patient CSF 2.85 ± 0.41 Hz, N = 3 CSFs, 15 coverslips, 1 neuron/coverslip (Fig. 4B; not significantly different, $t(22) = 0.68$, $p = 0.45$).

We analyzed the block of $GABA_B$ activation by 100 μM baclofen mediated by CSF from several controls and patients. Baclofen (100 μM) significantly reduced the firing rate of neurons pretreated for 24 hours with control CSF but was less efficacious in inhibiting the firing rate in neurons pretreated with anti-$GABA_B$ receptor encephalitis patient CSF (Fig. 4B). The efficacy of baclofen can be operationally defined as the relative reduction in the five minute average of firing rate during perfusion with baclofen relative to perfusion with artificial CSF. A 100 μM of baclofen was nearly maximally efficacious in inhibiting neurons treated with CSF from five different control patients similar to neurons that were not pretreated with CSF (efficacy ranges from 94.2% to 99.5%, $p>0.99$ for control CSF treated neurons compared to no treatment, N=5-8 coverslips, Dunn's multiple comparison post Kruskal-Wallis; Fig 4C). In contrast, neurons pretreated with CSF from patients 1, 2, and 3 were minimally silenced by 100 μM baclofen: -1.8 ± 5.4% (N = 16 coverslips, $p<0.001$), 10.3 ± 6.9% (N = 5 coverslips, $p=0.005$), and 26.6 ± 7.7%

(N = 10 coverslips, p = 0.001), respectively (mean percent of baseline ± SEM, p-values from Dunn's multiple comparison post Kruskal-Wallis non-parametric two factor analysis; Fig. 4C). These data indicate the patient GABABR1 autoantibodies are antagonists of $GABA_B$ receptors.

Patient GABABR1 antibodies do not inactivate $GABA_{B2}$ mediated signaling

The above data show that patient CSF abrogates $GABA_B$ function in neurons in vitro without reducing the number of surface $GABA_B$ receptors. We examined the possibility that patient antibodies disrupt $GABA_B$ activation by rendering them non-functional or inactivated by using a $GABA_{B2}$ agonist, CGP7930, which binds to the heptahelical domain of $GABA_{B2}$, directly activating it, which in turn activates G-protein signaling even in the absence of any agonist binding to $GABA_{B1}$ (Binet et al., 2004; Chen et al., 2005).

Neurons were pretreated with the most potent patient CSF (patient 1), then treated with 2, 10, or 100 μM baclofen, and then with 100 μM CGP7930. Compared to control CSF treated neurons, 2, 10, or 100 uM baclofen was less efficacious in patient CSF treated neurons by 99 ± 21% ($p < 0.001$, N = 3 coverslips), 69 ± 17% ($p = 0.001$, N = 5 coverslips) , and 55 ± 17% ($p = 0.006$, N = 9 coverslips), respectively (1 neuron/coverslip in each condition; Holm-Sidak multiple comparisons post two-way ANOVA). Despite the antagonistic effect on baclofen efficacy by $GABA_{B1}$ autoantibodies in patient CSF, the $GABA_{B2}$ agonist CGP7930 (100 μM) was equally efficacious in both control CSF or patient CSF treated neurons (Fig. 5A and 5B; control CSF: 99.5 ± 0.2%, patient CSF: 99.1 ± 0.3%; N = 4 coverslips, $p = 0.98$). These results indicate that patient $GABA_B$ autoantibodies prevent $GABA_{B1}$ activation by agonists, but do not affect the ability of $GABA_{B2}$ or downstream second messenger molecules to respond to $GABA_B$ receptor

mediated G protein activation.

Patient antibodies are competitive antagonists of $GABA_B$ receptors

To dissect the mechanism by which GABABR1 autoantibodies block $GABA_B$ receptor functions, we decreased the duration of exposure to autoantibodies before adding various doses of baclofen. In as little as 30 minutes of treatment with patient CSF, the efficacy of 3 μM baclofen was reduced to 34 ± 18% but the efficacy of 10 μM (84 ± 3%) or 100 μM (99%) baclofen remained largely unchanged. This represents a rightward shift in $logEC_{50}$ of baclofen-mediated neuron silencing from -6.4 ± 0.6 in the absence of patient CSF to -5.3 ± 0.02 after 30 minutes of treatment with patient CSF. By 2.5 hours, the block of $GABA_B$ activation by patient CSF is nearly maximal with a further shift in $logEC_{50}$ to -2.1 ± 0.5 (Fig. 6A). Statistical analysis confirms that the interaction of time x drug concentration has a significant effect on the baclofen block ($F(8,96) = 6.2$, $p<0.0001$). These data suggest that the block of $GABA_B$ receptors by autoantibodies in patient CSF is rapid compared to the 24 hours it takes for the surface density of NMDA receptors to reach half-maximal level after cross-linking and internalization in anti-NMDA receptor encephalitis (Moscato et al., 2014).

We evaluated the possibility that autoantibodies irreversibly bind to $GABA_B$ receptors and render them non-functional using a patient CSF of lower titer and thus potency (patient 4). The $GABA_B$ autoantibodies in patient 4 CSF inhibit baclofen-mediated silencing in a dose dependent manner (Fig. 6B; CSF concentration x baclofen concentration $F(4,194) = 29.5$, $p<0.0001$, N = 3 experiments 3 coverslips, 1 neuron/coverslip for each condition). After treatment with a 1:100 dilution of patient 4 CSF, the efficacy of 3 μM, 10 μM and 100 μM of baclofen was nearly maximal (94 ± 3%, 92 ± 3% and 96 ± 2%, respectively). This corresponds to a $logEC_{50}$ of approximately -6.6

$\pm$ 0.4. However, after treatment with a 1:20 or 1:5 dilution of patient 4 CSF, baclofen efficacy was greatly reduced in a CSF concentration dependent manner (1:20 CSF, logEC50 = -4.7 $\pm$ 0.07 and 1:5 CSF, logEC50 = -3.9 $\pm$ 0.1). Thus, increasing the baclofen concentration can overcome $GABA_B$ receptor block by patient $GABA_B$ autoantibodies, and an increasing titer of the patient CSF treatment result in a dextral shift in the dose-response curve of baclofen efficacy (Fig. 6B). These data suggest that patient GABABR autoantibodies may be competitive antagonists of $GABA_B$ receptors.

Discussion

Our work confirms that autoantibodies from anti-$GABA_B$ receptor encephalitis patients bind to $GABA_{B1}$ receptors (Lancaster et al., 2010), and extends it several ways. We show that the Sushi domain present in the presynaptically localized $GABA_{B1a}$ isoform is necessary and sufficient to bind patient anti-$GABA_B$ receptor antibodies, suggesting that the effects of $GABA_B$ receptor antibodies occur presynaptically. We demonstrate that $GABA_B$ receptor autoantibodies do not change surface $GABA_B$ receptor localization, in contrast to NMDA receptor autoantibodies from anti-NMDA receptor encephalitis patients. $GABA_B$ receptor autoantibodies block $GABA_B$ receptor activation by baclofen, suggesting that the clinical manifestations of $GABA_B$ receptor autoantibodies result from diminished $GABA_B$ receptor function, a mechanism which, to our knowledge, has never been observed in any other autoimmune disease. Patient $GABA_B$ receptor autoantibodies do not inactivate $GABA_B$ receptors, however, because the patient antibody mediated baclofen block was circumvented by directly activating the $GABA_{B2}$ subunit with a selective partial allosteric agonist, CGP7930. We conclude that patient $GABA_{B1}$ receptor autoantibodies are selective allosteric antagonists of presynaptic $GABAB_{1a,2}$ receptors. This work raises the clinically important hypothesis: GABABR2 agonists may treat the intractable seizures that are the primary cause of morbidity and mortality in patients with anti-$GABA_B$ receptor encephalitis.

The Sushi domain of GABAB1a is necessary and sufficient to bind autoantibodies from all anti-$GABA_B$ encephalitis patients we tested. The immunodominant domain recognized by anti-$GABA_B$ receptor antibodies is distinct from what has been identified in anti-NMDAR or anti-AMPAR encephalitides. In all anti-$GABA_B$ encephalitis patients tested here, autoantibodies bound to a 150 amino acid N-terminal portion of the receptor consisting of two Sushi domains, which are present in the

presynaptically localized $GABA_{B1a}$ subunit of $GABA_B$ receptors but not the $GABA_{B1b}$ subunit. Autoantibodies from patients with anti-NMDA receptor or anti-AMPA receptor encephalitis bind to the auxiliary amino-terminal domain that is homologous to bacterial periplasmic amino acid binding proteins (PBPs, Gleichman et al., 2012, 2014; O'Hara et al., 1993).

Similar to ionotropic glutamate receptors, Class III GPCRs, of which $GABA_B$ receptor is one, contain a domain homologous to bacterial periplasmic amino acid binding proteins, the Venus Flytrap domain, that binds ligand (Acher and Bertrand, 2005). Our results show that the VFT domain of $GABA_{B1}$ does not bind patient autoantibodies. That the immunodominant domain in patients with anti-$GABA_B$ encephalitis is structurally distinct from that recognized by antibodies from patients with anti-NMDA or anti-AMPA receptor encephalitis may account for the strikingly different effects of these patients' antibodies on surface receptor localization.

In anti-NMDA, anti-AMPA and anti-$GABA_A$ receptor encephalitides, patient autoantibodies decrease cognate receptors at synapses and in cell membranes, whereas $GABA_B$ receptor autoantibodies did not change surface $GABA_B$ receptor localization. The target antigens in the encephalitides associated with NMDA, AMPA or $GABA_A$ receptors are generally present in two copies in an oligomeric configuration, e.g., two subunits of GluN1, GluA1, GluA2 or $GABA_{A\beta3}$ in heterooligomeric receptors. Thus two autoantibody binding sites are present on each ionotropic receptor, perhaps facilitating their clustering or aggregation by divalent autoantibodies. NMDA receptor internalization caused by antibodies from anti-NMDA receptor encephalitis patients is dependent on the divalency of patient IgGs (Hughes et al., 2010; Moscato et al., 2014). In contrast, $GABA_B$ receptors are most often present in neurons as heterodimers; autoantibodies thus bind to one epitope for every assembled receptor

on the cell surface. This difference may explain why treatment with $GABA_{B1}$ autoantibodies does not result in $GABA_B$ receptor internalization.

Identification of the immunodominant domain may also shed light on the pathophysiologic mechanism mediated by $GABA_B$ receptor autoantibodies. The $GABA_{B1a}$ subunit containing $GABA_{B(1a,2)}$ heterodimeric receptor is preferentially trafficked to pyramidal neuron axons (Vigot et al., 2006; Laviv et al., 2011; Degro et al., 2015). When activated, this receptor inhibits glutamate release from glutamatergic terminals (Biermann et al., 2010). Either of the two Sushi domains present in $GABA_{B1a}$ is sufficient to traffic the subunit to the axonal domain (Vigot et al., 2006; Hannan et al., 2012). While patient autoantibodies are functional $GABA_B$ receptor antagonists, it is possible that autoantibodies also affect receptor trafficking and presynaptic localization by blocking the two Sushi domains.

Taken together, autoantibody binding to Sushi domains, failure of autoantibodies to decrease surface $GABA_B$ receptors and autoantibody-mediated block of baclofen-mediated inhibition of neuronal excitability is consistent with $GABA_{B1}$ autoantibodies being allosteric antagonists of $GABA_B$ receptors. It is possible that antibody bound $GABA_B$ receptors are inactivated and cannot signal through G proteins. This seems unlikely, because even in saturating doses of baclofen, $GABA_{B2}$ could be activated by a partial allosteric agonist in cells treated with patient autoantibodies. Our data can't exclude the possibility that patient autoantibodies disrupt heterodimerization of ligand binding $GABA_{B1}$ and the G-protein activating $GABA_{B2}$ subunits. However, this possibility seems unlikely because receptors remain activatable by saturating doses of baclofen, a $GABA_{B1}$ agonist. The regions of GABAB1and $GABA_{B2}$ that are necessary for dimerization are the C-terminal coiled-coil domain and the upper lobe of the VFTs. Moreover, interaction between the VFTs of $GABA_{B1}$ and $GABA_{B2}$ increase

the affinity of $GABA_{B1}$ for agonists and antagonists 10-100 fold (Pin et al., 2004). It is possible that the steric bulk of an IgG bound to the Sushi domains in GABAB1a subunit affects the interaction of the $GABA_{B1}$ and $GABA_{B2}$ VFTs, reducing the affinity of $GABA_B$ receptors to baclofen. This is supported by our observation that the block of baclofen-mediated inhibition of neuronal excitability by patient autoantibodies is surmountable by increasing the baclofen concentration. Together, our data suggest that patient $GABA_B$ autoantibodies are surmountable allosteric antagonists of $GABA_B$ receptors.

In anti-$GABA_B$ receptor encephalitis patients, autoantibodies may contribute to seizures by interfering with GABA-mediated inhibition of neuronal firing. Development of CNS penetrant $GABA_{B2}$ heptahelical domain agonists that rescue autoantibody antagonism in vitro may provide a viable therapeutic intervention to control the intractable seizures that are often the cause of death in anti-$GABA_B$ receptor encephalitis patients.

Figures

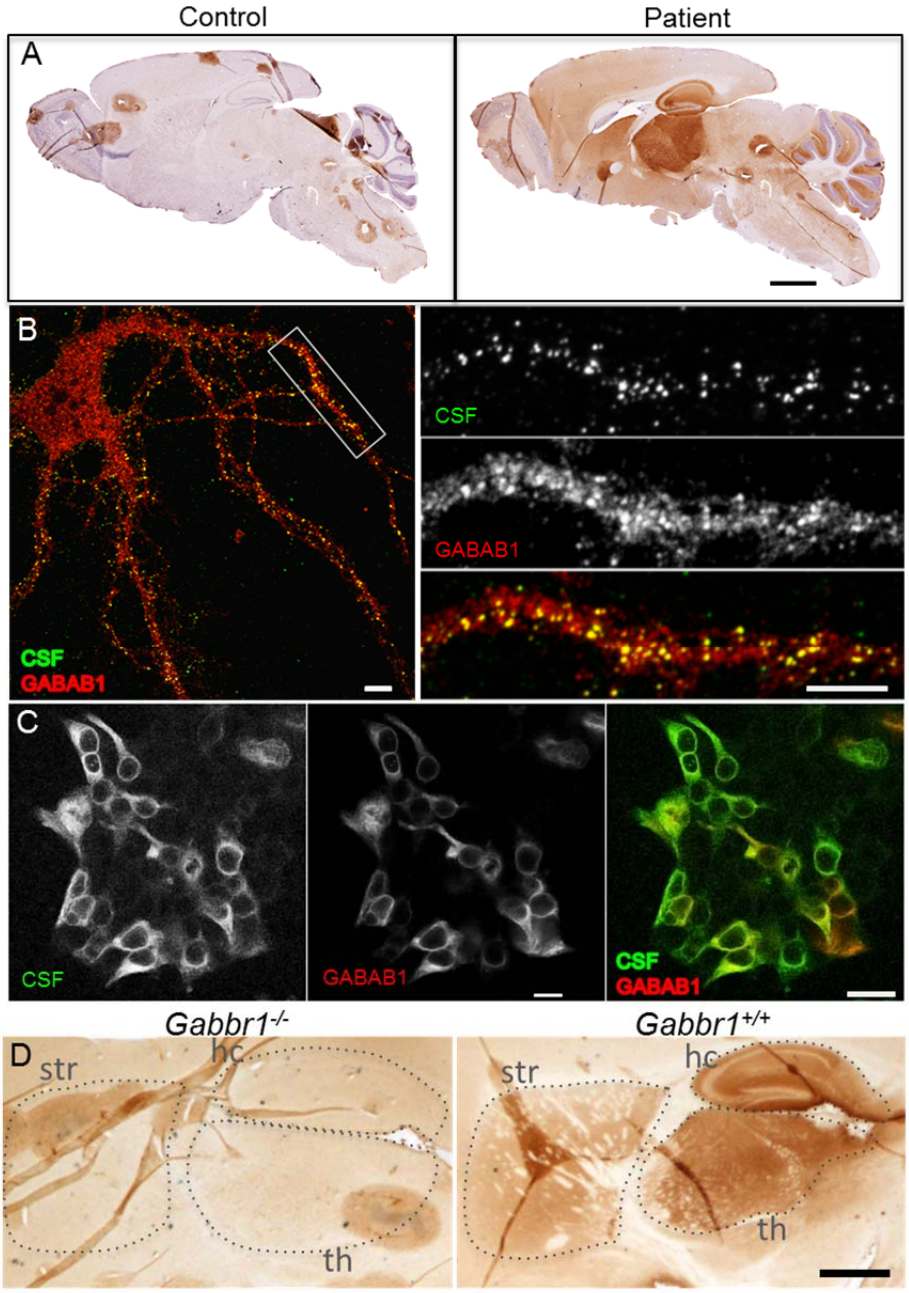

Control
Patient
A
B
CSF
GABAB1
C
CSF
GABAB1
CSF
GABAB1
Gabbr1-/-
Gabbr1+/+
D
str
hc
th
str
hc
th

Figure 1. Cerebrospinal fluid (CSF) from patients contains antibodies that bind to GABAB1 receptors.

(A) Immunostaining of mouse saggital brain sections with patient CSF shows binding in hippocampus, thalamus, striatum and cerebellum (brown); sections counterstained with hematoxylin (purple). Scale bar = 1mm.

(B) Immunostaining of unfixed and unpermeabilized 14 div hippocampal neurons with patient CSF (green), followed by permeabalization and staining with commercial antibodies to an intracellular GABAB1 epitope (red). Boxed area in neuron on left shown at higher magnification to right (scale bars = 5 um).

(C) Immunostaining of fixed, permeabalized HEK293T cells expressing GABAB1a and GABAB2 with patient CSF (green) and commercial GABAB1 antibody (red). Scale bar = 20 um.

(D) Immunohistochemical staining of mid saggital brain sections from GABAB1 knockout mice (left) or wild type littermates (right) with patient CSF. In wildtype mice, patient CSF prominently stains hiccpocampus (hc), thalamus (th) and the striatum (str), while in knockout mice, patient CSF does not stain any brain structure above background. Scale bar = 500um.

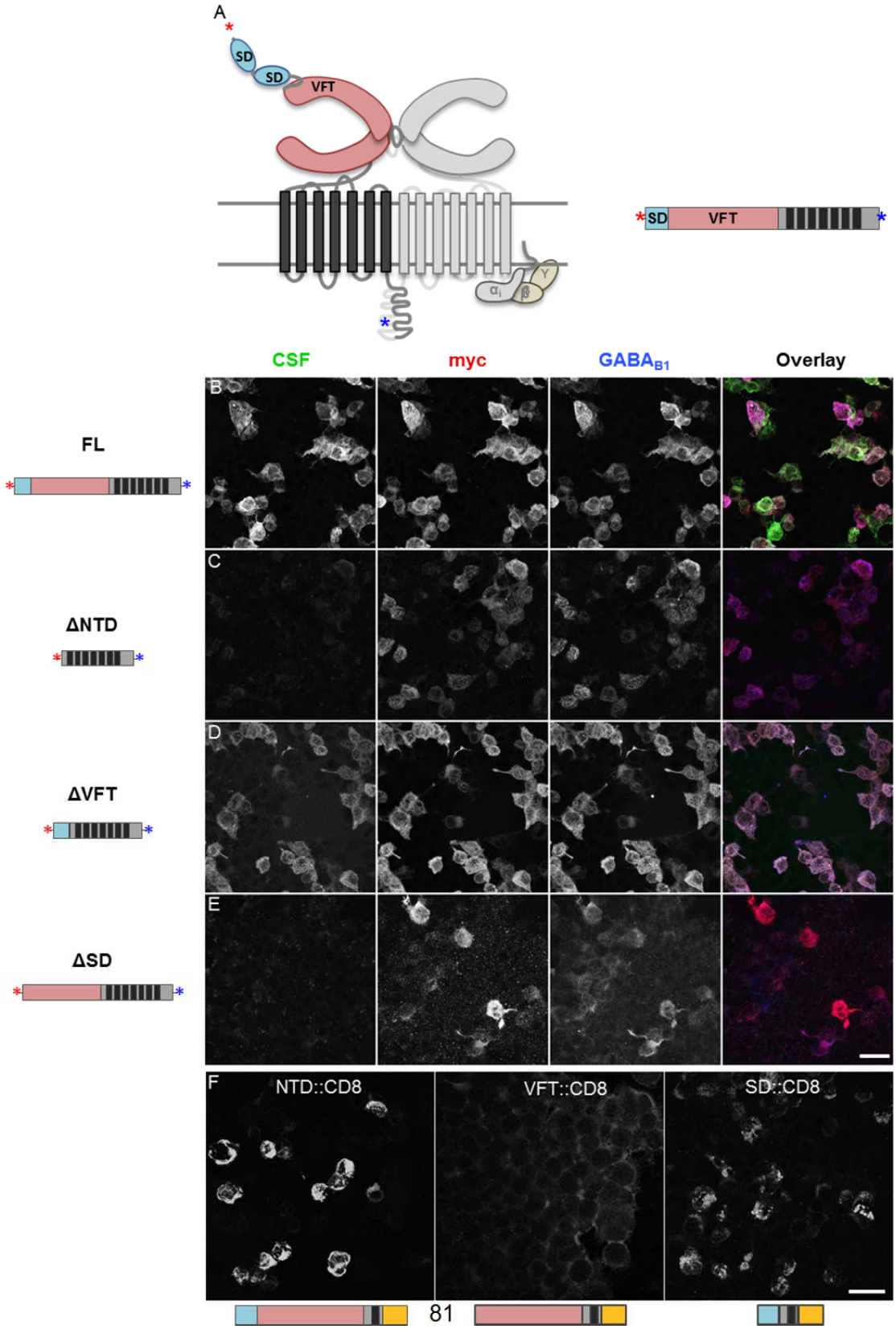
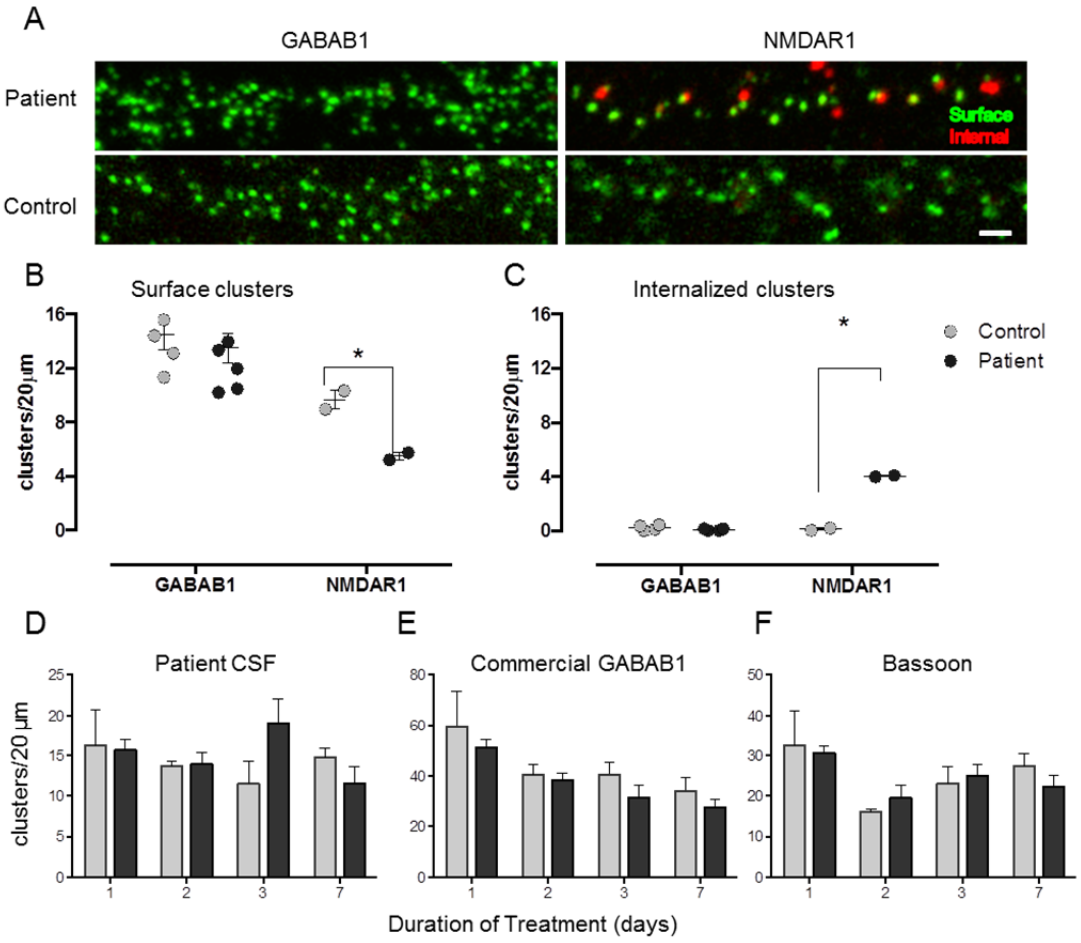

A
SD
SD
VFT
SD
VFT
CSF
myc
GABA$_{B1}$
Overlay
FL
ΔNTD
ΔVFT
ΔSD
B
C
D
E
F
NTD::CD8
VFT::CD8
SD::CD8

Figure 2. Sushi domains in GABAB1a are the dominant epitope for autoantibodies in patient CSF.

(A) left: Diagram of the tertiary structure of GABAB receptor comprised of GABAB1 and GABAB2. right: Domain structure of GABAB1 comprised of Sushi domains (blue), ligand binding Venus Flytrap domain (pink) and seven transmembrane domain (black). The myc epitope in the transfected construct and the epitope recognized by a commercial GABAB1 antibody are represented by a red and a blue asterisk, respectively.

(B-E) HEK293T cells expressing wildtype (A) or deletion (B-D) GABAB1a and GABAB2 immunostained with patient CSF (green, left column), N-terminal myc antibody (red, middle left column) or C-terminal intracellular GABAB1 antibody (blue, middle right column). Cells expressing each deletion construct were immunostained for the N-terminal myc and C-terminal GABAB1 epitope. (B) Immunostaining is present in cells expressing full length GABAB1. (C) Immunostaining is absent in cells expressing NTD deleted GABAB1. C: Immunostaining is present in cells expressing VFT deleted GABAB1, although staining appears qualitatively reduced compared to cells expressing full length receptors. (D) Immunostaining is absent in cells expressing GABAB1 lacking both Sushi domains. (E) HEK293T cells expressing fusion proteins of GABAB1a and a mouse CD8 receptor fragment.

(F) The GABAB1a N-terminal domain alone is sufficient to bind patient GABAB1 antibodies (left). The VFT alone does not bind patient GABAB1 antibodies (middle; image was gamma shifted so background staining is apparent). The Sushi domains (SD) alone are sufficient to bind to human autoantibodies (right). These data demonstrate that the Sushi domains are necessary and sufficient to bind patient GABAB1 antibodies. Scale bars = 20 um.


A
GABAB1
NMDAR1
Patient
Control
Surface
Internal
B
Surface clusters
clusters/20µm
16
12
8
4
0
*
GABAB1
NMDAR1
C
Internalized clusters
clusters/20µm
16
12
8
4
0
*
Control
Patient
GABAB1
NMDAR1
D
Patient CSF
clusters/20 µm
25
20
15
10
5
0
1
2
3
7
E
Commercial GABAB1
80
60
40
20
0
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Bassoon
50
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30
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7
Duration of Treatment (days)

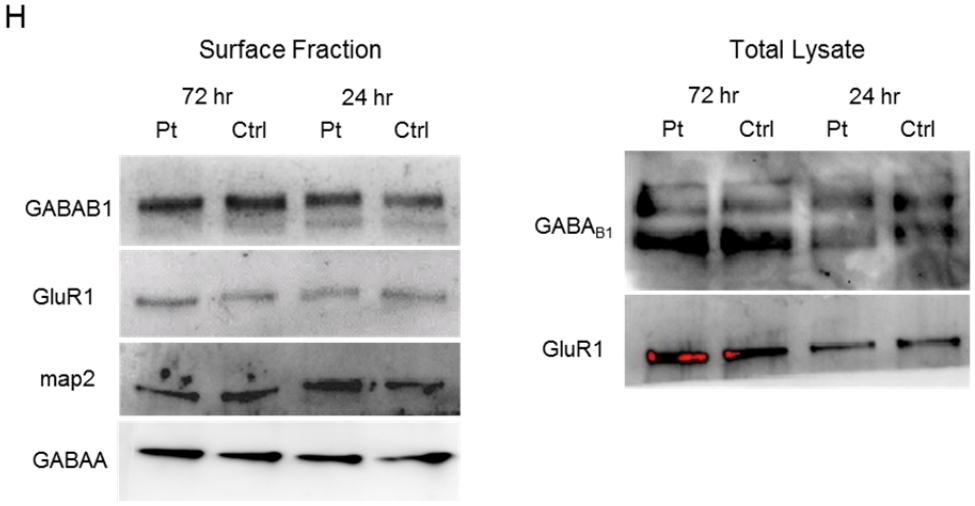
H
Surface Fraction
72 hr
24 hr
Pt
Ctrl
Pt
Ctrl
GABAB1
GluR1
map2
GABAA
Total Lysate
72 hr
24 hr
Pt
Ctrl
Pt
Ctrl
GABA$_{B1}$
GluR1

Figure 3. Patient GABAB1 antibodies from patients with anti-GABAB receptor encephalitis do not reduce surface receptor clusters.

(A) Unfixed, unpermeabalized hippocampal neurons treated with patient or control CSF for 24 hours followed by immunostaining with patient CSF and anti-human Alexa 488 conjugated secondary antibody (green). Internalized patient IgG was visualized after fixation, permeabilization and immunostaining with anti-human Alexa 594 conjugated secondary antibody (red). Left: After 24 h treatment, the density of surface clusters is the same in patient and control CSF treated neurons, and no internalized clusters are detected (N = 3 patient CSF, 4-6 coverslips/patient, 4 cells/coverslip). Right: In contrast, after 24 h treatment with CSF from anti-NMDAR encephalitis patients, a significant reduction in surface clusters and a significant increase in internalized clusters are detected, as previously reported (Hughes et al., 2010; Moscato et al., 2014; N = 2 patient CSF, 4-6 coverslips/patient, 4-5 cells/coverslip). Scale bar = 5 um.

(B) Quantification of immunostaining shows that after 24 h treatment, the density of surface GABAB1 clusters are not significantly different in patient or control CSF treated neurons (Student's t test, $p = 0.85$), while 24 h treatment with CSF from anti-NMDA receptor encephalitis results in a significant reduction in surface clusters compared to control CSF treated neurons (Student's t test $p < 0.05$).

(C) Quantification of immunostaining shows that after 24 h treatment, few if any internalized GABAB1 clusters are detected in patient or control CSF (Student's t test, $p = 0.21$), while the density of internalized clusters is significantly higher in cells treated with CSF from anti-NMDA receptor encephalitis patients compared to controls (Student's t test, $p < 0.05$).

(D-F) Duration of patient CSF treatment for up to 7 days does not affect surface

GABAB1 receptor cluster or presynaptic terminal density. Quantification of surface GABAB1 (D), total GABAB1 (E) and presynaptic Bassoon (F) cluster density in neurons treated for 1, 2, 3 and 7 days with anti-GABAB1 patient or control CSF (N = 5 patient CSF and control samples, 8-15 coverslips/patient, 4 neurons/coverslip).

(H) Surface biotinylation and Western blot of surface and total protein fractions isolated from patient or control CSF treated neurons. Surface GABAB 1 receptor protein level is not different in patient or control treated neurons after 24 or 72 hrs (N = 1 patient sample, same samples used for 24 and 72 h treatment; 5 coverslips/patient). GABAA protein band controls for quantity of protein in surface fraction of protein lysates. No change in surface AMPA receptor protein (GluR1) or total GluR1 was observed. MAP2 protein band controls for the quantity of protein in the total lysate. (Right) There is no difference in total GABAB1 protein levels in patient or control treated neurons after 24 or 72 hrs (N = 1 patient sample, same samples used for 24 and 72 h treatment; 5 coverslips/patient).

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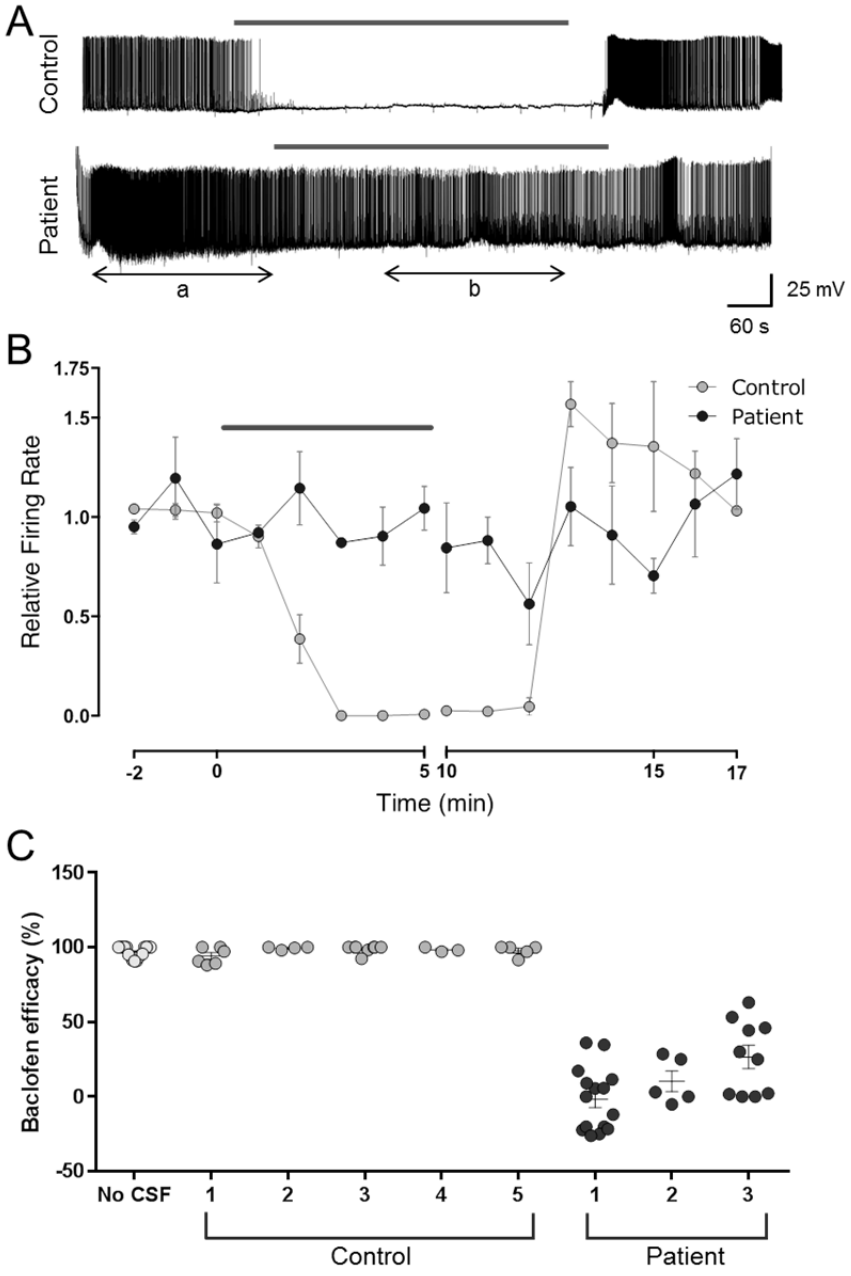
A
Control
Patient
a
b
25 mV
60 s
B
Relative Firing Rate
1.75
1.5
1.0
0.5
0.0
Control
Patient
-2
0
5
10
15
17
Time (min)
C
Baclofen efficacy (%)
150
100
50
0
-50
No CSF
1
2
3
4
5
1
2
3
Control
Patient

Figure 4. Patient GABAB1 antibodies block GABAB receptor activation.

(A) Whole cell current clamp traces before, in presence of 100 um baclofen (solid bar) and after washout. Top: Cells treated with control CSF show block of action potential firing by baclofen. Bottom: Cells treated with patient CSF for 24 hours show no block of action potential firing by baclofen. Scale bar = 60 s, 25 mV.

(B) Quantification of firing rate in one minute bins, normalized to the mean firing rate between t=-3 min to t=0 min. Solid bar indicates infusion of 100 uM baclofen for 10 mins, beginning at t=0 min. Data between t = 5-10 min was excluded to illustrate baclofen wash-on and off. After the onset of baclofen infusion, neurons stop firing action potentials within 1-2 min in control CSF treated neurons (open circles). Firing rate is not altered in patient CSF treated neurons (closed circles). Repeated measures ANOVA; treatment X time $F(13, 65) = 11.82$; $p < 0.05$.

(C) Efficacy of 100 uM baclofen in neurons treated for 24 hours with 1:100 of CSF from patients or controls. Efficacy is defined as the relative reduction in the five minute average of firing rate of neurons during perfusion with baclofen relative to perfusion with artificial CSF. Numerically, it is [1 – (average firing rate in region b/ average firing rate in region a)] where regions a and b are labelled in Fig 4A. In 100 uM baclofen, baclofen is maximally efficacious in control CSF treated neurons (gray circles, N = 6 control CSF, 5-20 coverslips/sample, 1 neuron/coverslip). Efficacy of 100 uM baclofen is reduced after treatment with patient CSF (N = 3 patient CSF, 5-15 coverslips/sample, 1 neuron/coverslip; significantly different from control CSF, Dunn's multiple comparison post Kruskal-Wallis non-parametric two factor analysis of variation, $p = 0.001$).

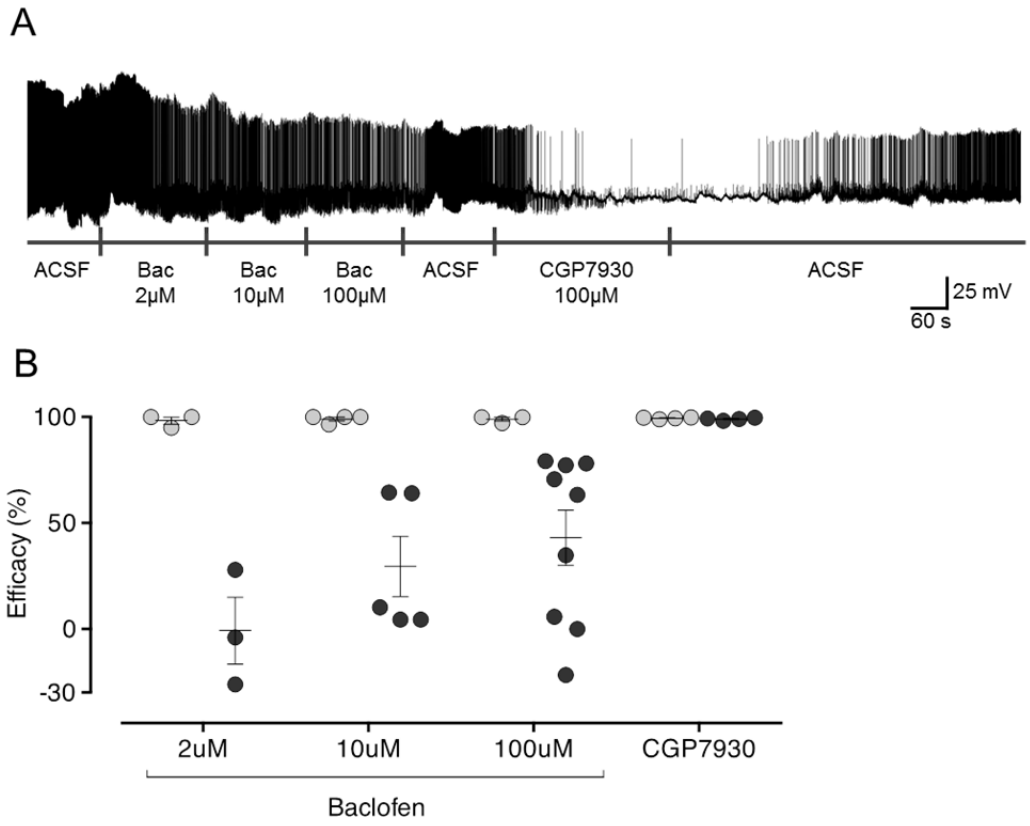
A
ACSF
Bac
2μM
Bac
10μM
Bac
100μM
ACSF
CGP7930
100μM
ACSF
25 mV
60 s
B
100
50
0
-30
Efficacy (%)
2uM
10uM
100uM
CGP7930
Baclofen

Figure 5. Patient GABABR1 antibodies do not inactivate GABAB2 mediated signaling

(A) Whole cell current clamp trace during perfusion with the indicated concentrations of either the GABAB1 binding agonist, baclofen or the GABAB2 binding agonist, CGP 7930 from a cultured hippocampal neuron treated for 24 hours with 1:100 of CSF from patient 1. Scale bar = 60 s, 25 mV.

(B) Efficacy of baclofen or CGP7930 at inhibiting firing in cultured neurons treated with 1:100 of CSF from patient 1. Despite being able to reduce the efficacy of baclofen even at a concentration of baclofen at 100uM, treatment with patient CSF (black circles) is unable to reduce the ability of CGP9730 at 100uM to silence neurons identically whether they are treated with patient or control CSF ($p = 0.40$; Holm-Sidak multiplicity correction)

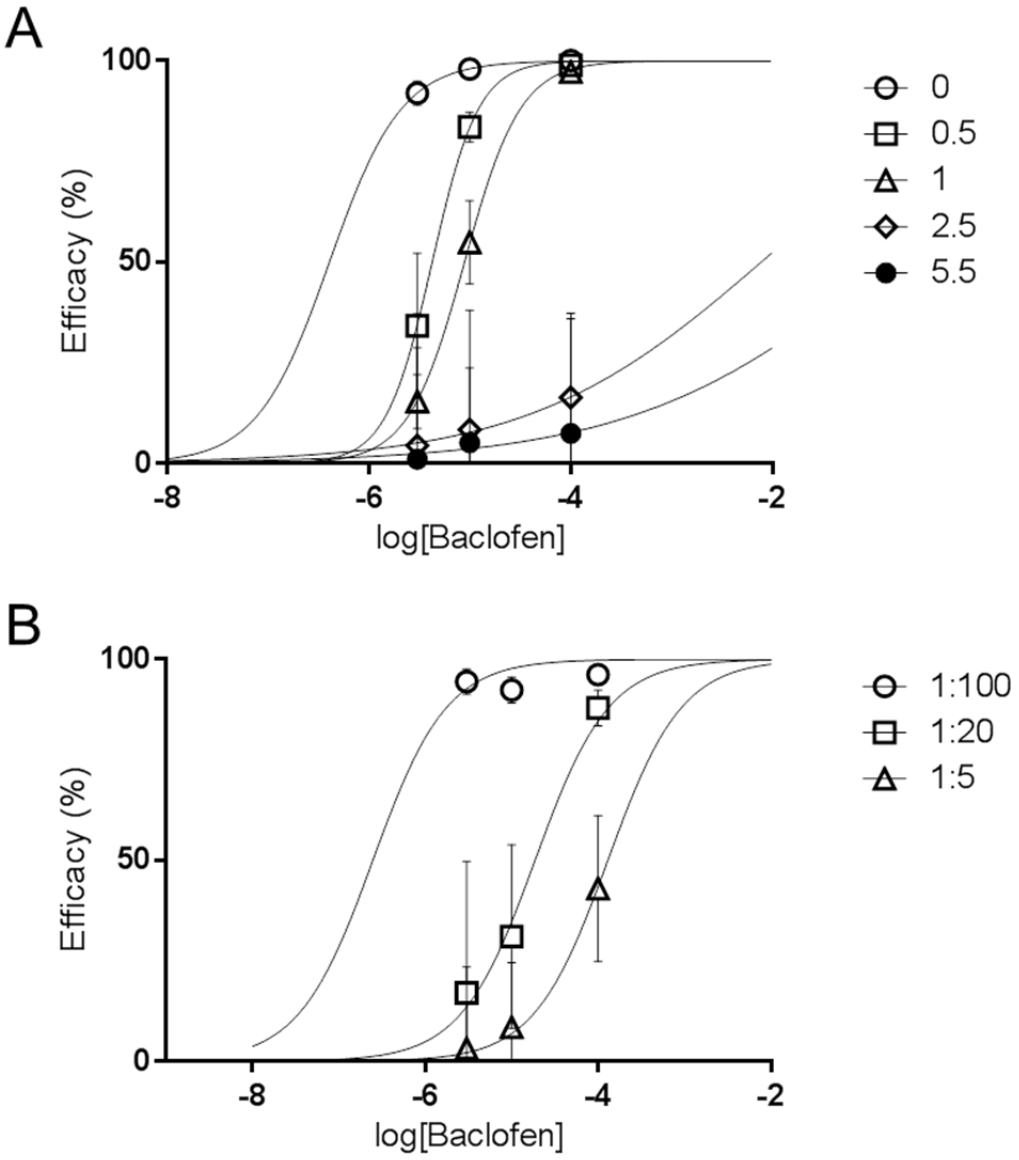
A
100
50
0
Efficacy (%)
-8
-6
-4
-2
log[Baclofen]
0
0.5
1
2.5
5.5
B
100
50
0
Efficacy (%)
-8
-6
-4
-2
log[Baclofen]
1:100
1:20
1:5

Figure 6. GABAB1 autoantibodies are allosteric antagonists of GABAB receptors.

(A) Time course of onset of GABAB1 autoantibody mediated block of GABAB1 activation. Neurons were recorded after indicated duration of treatment with CSF from case 1 in the presence of 3uM (light gray circles), 10uM (dark gray circles) or 100uM (black circles) of baclofen. With increasing duration of exposure to GABAB1 autoantibodies, neurons required increasingly higher doses of baclofen to circumvent the block on GABAB1 activation by antibodies. By 2.5 hours or treatment, even 100 uM of baclofen was insufficient to overcome the antibody block of GABAB activation (ANOVA Treatment duration X baclofen concentration $F(8, 96) = 6.271$; $p<0.05$) .

(B) Dose response of increasing concentration of GABAB1 autoantibodies. A lower titer CSF (case 4) was used, which when used for treating neurons at 1:100 does not block GABAB activation by baclofen. But after treatment with higher concentrations of CSF, neurons are inhibited by baclofen only at 100uM (ANOVA, CSF concentration X baclofen concentration $F(4, 194) = 29.49$; $p<0.05$)

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CHAPTER 3. Passive transfer model of anti-NMDA receptor encephalitis

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Human N-methyl D-aspartate receptor antibodies alter memory and behaviour in mice

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Abstract

Anti-N-methyl D-aspartate receptor (NMDAR) encephalitis is a severe neuropsychiatric disorder that associates with prominent memory and behavioral deficits. Patients' antibodies react with the N-terminal domain of the GluN1 (previously known as NR1) subunit of NMDAR causing in cultured neurons a selective and reversible internalization of cell-surface receptors. These effects and the frequent response to immunotherapy have suggested an antibody-mediated pathogenesis, but to date there is no animal model showing that patients' antibodies cause memory and behavioral deficits. To develop such a model, C57BL6/J mice underwent placement of ventricular catheters connected to osmotic pumps that delivered a continuous infusion of patients' or control cerebrospinal fluid (flow rate 0.25 μl/h, 14 days). During and after the infusion period standardized tests were applied, including tasks to assess memory (novel object recognition in open field and V-maze paradigms), anhedonic behaviors (sucrose preference test), depressive-like behaviors (tail suspension, forced swimming tests), anxiety (black and white, elevated plus maze tests), aggressiveness (resident-intruder test), and locomotor activity (horizontal and vertical). Animals sacrificed at Days 5, 13, 18, 26 and 46 were examined for brain-bound antibodies and the antibody effects on total and synaptic NMDAR clusters and protein concentration using confocal microscopy and immunoblot analysis. These experiments showed that animals infused with patients' cerebrospinal fluid, but not control cerebrospinal fluid, developed progressive memory deficits, and anhedonic and depressive-like behaviors, without affecting other behavioral or locomotor tasks. Memory deficits gradually worsened until Day 18 (4 days after the infusion stopped) and all symptoms resolved over the next week. Accompanying brain tissue studies showed progressive increase of brain-bound human antibodies, predominantly in the hippocampus (maximal on Days 13–18), that

after acid extraction and characterization with GluN1-expressing human embryonic kidney cells were confirmed to be against the NMDAR. Confocal microscopy and immunoblot analysis of the hippocampus showed progressive decrease of the density of total and synaptic NMDAR clusters and total NMDAR protein concentration (maximal on Day 18), without affecting the post-synaptic density protein 95 (PSD95) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. These effects occurred in parallel with memory and other behavioral deficits and gradually improved after Day 18, with reversibility of symptoms accompanied by a decrease of brain-bound antibodies and restoration of NMDAR levels. Overall, these findings establish a link between memory and behavioral deficits and antibody-mediated reduction of NMDAR, provide the biological basis by which removal of antibodies and antibody-producing cells improve neurological function, and offer a model for testing experimental therapies in this and similar disorders.

Introduction

Memory, learning, and behaviour depend on the proper function of the excitatory glutamate N-methyl D-aspartate receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and underlying mechanisms of synaptic plasticity (Lau and Zukin, 2007; Shepherd and Huganir, 2007). The critical role of NMDAR in these functions has been shown in animal models in which the NMDAR are altered genetically (Mohn et al., 1999; Belforte et al., 2010) or pharmacologically (Jentsch and Roth, 1999; Mouri et al., 2007). In humans this evidence comes from more indirect observations such as studies investigating the effects of phencyclidine or ketamine (non-competitive antagonists of NMDAR that cause psychosis) (Weiner et al., 2000; Gunduz-Bruce, 2009), and brain tissue studies of patients with schizophrenia or

Alzheimer's disease in which several molecular pathways that modulate glutamate receptor trafficking or function are affected (Snyder et al., 2005; Hahn et al., 2006). In 2007 we identified a novel disorder (anti-NMDAR encephalitis) that occurs with highly specific antibodies against extracellular epitopes located at the amino terminal domain of the GluN1 subunit of NMDAR (Dalmau et al., 2007; Gleichman et al., 2012). The resulting syndrome resembles the spectrum of symptoms that occurs in genetic or pharmacologic models of NMDAR hypofunction, including memory loss and neuropsychiatric alterations ranging from psychosis to coma (Dalmau et al., 2008; Irani et al., 2010; Viaccoz et al., 2014). Regardless of the type of presentation, most patients develop severe problems forming new memories and amnesia of the disease. Symptoms are usually accompanied by systemic and intrathecal synthesis of antibodies, the latter likely produced by plasma cells contained in brain inflammatory infiltrates (Dalmau et al., 2008; Martinez-Hernandez et al., 2011). These long-lived plasma cells and persistent antibody synthesis may explain the lengthy symptoms of most patients (average hospitalization □3 months) (Dalmau et al., 2008). Yet, despite the severity and duration of the disease, 80% of the patients have substantial recovery after immunotherapy (accompanied by removal of an underlying tumor, usually an ovarian teratoma, when appropriate), or sometimes spontaneously (Iizuka et al., 2008; Titulaer et al., 2013).

Investigations on the potential pathogenic role of patients' antibodies using cultured neurons showed that the antibodies caused crosslinking and selective internalization of NMDARs that correlated with the antibody titers, and these effects were reversible after removing the antibodies (Hughes et al., 2010; Mikasova et al., 2012). In contrast, patients' antibodies did not alter the localization or expression of other synaptic proteins, number of synapses, dendritic spines, dendritic complexity, or cell

survival (Hughes et al., 2010). In parallel experiments, the density of NMDAR was also significantly reduced in the hippocampus of rats infused with patients' antibodies, a finding comparable to that observed in the hippocampus of autopsied patients (Hughes et al., 2010). Overall, these studies suggested an antibody-mediated pathogenesis, but the demonstration that patients' antibodies caused symptoms remained pending. Modelling symptoms and showing that these correlate with antibody-mediated reduction of NMDAR would prove the pathogenicity of patients' antibodies, support the use of treatments directed toward decreasing the levels of antibodies or antibody-producing cells, and help to investigate experimental therapies in this and similar disorders. We report here such a model using continuous 14-day cerebroventricular infusion of patients' CSF in mice. The aims were to determine (i) if patients' antibodies altered memory and behaviour; (ii) whether mice symptoms correlated with brain antibody-binding and reduction of NMDAR; and (iii) whether the clinical and molecular alterations recovered after stopping the antibody infusion.

Materials and methods

Animals

Male C57BL6/J mice (Charles River), 8–10 weeks old (25–30 g) were housed in cages of five until 1 week before surgery when they were housed individually. The room was maintained at a controlled temperature (21 ± 1°C) and humidity (55 ± 10%) with illumination at 12-h cycles; food and water were available ad libitum. All experiments were performed during the light phase, and animals were habituated to the experimental room for 1 week before starting the tests. All procedures were conducted in accordance with standard ethical guidelines (European Communities Directive 86/609/EU) and approved by the local ethical committees: Comitè Ètic d'Experimentació Animal, Institut

Municipal d'Assistència Sanitària (Universitat Pompeu Fabra), and Institutional Animal Care and Use Committee (University of Pennsylvania).

Patients' CSF samples

CSF from 25 patients with high titre NMDAR antibodies (all >1:320) were pooled and used for cerebroventricular infusion. CSF from 25 subjects without NMDAR antibodies (11 with normal pressure hydrocephalus and 14 with non-inflammatory CNS disorders) were similarly pooled and used as controls. Before loading the osmotic pumps (discussed below), the pooled CSF samples from patients and controls were dialyzed (Slide-A-Lyzer 7K, Thermo) against sterile phosphate-buffered saline (PBS) overnight at 4°C, and the concentration of total IgG normalized to the CSF physiologic concentration of 2 mg/dl. All mice received the same pooled CSF either from patients or controls. Studies were approved by the institutional review board of Hospital Clínic and Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona.

Surgery, placement of ventricular catheters and osmotic pumps

Cerebroventricular infusion of CSF was performed using osmotic pumps (model 1002, Alzet) with the following characteristics: volume 100 µl, flow rate 0.25 µl/h, and duration 14 days. Twenty-four hours before surgery, two osmotic pumps per animal were each loaded with 100 µl of patient or control CSF. The pumps were then connected to a 0.28 mm IM (internal diameter) polyethylene tube (C314CT, PlasticsOne) and left overnight in sterile PBS at 37°C. The next day, mice were deeply anaesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) along with subcutaneous administration of the analgesic meloxicam (1 mg/kg). Mice were then placed in a stereotaxic frame, and a bilateral catheter (PlasticsOne, model 3280PD-2.0/SP) was inserted into the ventricles (0.02 mm anterior and 1.00 mm lateral from bregma, depth 0.22 mm) and secured with dental cement. Each arm

of the catheter was connected to one osmotic pump, which was subcutaneously implanted on the back of the mice. Appropriate ventricular placement of the catheters was assessed in randomly selected mice injecting methylene blue through the catheters (Fig. 1A–C).

Cognitive tasks

All behavioral tasks were performed by researchers blinded to experimental conditions using standardized tests reported by us (Maldonado et al., 1970; Filliol et al., 2000; Berrendero et al., 2005; Bura et al., 2007, 2010, 2013; Aso et al., 2008; Puighermanal et al., 2009; Burokas et al., 2012; Llorente-Berzal et al., 2013) and others (Porsolt et al., 1977; Crawley and Goodwin, 1980; Handley and Mithani, 1984; Steru et al., 1985; Konig et al., 1996; Caille et al., 1999; Strekalova et al., 2006; Taglialatela et al., 2009; Ennaceur, 2010) and following the schedule summarized in Fig. 1D. The tasks were aimed to assess memory (novel object recognition in open field and V-maze), anhedonic behaviours (sucrose preference test), depressive-like behaviours (tail suspension, and forced swimming tests), anxiety (black and white and elevated plus maze tests), aggressiveness (resident-intruder test) and locomotor activity (horizontal and vertical activity assessment). A brief description of each task is provided in the Supplementary material.

Brain tissue processing

To determine the effects of patients' antibodies on mouse brain, animals were sacrificed at the indicated time points (Fig. 1D, Days 5, 13, 18, 26 and 46) with CO2. Brains were harvested, sagittally split, and transferred to ice-cold PBS. Half of the brain

was fixed by immersion in 4% paraformaldehyde (PFA) for 1 h at 4°C, cryoprotected with 40% sucrose for 48 h at 4°C, embedded with freezing media, and snap-frozen with isopentane chilled with liquid nitrogen. The other half-brain was used for dissection of hippocampus and cerebellum for IgG and protein extraction (see below).

Immunohistochemistry and quantitative peroxidase staining

For determination of antibodies bound to brain tissue using immunoperoxidase staining, 7-µm thick tissue sections were sequentially incubated with 0.25% H2O2 for 10 min at 4°C, 5% goat serum for 15 min at room temperature, biotinylated goat anti-human IgG (1:2000, Vector labs) overnight at 4°C, and the reactivity developed using avidin-biotin-peroxidase and diaminobenzidine. Sections were mildly counterstained with haematoxylin, and results photographed under a Leica DMD108 microscope. Images were prepared creating a mask for diaminobenzidine color, converting the mask to greyscale intensities, and inverting the pixels using Adobe Photoshop CS6 package. Hippocampal, frontal cortex, striatum and cerebellar regions were manually outlined; intensity and area were quantified in two serial sections using the public domain Fiji ImageJ software (http://fiji.sc/Fiji). Values were divided by area and normalized to the group with the highest mean (defined as 100%, patients' CSF treated animals sacrificed at Day 18).

Immunofluorescence and confocal microscopy with brain tissue

For determination of antibodies bound to brain tissue using immunofluorescence, 5 µm-thick tissue sections were blocked with 5% goat serum and 1% bovine serum albumin for 60 min at room temperature, and incubated overnight at 4°C with Alexa Fluor® 488 goat anti-human IgG (A11013, diluted 1:1000, Molecular Probes/ Life Technologies). Slides were then mounted with ProLong® Gold (P36930, Molecular Probes) and results scanned under a LSM710 Zeiss confocal microscope.

Sections from all animals were analyzed in parallel. Quantification of fluorescent intensity in areas of CA1, CA3 and dentate gyrus was done using Fiji ImageJ software. Background was subtracted and intensity divided by area. Mean intensity of IgG immunostaining in animals treated with patients' CSF and sacrificed at Day 18 was defined as 100%.

To determine the effects of patients' antibodies on total and synaptic NMDAR clusters and PSD95, non-permeabilized 5-µm thick sections were blocked with 5% goat serum and 1% bovine serum albumin as above, incubated with human CSF antibodies for 2 h at room temperature, washed with PBS, permeabilized with Triton™ X-100 0.3% for 10 min at room temperature, and incubated with rabbit polyclonal antibody against PSD95 (diluted 1:250, Clone 18258 Abcam) overnight at 4°C. Next day, the slides were washed and incubated with the corresponding secondary antibodies, Alexa Fluor® 594 goat anti-human IgG and Alexa Fluor® 488 goat anti-rabbit IgG (A-11014, A-11008, both diluted 1:1000, Molecular Probes) for 1 h at room temperature. Slides were mounted as above and results scanned with a confocal microscope (Zeiss LSM710) with EC-Plan NEOFLUAR CS ×100/1.3 NA oil objective. Standardized z-stacks including 50 optical images were acquired from five different, equally spaced areas of CA1, CA3 and dentate gyrus of hippocampus using sequential scanning, 1024 × 1024 lateral resolution, and Nyquist optimized z-sampling frequency. Images were deconvolved with 20 iterations using theoretical point spread functions and maximum likelihood estimation algorithms of Huygens Essential software (Scientific Volume Imaging). For cluster density analysis a spot detection algorithm from Imaris suite 7.6.4 (Bitplane) was used based on automatic segmentation of the images to spots (Banovic et al., 2010). Density of clusters was expressed as spots/µm3. Three-dimensional colocalization of clusters (e.g. NMDAR and PSD95) was done using a spot co- localization algorithm implemented in

Imaris suite 7.6.4. Synaptic localization was defined as co-localization of NMDAR or AMPAR with post-synaptic PSD95. Synaptic cluster density was expressed as colocalized spots/μm3. For each animal, five identical image stacks in each hippocampal area (CA1, CA2 and dentate gyrus) were acquired and the mean densities calculated for total and synaptic NMDAR and AMPAR. Densities were normalized to the mean density of control CSF treated animals (100%). For the AMPAR the antibody used was guinea pig GluA1 antibody (1:100, clone AGP-009, Alomone), and as secondary antibody Alexa Fluor® 594 goat anti-guinea pig IgG (A11076, 1:1000, Molecular Probes).

The presence of apoptosis, cellular infiltrates, and complement was assessed in the hippocampal region (CA3) in mice sacrificed on Day 18 and corresponding controls. Apoptosis was determined by standard terminal deoxynucleotidyl transferase mediated biotinylated UTP nick end labelling (TUNEL) using the TACS 2TdT-Fluor in situ apoptosis detection kit (Trevigen), and immunolabelling of cleaved caspase 3 (1:200, #9661 Cell Signalling, Technologies) using a goat anti-rabbit Alexa Fluor® 488 as secondary antibody (1:1000 Molecular Probes). The presence of complement was assessed using rabbit anti-mouse C5b-9 (1:500, Abcam) and Alexa Fluor® 488 goat anti-rabbit IgG (1:500, #A11008, Molecular Probes). Immunolabelling for T and B lymphocytes was done using rabbit anti-mouse CD3 (1:1000, #ab16669 Abcam) followed by secondary antibody goat anti-rabbit Alexa Fluor® 488 (1:1000, Molecular Probes), and rat anti-CD45R (1/10000, #ab64100) followed by goat anti-rat Alexa Fluor® 594 (1/1000, #A-11007 Molecular Probes). Results were scanned with a confocal microscope Zeiss LSM710.

Extraction of human IgG bound to mice brain

Under a dissection microscope (Zeiss stereomicroscope, Stemi 2000), the hippocampus and cerebellum were isolated, weighed, snap-frozen, and

stored at −80°C. Tissue (10 mg) was homogenized in 0.5 ml ice-cold PBS with protease inhibitors (Sigma-Aldrich) and centrifuged at 16 000g for 5 min. All steps were performed at 4°C. Washing was repeated four times to remove unbound IgG. The last wash was done in 100 µl and the supernatant saved as pre-extraction fraction. To extract the specifically bound antibodies, the pellet was solubilized for 5 min in acid (86 µl 0.1 M Na-citrate buffer pH 2.7), centrifuged at 16 000g for 5 min, and the supernatant neutralized with 14 µl 1.5 M Tris pH 8.8, and used to determine the presence of NMDAR (GluN1) antibodies (see below).

Immunofluorescence with HEK293 cells expressing GluN1

The presence of GluN1 antibodies in IgG extracts from brain was determined using a HEK293 cell-based assay expressing GluN1, as reported (Dalmau et al., 2008). After fixation with 4% paraformaldehyde and permeabilization with 0.3% Triton™ X-100, cells were blocked with 1% bovine serum albumin for 90 min, and incubated with undiluted acid-extracted IgG or pre-extraction fraction from brain of infused mice, at 4°C overnight. The next day, cells were washed and incubated with a mouse monoclonal antibody against a non-competing GluN1 epitope located at amino acid 660-811 (1:20 000; clone MAB363, Millipore) for 1 h at room temperature, followed by the corresponding Alexa Fluor® secondary antibodies (A11013, A11032, both diluted 1:1000, Molecular Probes) for 1 h at room temperature. The titre of positive samples was calculated by serial dilutions until the reactivity was no longer visible. Results were photographed under a fluorescence microscope using Zeiss Axiovision software.

Immunoblot analyses

Total protein from hippocampus and cerebellum was obtained by dissecting these regions from 20-µm thick paraformaldehyde-fixed sagittal mouse brain sections on glass slides at 4°C under a Zeiss stereomicroscope (Stemi 2000). Two

consecutive sections of isolated hippocampus or cerebellum were then transferred to an Eppendorf tube in PBS supplemented with protease inhibitors. Loading buffer (RotiLoad) was added, the solubilized tissue boiled for 5 min, and the proteins separated in a 10% SDS gel electrophoresis with semi-dry blotting on PVDF membranes. Membranes were blocked in 5% non-fat skimmed milk and incubated overnight at 4°C with the following polyclonal rabbit antibodies: GluN1 (1:1000, Sigma-Aldrich), GluR2/3 (1:1000, Abcam), and PSD95 (1:1000, Synaptic Systems), or a monoclonal mouse anti-β-actin (1:20 000, Sigma-Aldrich). Membranes were incubated with secondary antibodies for 1 h at room temperature (anti-rabbit IgG HRP 1:1000, anti-mouse IgG HRP 1:10 000) and analysed by enhanced chemiluminescence (all Amersham GE Healthcare) on a LAS4000 (GE Healthcare). All studies were done in duplicate. Analysed films were in the linear range of exposure, digitally scanned, and signals quantified using Fiji ImageJ software. The signal intensity of each antigen was normalized to that of actin in the same lane. The mean intensity of signal in control CSF treated animals was defined as 100% and all other intensities expressed in per cent relative to this value.

Statistics

Behavioural tests were analysed using repeated measures two-way ANOVA for tests with multiple time points (novel object recognition, sucrose preference test, resident-intruder test, locomotor activity), independent sample t-tests for tests with single time points (forced swimming test, black and white test, elevated plus maze test) or by Mann Whitney-U for skewed distributions (tail suspension test). Non-normally distributed parameters were log-transformed (black and white test, elevated plus maze test). Significance of NMDAR antibody titre in acid-extracted IgG fractions was calculated using the Kruskal-Wallis test and Dunn's post hoc test compared to titres at Day 46. Human IgG intensity, confocal cluster density and immunoblot data (GluN1,

PSD95) from different time points or regions were analysed using two-way ANOVA with Sidak-Holm post hoc testing to calculate multiplicity-adjusted P-values. Confocal cluster density in the different hippocampal subregions (CA1, CA3, dentate gyrus) were not significantly different and were analysed pooled. All experiments were assessed visually for outliers (e.g. one animal with very different results from the other animals at the same time point), but none were identified, so measurements were pooled per time point and treatment (patient or control CSF). For confocal AMPAR cluster density measured at single time points, independent sample t-tests were used. A P-value of <0.05 was considered significant in post hoc testing after correction for multiple testing (Sidak-Holm). In the two-way ANOVA the cut-off for interaction between two factors was set at 0.10; if the P-value for interaction was <0.10, the effects of treatment were considered for the separate time points (post hoc analysis). All tests were done using GraphPad Prism (Version 6).

Results

One-hundred and eleven mice were included in the studies, 56 for cognitive and behavioural tests, and 55 for assessment of antibody binding to brain and the effects on total and synaptic NMDAR (Fig. 1).

Cerebroventricular infusion of patients' CSF alters memory and behaviour in mice

The most robust effect during the 14-day infusion of patients' CSF was on the novel object recognition test in both the open field and V-maze paradigms (Fig. 2A and B). Compared with animals infused with control CSF, those infused with patients' CSF showed a progressive decrease of the object recognition index, indicative of a memory deficit (Bura et al., 2007; Puighermanal et al., 2009; Taglialatela et al., 2009). The memory deficit became significant on Day 10 and was maximal on Day 18 (4 days after

the infusion of CSF had stopped). On Day 25, the object recognition index had normalized and was similar to that of animals treated with control CSF (Fig. 2A and B). For all time-points, the total time spent exploring both objects (internal control) was similar in animals infused with control or patients' CSF (Supplementary Table 1).

The preference to drink sweetened water (sucrose preference test) was used as a measure of anhedonic behaviour. Mice infused with patients' CSF and tested during the infusion period (Day 10) had less preference for sucrose compared with mice infused with control CSF (Fig. 2C). In contrast, the same mice tested 10 days after the infusion of CSF had stopped (Day 24) showed a preference for sucrose similar to that of the control mice. The total consumption of water with and without sucrose was similar in both groups (internal control, Supplementary Table 1). In addition, two tests of depressive-like behaviour were performed. The tail suspension test, performed on Day 12, showed that animals infused with patients' CSF had longer periods of immobility compared with those infused with control CSF (Fig. 2D). In contrast, 6 days after the infusion of CSF had stopped (Day 20), no differences were noted with the forced swimming test (examining immobility in inescapable situations; Fig. 2E and Supplementary Table 1). Overall, these findings suggest that the infusion of NMDAR antibodies was associated with anhedonic and depressive-like behaviours.

In contrast to the prominent memory deficit, along with anhedonia and depressive behaviour, no significant differences were noted in tests of anxiety (black and white test, elevated plus maze test), aggression (resident-intruder test) and locomotor activity (Fig. 3A–D).

Patients' antibodies bind to NMDAR in mouse brain

Animals infused with patients' CSF, but not control CSF, had progressively

increasing human IgG immunostaining (representing IgG bound to brain) that correlated with the duration of the infusion. The distribution of IgG immunostaining predominated in regions with high density of NMDAR, mainly the hippocampus (Fig. 4A), resembling that obtained with brain sections directly incubated with patients' CSF or a monoclonal antibody against GluN1 (Dalmau et al., 2008). Upon quantification of immunostaining, the maximal antibody binding was identified in mice sacrificed on Day 18, which had received 14 days of CSF infusion, compared with mice sacrificed on Days 5 or 13 (Fig. 4B and C). In animals sacrificed on Days 26 and 46 the presence of IgG immunostaining progressively decreased. In frontal cortex the dynamics of IgG binding were similar to those of the hippocampus (Supplementary Fig. 1), but the amount of IgG was substantially less; in other brain regions such as the cerebellum and striatum, the IgG immunostaining was sparse and not significantly different between animals infused with patients' CSF or control CSF (data not shown).

Studies with immunofluorescence and confocal microscopy showed that in animals infused with patients' CSF the presence of hippocampal IgG was visible as a punctate immunolabelling on the surface of neurons and neuronal processes in contrast to mice infused with control CSF where minor amounts of IgG reactivity without preference for neuronal structures were noted (Fig. 4D–G). In addition, the amount of human IgG bound to all selected regions of hippocampus was significantly higher than in the control group (Fig. 4H).

To determine if the IgG immunostaining represented brain-bound NMDAR antibodies, IgG was extracted from several brain regions and examined for reactivity with HEK cells expressing GluN1. These studies showed that the IgG extracted from

hippocampus of mice infused with patients' CSF reacted specifically with GluN1 (Fig. 5A). The NMDAR antibody concentration in the extracts correlated with the duration of infusion of CSF; it increased until Day 13, reached the maximal concentration on Days 13–18, and decreased afterwards (Fig. 5A and C). NMDAR antibodies were also detected in IgG extracts from other brain regions (frontal cortex, cerebellum) but at lower concentration to that obtained from hippocampus (Fig. 5D). Demonstration that the extracted antibodies were specifically bound to the NMDAR was provided by the lack of GluN1 reactivity in the pre-extraction fractions (Fig. 5B and E). Parallel studies with tissue from animals infused with control CSF did not show NMDAR antibodies (Supplementary Fig. 2).

Effects of patients' antibodies on NMDAR

To determine the effects of patients' antibodies on NMDAR, we focused on the hippocampus, which was the region with maximal concentration of NMDAR-bound antibodies. Compared with animals infused with control CSF, those infused with patients' CSF had on Days 13 and 18 a significant decrease of the density of total and synaptic hippocampal NMDAR clusters followed by a gradual recovery after Day 18 (pooled analysis of CA1, CA3 and dentate gyrus; Fig. 6A–D). No significant differences in between hippocampal subregions (CA1, CA3, dentate gyrus) were observed (not shown). In contrast, patients' antibodies did not alter the density of PSD95 or AMPAR clusters (Fig. 6E and F).

Immunoblot analysis of total protein extracted from hippocampus showed that on Days 13 and 18, mice infused with patients' CSF had a significant decrease of total NMDAR protein concentration compared with mice infused with control CSF (Fig. 7A and B). The magnitude of this effect was greater in animals with higher concentration of IgG bound to hippocampus (Fig. 7C). Parallel studies examining the effect

on the protein concentrations of PSD95 (Fig. 7A and E) and AMPAR (Fig. 7D) demonstrated no significant differences between mice infused with patients' CSF or control CSF.

In cerebellum, no significant effects on the cluster density or total protein concentration of NMDAR, PSD95 and AMPAR were noted in animals infused with patients' CSF compared to those infused with control CSF (data not shown).

Immunohistochemical studies for neuronal apoptosis, infiltrates of T or B cells, and deposits of complement in hippocampus of animals infused with patients' or control CSF, examined on Day 18, showed no abnormalities (Fig. 8).

Discussion

We report that passive transfer of NMDAR antibodies by continuous ventricular infusion of CSF from patients with anti-NMDAR encephalitis causes memory and behavioural deficits in mice, and that the effects are likely mediated by the binding of antibodies to NMDAR resulting in a specific decrease of the density of these receptors. Data from earlier reports showing that despite the severity and duration of symptoms, most patients with anti-NMDAR encephalitis respond to immunotherapy (Gresa-Arribas et al., 2014), and findings at the cellular level demonstrating that patients' antibodies cause a titre-dependent decrease of synaptic NMDAR receptors fulfilled most of the Witebsky's criteria for an antibody-mediated disease (Rose and Bona, 1993), but the transfer of symptoms to animals was pending. In the current study, four sets of experiments satisfy this postulate: (i) the development of symptoms in animals infused with patients' CSF, but not control CSF; (ii) the demonstration that the infused antibodies reacted predominantly with brain regions with high density of NMDAR (e.g. hippocampus) and specifically recognized these receptors; (iii) the

identification of a selective decrease of the density of total and synaptic NMDAR clusters and total NMDAR protein concentration without affecting PSD95, and that these effects correlated with the concentration of brain-bound antibodies; and (iv) the correlation noted between the intensity of the abovementioned findings and time-course of patients' antibody infusion, as well as between the reversibility of symptoms and restoration of NMDAR levels after stopping the infusion of CSF antibodies.

Approximately 75% of patients with anti-NMDAR encephalitis present with mood and psychiatric alterations ranging from manic or depressive behaviour to psychosis, often followed by stereotyped movements, seizures, or decreased level of consciousness (Kayser et al., 2013; Titulaer et al., 2013). Regardless of the presentation, most patients develop severe problems forming new memories and have amnesia of the disease. Close examination during the phase of recovery shows, in some patients, impairment in the visual recognition of objects or faces (e.g. physicians, nurses) (Frechette et al., 2011). Owing to the wide range of symptoms of the disease and lack of previous studies examining the distribution of brain tissue NMDAR-antibody binding when these antibodies are infused intraventricularly, we used standardized memory and behavioural tests. The most notable effects were observed in the tests of memory (novel object recognition) using different groups of animals in two different paradigms (open field and V-maze). While the first depends predominantly on normal hippocampal function, the second is dependent of perirhinal-hippocampal structures (Winters et al., 2004). Compared with animals infused with control CSF, those infused with patients' CSF developed progressive memory deficits, which were maximal on Days 13–18 when the highest concentration of brain-bound NMDAR antibodies and lowest density of NMDAR occurred. Other paradigms affected were related to depressive-like behaviours (tail suspension test) and anhedonic behaviours (sucrose preference test).

We did not find significant abnormalities in the tests of aggression and anxiety, which are often present in the human disease, or in locomotor activity (an expected finding given that paralysis rarely occurs in patients).

The high levels of brain-bound NMDAR antibodies between Days 13–18 suggests that after stopping the infusion of patients' CSF on Day 14, the NMDAR antibodies continued being distributed from mice cerebroventricular system to parenchyma. This distribution occurred slowly; for example, 5 days after starting the infusion of patients' CSF the amount of NMDAR antibodies that had reached the hippocampus was very limited compared to that seen on Days 13–18 (shown in Fig. 4B). Moreover, previous studies using cultured neurons treated with patients' CSF showed that once the antibodies bound to the NMDARs, the reduction of receptors was microscopically visible in 2 h but it took 12 h to result in the lowest receptor density. Subsequently, there was a steady state of low NMDAR density for as long as the neurons were exposed to patients' antibodies (Moscato et al., 2014). Together, these findings explain the progressive worsening of symptoms along with continued antibody binding and decrease of NMDAR for at least 4 days after the ventricular infusion stops and the subsequent recovery starts.

Although the hippocampus was the region with the highest concentration of brain-bound NMDAR antibodies, these antibodies were also extracted from cerebral cortex or cerebellum though at much lower levels. The higher concentration of antibodies and predominant decrease of NMDAR in the hippocampus are consistent with the predominant binding of human antibodies to this brain region when sections of rodent brain are directly incubated with patients' antibodies (Dalmau et al., 2007; Moscato et al., 2014). Additionally, because of the close spatial relationship to the ventricles, the intraventricular infusion of human CSF antibodies might have contributed

to the preferential binding to the hippocampus.

The correlation between the concentration of brain-bound antibodies and selective reduction of NMDAR cluster density and protein concentration was similar to that reported using in vitro studies with cultured rat hippocampal neurons (Hughes et al., 2010; Moscato et al., 2014). Moreover, autopsies of patients with anti-NMDAR encephalitis showed that the hippocampal regions with highest concentration of brain-bound antibodies were also the areas with lower expression of NMDAR (Dalmau et al., 2007). In the current model, patients' antibodies did not alter AMPAR cluster density or protein concentration; these findings are in line with those reported with cultured neurons where the clusters of AMPAR and AMPAR-mediated currents were not directly affected (Hughes et al., 2010). These experiments, however, did not explore whether paradigms that normally induce long-term potentiation, and therefore increase the number of synaptic AMPAR, were altered by patients' antibodies. Mikasova et al. (2012) showed that neurons exposed to patients' NMDAR antibodies failed to show an increase in cell surface AMPAR after induction of chemical long-term potentiation. Another study examining the acute metabolic effects of patients' antibodies after injection into rat brain showed impairment of NMDA and AMPA-mediated synaptic function (Manto et al., 2010). In the present model, we did not perform electrophysiological studies on acute slices of brain (a goal of future studies); however, there is reported evidence that patients' NMDAR antibodies suppress induction of long-term potentiation when directly applied to mouse hippocampal slices (Zhang et al., 2012). Work with cultured neurons indicates that the decrease of synaptic NMDAR currents is likely a result of the antibody-mediated low receptor levels, as no direct antibody blockade was detected (Moscato et al., 2014).

Our study has limitations related to the type of disease and symptoms

to model. For example, different from other models of antibody-mediated CNS disorders where the antibodies result in characteristic symptoms (e.g. amphiphysin antibodies and visible muscle spasms) (Sommer et al., 2005) or focal deficits with visible tissue changes (e.g. AQP4 antibodies and neuromyelitis optica) (Hinson et al., 2012; Bradl and Lassmann, 2014), anti-NMDAR encephalitis results in a broader spectrum of symptoms where memory and behavioural deficits occur early, and the structural alterations are not visible unless the NMDAR clusters or protein concentration are measured. It is not surprising that in the current model the full spectrum of symptoms, such as seizures, dyskinesias or coma, did not occur. Studies with NMDAR antagonists have shown that the progression of symptoms (from behavioural and memory deficits to unresponsiveness with catatonic features and coma) correlated with the intensity of the decrease of receptor function (Javitt and Zukin, 1991). Therefore, it is likely that prolonged infusion or higher concentration of patients' antibodies would cause additional symptoms. This is supported by the current model, in which the time course of symptom development, brain-bound antibody concentration, and decrease of synaptic NMDAR correlated well with each other. Future experiments using prolonged infusion or higher concentration of patients' antibodies may also result in symptoms beyond hippocampal-parahippocampal regions. Compared with the hippocampus, other brain regions normally have lower density of NMDAR, and appeared to be less accessible to the ventricularly infused antibodies. Direct injection of antibodies into those brain regions can be considered, but we previously tried bilateral hippocampal infusion using the same osmotic pump approach, resulting in more limited antibody diffusion and no symptoms (data not published). Moreover, the phenotype of the current model is likely influenced by the strain of mice. In this study we used C57BL6/J mice because we were interested in the effects on memory and behaviour, but this strain is one of the

most resistant to develop seizures (Ferraro et al., 2002).

The antibody-induced depletion of synaptic NMDAR along with the similarities between the human disease and the phenotypes induced by NMDAR antagonists (phencyclidine, ketamine or MK801) have suggested points of convergence with one of the most influential theories of schizophrenia, the NMDA-hypofunction model (Olney and Farber, 1995; Kehrer et al., 2008). The presence of positive (hallucinations, delusions, hyperactivity) and negative (decreased motivation, flat affect, deficit of memory and learning) symptoms is, however, not identical among the drug-induced phenotypes and also varies among animal species (Javitt and Zukin, 1991). It has been suggested that NMDAR-bearing parvalbumin-positive GABAergic interneurons are disproportionally more sensitive to NMDAR antagonists than other neurons (Li et al., 2002). Interestingly, a genetic model of partial ablation of the GluN1 subunit of NMDAR in corticolimbic GABAergic interneurons resulted in symptoms partially resembling our GluN1 immunological model of receptor depletion, including memory deficits and anhedonic behaviours (Belforte et al., 2010). Differences related to the underlying mechanisms (pharmacologic blockade, genetic or immunologic NMDAR depletion) and regions where the NMDAR function is depleted (general, corticolimbic, or hippocampal-parahippocampal) likely influence the clinical phenotypes.

Overall, the current findings provide robust evidence that antibodies from patients with anti-NMDAR encephalitis alter memory and behaviour through reduction of cell-surface and synaptic NMDAR, and therefore support the use of treatments directed at decreasing the levels of antibodies or antibody-producing cells. This approach can now be adapted to (i) model other aspects of the disease by changing the duration and dosing of antibody infusion, or strain of mice; (ii) investigate other disorders of memory and behaviour that occur in association with antibodies against

other cell surface or synaptic proteins, such as AMPAR or GABA(B)R (Lai et al., 2009; Lancaster et al., 2010); and (iii) determine whether compounds such as Ephrin-B2 ligand that has been shown to prevent the destabilizing NMDAR crosslinking effects of patients' antibodies improve or alter the course of the disease (Mikasova et al., 2012).

Figures

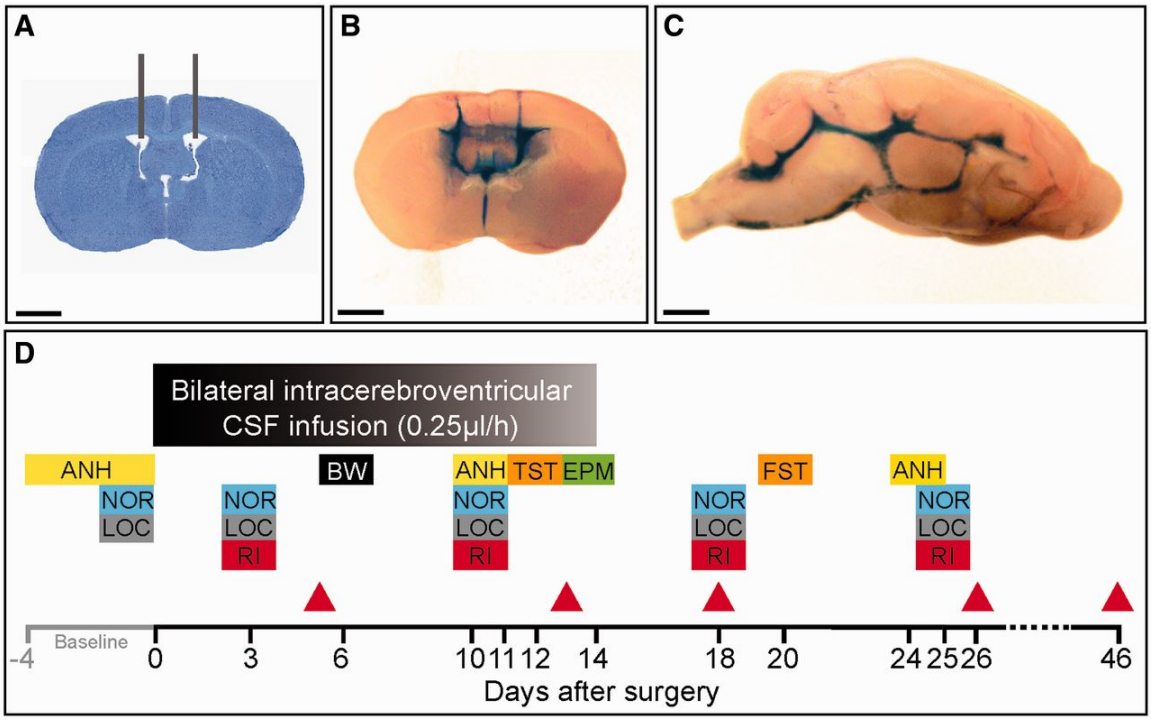
A
B
C
D
Bilateral intracerebroventricular
CSF infusion (0.25µl/h)
ANH
NOR
LOC
BW
NOR
LOC
RI
ANH
TST
EPM
NOR
LOC
RI
NOR
LOC
RI
FST
ANH
NOR
LOC
RI
Baseline
-4
0
3
6
10
11
12
14
18
20
24
25
26
46
Days after surgery

Figure 1. Experimental design and placement of ventricular catheters.

(A) Representative coronal mouse brain section with catheter placement. Scale bar = 2 mm. (B and C) Coronal and sagittal mouse brain sections demonstrating cerebroventricular diffusion of methylene blue after ventricular infusion. Scale bars = 2 mm. (D) Schedule of cognitive testing and animal sacrifice. At Day 0, catheters and osmotic pumps were placed and bilateral ventricular infusion of patients' or control CSF started. Infusion lasted for 14 days. Memory [novel object recognition (NOR)], anhedonia [sucrose preference test (ANH)], depressive-like behaviour [tail suspension test (TST) and forced swimming test (FST)], anxiety [black and white test (BW) and elevated plus maze test (EPM)], aggressiveness [resident intruder test (RI)] and locomotor activity (LOC) were assessed blinded to treatment at the indicated days. The novel object recognition was assessed in open field and V-maze paradigms in two different cohorts of mice. Animals were habituated for 1 to 4 days before surgery (baseline) to novel object recognition, anhedonia, and locomotor activity. Red arrowheads indicate the days of sacrifice for studies of effects of antibodies in brain.

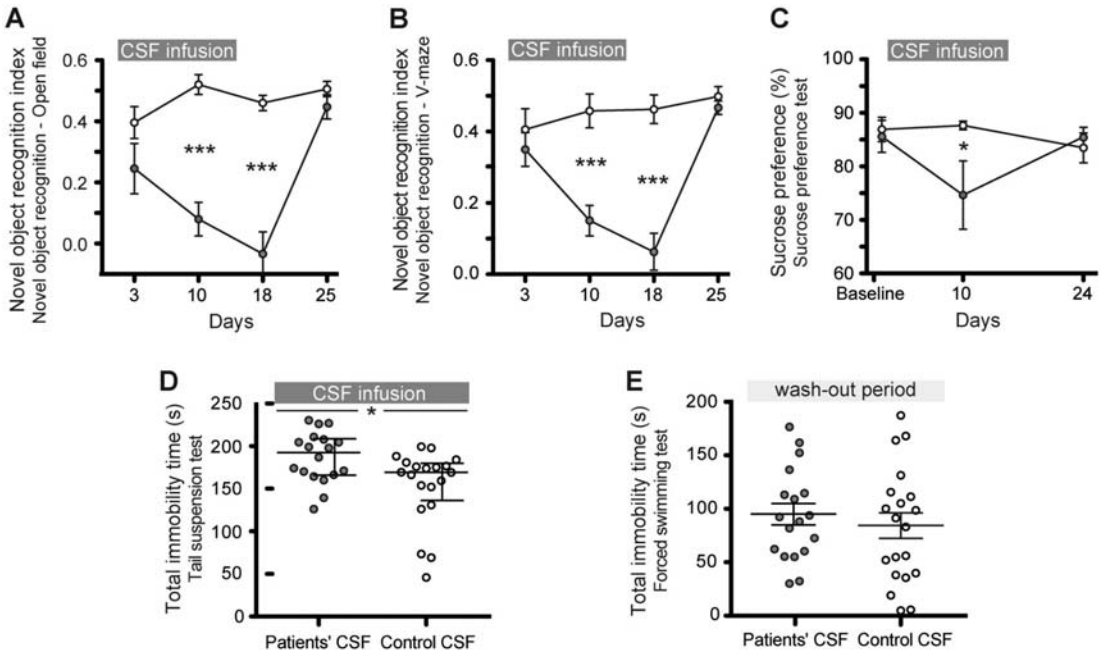
A
Novel object recognition index
Novel object recognition - Open field
CSF infusion
0.6
0.4
0.2
0.0
***
***
3
10
18
25
Days
B
Novel object recognition index
Novel object recognition - V-maze
CSF infusion
0.6
0.4
0.2
0.0
***
***
3
10
18
25
Days
C
Sucrose preference (%)
Sucrose preference test
CSF infusion
100
90
80
70
60
*
Baseline
10
24
Days
D
Total immobility time (s)
Tail suspension test
CSF infusion
250
200
150
100
50
0
*
Patients' CSF
Control CSF
E
Total immobility time (s)
Forced swimming test
wash-out period
200
150
100
50
0
Patients' CSF
Control CSF

Figure 2. Infusion of CSF from patients with NMDAR antibodies causes deficits in memory, anhedonia and depressive-like behaviour.

(A and B) Novel object recognition index in open field (A) or V-maze paradigms (B) in animals treated with patients' CSF (grey circles) or control CSF (white circles). A high index indicates better object recognition memory. (C) Preference for sucrose-containing water in animals infused with patients' CSF (grey) or control CSF (white). Lower percentages indicate anhedonia. (D and E) Total time of immobility in tail-suspension test during the infusion period (D, Day 12) and in forced swimming test after the infusion period (E, Day 20). Data are presented as mean ± SEM (median ± IQR in D). Number of animals: patients' CSF n = 18 (open field novel object recognition n = 8), control CSF n = 20 (open field novel object recognition n = 10). Significance of treatment effect was assessed by two-way ANOVA (A–C) with an α-error of 0.05 and post hoc testing with Sidak-Holm adjustment (asterisks), unpaired t-test (E) or Mann-Whitney U test (D). *P < 0.05, ***P < 0.001. See Supplementary Table 1 for detailed statistics.

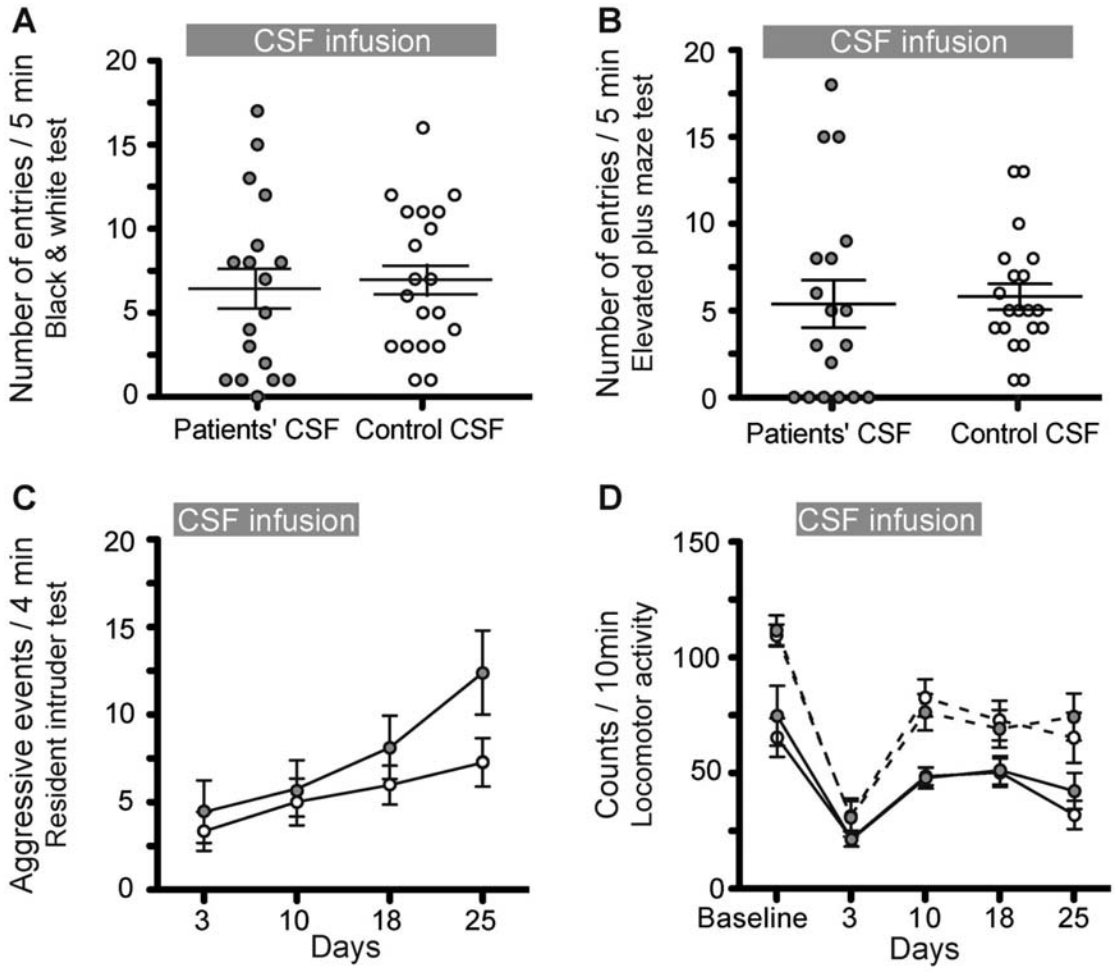
A
CSF infusion
Number of entries / 5 min
Black & white test
20
15
10
5
0
Patients' CSF
Control CSF
B
CSF infusion
Number of entries / 5 min
Elevated plus maze test
20
15
10
5
0
Patients' CSF
Control CSF
C
CSF infusion
Aggressive events / 4 min
Resident intruder test
20
15
10
5
0
3
10
18
25
Days
D
CSF infusion
Counts / 10min
Locomotor activity
150
100
50
0
Baseline
3
10
18
25
Days

Figure 3. Infusion of CSF from patients with NMDAR antibodies does not alter the tests of anxiety, aggression and locomotor activity.

(A and B) Number of entries into bright/open compartments during a 5 min period in a standard black and white (A, Day 6) or elevated plus maze test (B, Day 14) in animals treated with patients' CSF (filled circles) or control CSF (open circles). (C) Number of aggressive events over a 4-min period in a resident intruder paradigm in both treatment groups. (D) Horizontal (solid lines) and vertical (dashed lines) movement count over a 10 min period in both treatment groups. Data are presented as mean ± SEM. Number of animals: patients' CSF n = 18, control CSF n = 20. Statistical assessment as indicated in Fig. 2 and Supplementary Table 1.

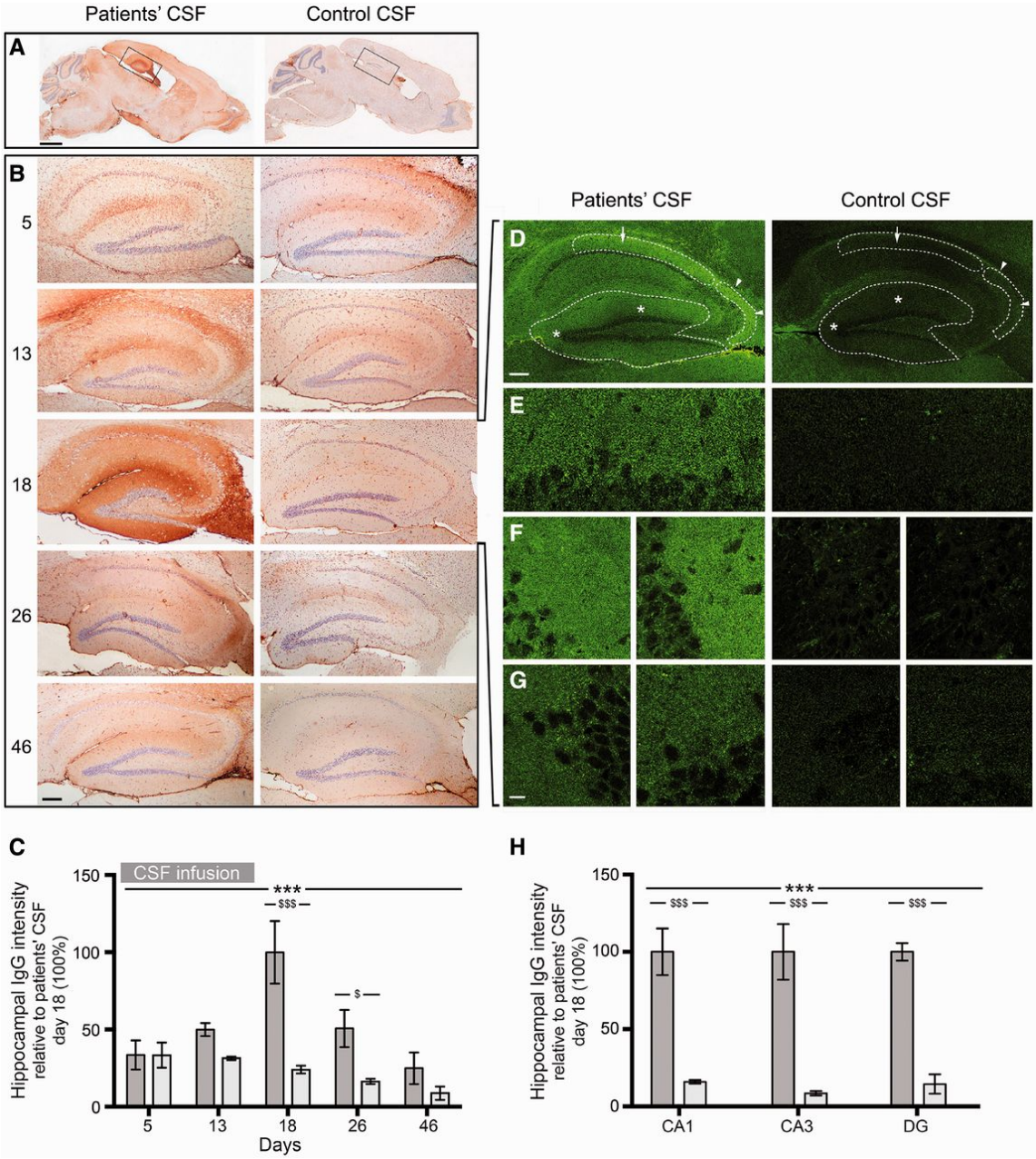
Patients' CSF
Control CSF
A
B
5
13
18
26
46
D
E
F
G
C
CSF infusion
Hippocampal IgG intensity relative to patients' CSF day 18 (100%)
150
100
50
0
***
$$$
$
5
13
18
26
46
Days
H
CA1
CA3
DG

Figure 4. Animals infused with patient's CSF have a progressive increase of human IgG bound to hippocampus.

(A and B) Immunostaining of human IgG in sagittal brain sections (A) and hippocampus (B) of representative animals infused with patients' CSF (left) and control CSF (right), sacrificed at the indicated experimental days. In animals infused with patients' CSF there is a gradual increase of IgG immunostaining until Day 18, followed by decrease of immunostaining. Scale bars: A = 2 mm; B = 200 μm. (C) Quantification of intensity of human IgG immunolabelling in hippocampus of mice infused with patients' CSF (dark grey columns) and control CSF (light grey columns) sacrificed at the indicated time points. (D–H) Confocal microscopy analysis of IgG bound to the hippocampus on Day 18. (D) Sagittal section of the hippocampus with areas examined at higher magnification in E (arrow in CA1), F (arrow heads in CA3) and G (asterisks in dentate gyrus). Note the fine punctate IgG immunolabelling surrounding neuronal bodies in mice infused with patients' CSF; this immunolabelling is similar to that reported in brain sections directly incubated with patients' antibodies, as in Dalmau et al. (2008). Scale bars: D = 200 μm; E–G = 10 μm. (H) Quantification of the intensity of human IgG immunofluorescence in the indicated areas in animals infused with patients' CSF (dark grey columns) or control CSF (light grey columns). For all quantifications, mean intensity of IgG immunostaining in the group with the highest value (animals treated with patients' CSF and sacrificed at Day 18) was defined as 100%. All data are presented as mean ± SEM. For each time point five animals infused with patients' CSF and five with control CSF were examined. Significance of treatment effect was assessed by two-way ANOVA with an α-error of 0.05 (*) and post hoc testing with Sidak-Holm adjustment ($). ***, $$$P < 0.001; $P < 0.05. See Supplementary Table 2 for detailed statistics.

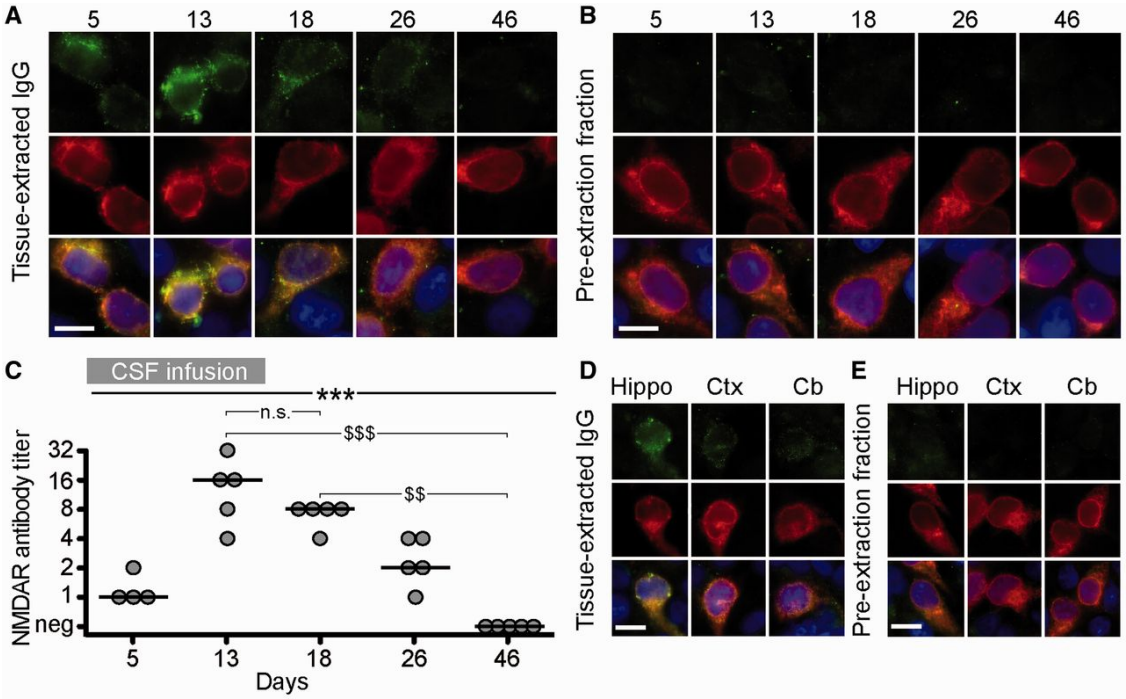
A
5
13
18
26
46
Tissue-extracted IgG
B
5
13
18
26
46
Pre-extraction fraction
C
CSF infusion
***
n.s.
$$$
$$
NMDAR antibody titer
32
16
8
4
2
1
neg
5
13
18
26
46
Days
D
Hippo
Ctx
Cb
Tissue-extracted IgG
E
Hippo
Ctx
Cb
Pre-extraction fraction

Figure 5. The human IgG extracted from brain of mice infused with patients' CSF is specific for NMDARs.

(A and B) HEK293 cells expressing the GluN1 subunit of the NMDAR immunolabelled with acid-extracted IgG fractions (top row in A) or pre-extraction fractions (top row in B) from hippocampus of mice infused with patients' CSF and sacrificed on the indicated days. The maximal reactivity with GluN1-expressing cells was noted in acid-extracted IgG fractions from Days 13 and 18 (A); none of the pre-extraction fractions showed GluN1 reactivity (B) indicating that the reactivity of acid-extracted fractions corresponds to IgG antibodies that were bound to brain NMDAR receptors. The second row in A and B shows the reactivity with a monoclonal GluN1 antibody, and the third row the colocalization of immunolabelling. Scale bars = 10 μm. (C) Quantification of NMDAR antibody titre in IgG-extracted fractions from hippocampus of animals treated with patients' CSF (n = 5 mice per each time point, except four mice for Day 5). Solid line = median. Significance was tested by Kruskal-Wallis with an α-error of 0.05 (asterisks) and post hoc testing with Dunn's test ($). **, $$P < 0.01, ***, $$$P < 0.001. See Supplementary Table 2 for detailed statistics. (D and E) HEK293 cells expressing the GluN1 subunit of the NMDAR immunolabelled with acid-extracted IgG fractions (D) and pre-extraction fractions (E) from hippocampus (Hippo), cerebral cortex (Ctx) and cerebellum (Cb) of mice infused with patients' CSF (Day 18). The acid-extracted IgG fraction from hippocampus showed higher level of NMDAR antibodies than those extracted from cerebral cortex (Ctx) and cerebellum (Cb). Scale bars = 10 μm. n.s = not significant.

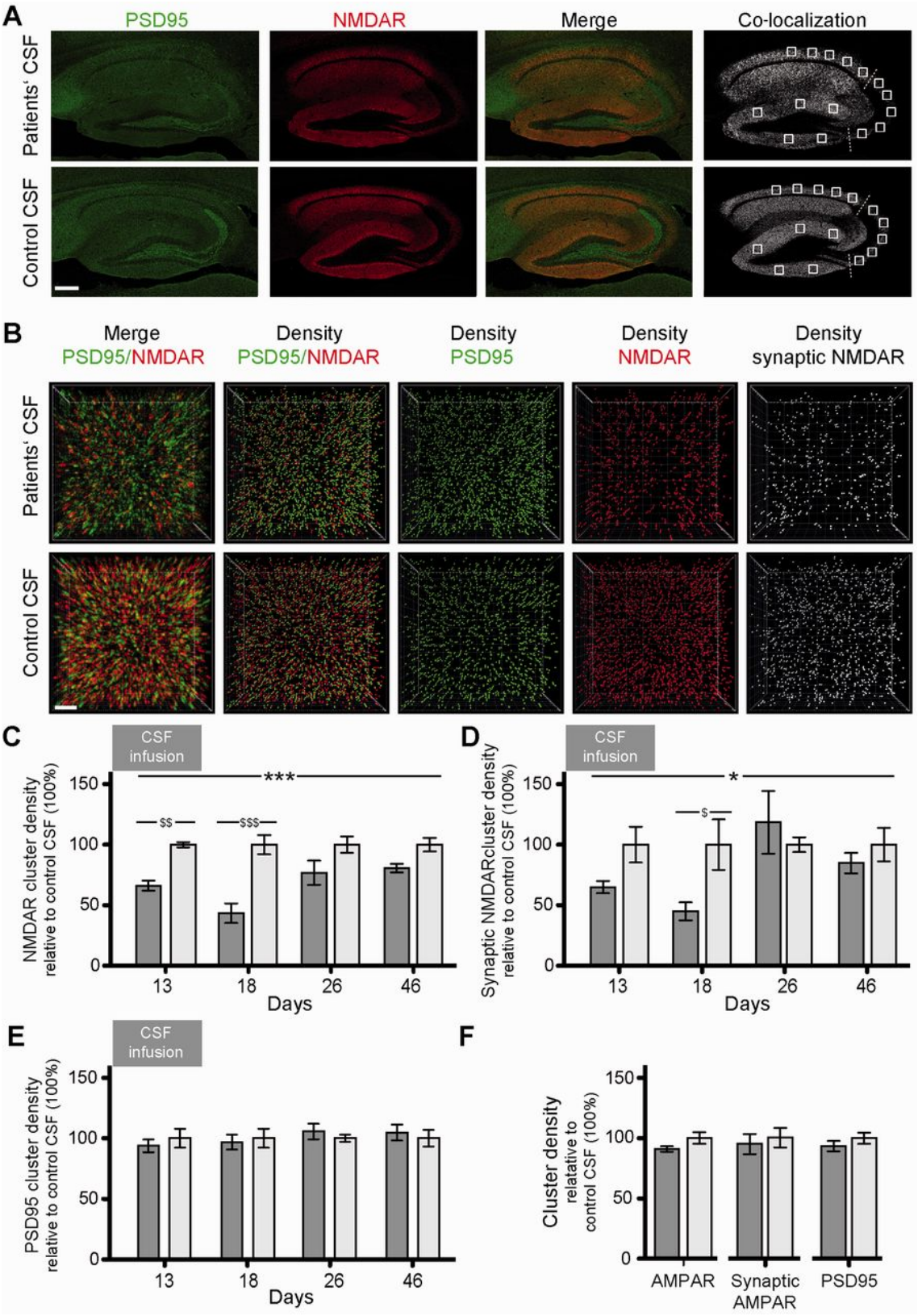
A
PSD95
NMDAR
Merge
Co-localization
Patients' CSF
Control CSF
B
Merge PSD95/NMDAR
Density PSD95/NMDAR
Density PSD95
Density NMDAR
Density synaptic NMDAR
Patients' CSF
Control CSF
C
CSF infusion
***
$$
$$$
NMDAR cluster density relative to control CSF (100%)
150
100
50
0
13
18
26
46
Days
D
CSF infusion
*
$
Synaptic NMDARcluster density relative to control CSF (100%)
150
100
50
0
13
18
26
46
Days
E
CSF infusion
PSD95 cluster density relative to control CSF (100%)
150
100
50
0
13
18
26
46
Days
F
Cluster density relatative to control CSF (100%)
150
100
50
0
AMPAR
Synaptic AMPAR
PSD95

Figure 6. Patients' NMDAR antibodies selectively reduce the density of total and synaptic NMDAR clusters in hippocampus of mice.

(A) Hippocampus of mice infused for 14 days (Day 18) with patients' CSF (upper row) or control CSF (lower row) immunolabelled for PSD95 and NMDAR. Images were merged (merge) and post-processed to demonstrate co-localizing clusters (co-localization). Squares in 'co-localization' indicate the analysed areas in CA1, CA3 and dentate gyrus. Scale bar = 200 μm. (B) Three-dimensional projection and analysis of the density of total clusters of PSD95 and NMDAR, and synaptic clusters of NMDAR (defined as NMDAR clusters colocalizing with PSD95) in a representative CA3 region (square in A 'co-localization'). Merged images (merge, PSD95 green, NMDAR red) were post-processed and used to calculate the density of clusters (density = spots/μm3). Scale bar = 2 μm. (C–F) Quantification of the density of total (C) and synaptic (D) NMDAR clusters, PSD95 clusters (E), and total/synaptic AMPAR and PSD95 clusters (Day 18 only, F) in a pooled analysis of hippocampal subregions (CA1, CA3, dentate gyrus) in animals treated with patients' CSF (dark grey) or control CSF (light grey) on the indicated days. Mean density of clusters in control CSF treated animals was defined as 100%. Data are presented as mean ± SEM. For each time point five animals infused with patients' CSF and five with control CSF were examined. Significance of treatment effect was assessed by two-way ANOVA with an α-error of 0.05 (asterisks) and post hoc testing with Sidak-Holm adjustment ($) (C–E) or unpaired t-test (F). *, $P < 0.05; **, $$P < 0.01; ***, $$$P < 0.001. See Supplementary Table 2 for detailed statistics.

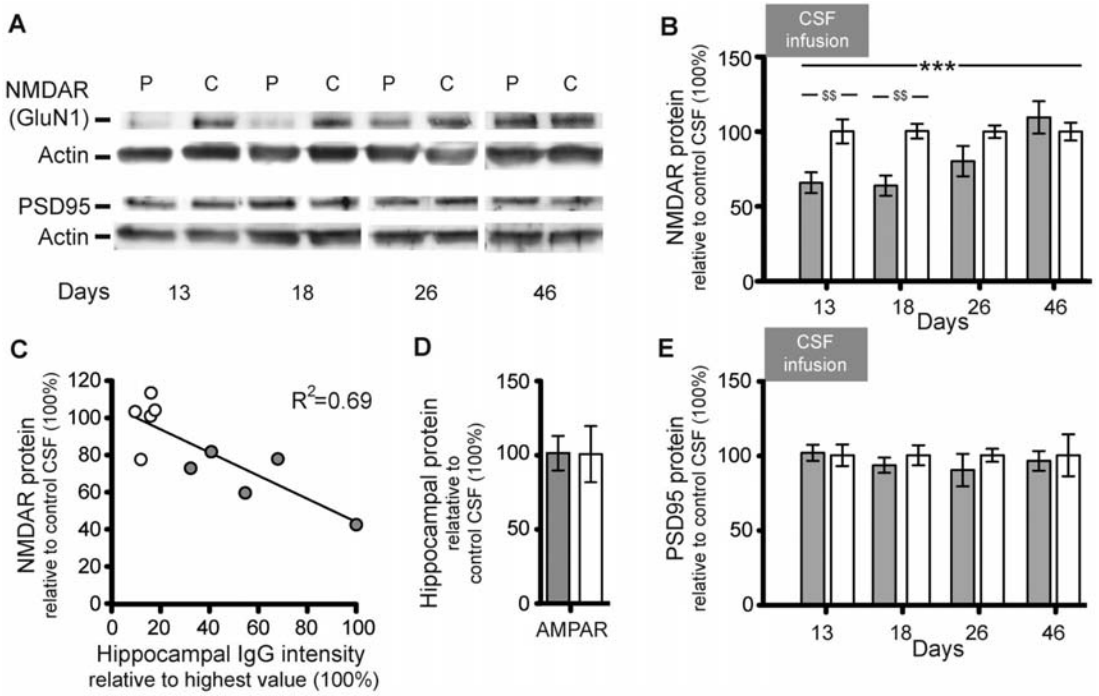
A
NMDAR (GluN1)
Actin
PSD95
Actin
P C P C P C P C
Days 13 18 26 46
B
CSF infusion
***
$$ $$
NMDAR protein relative to control CSF (100%)
Days
C
R²=0.69
NMDAR protein relative to control CSF (100%)
Hippocampal IgG intensity relative to highest value (100%)
D
Hippocampal protein relatative to control CSF (100%)
AMPAR
E
CSF infusion
PSD95 protein relative to control CSF (100%)
Days

Figure 7. Patients' NMDAR antibodies selectively reduce the protein concentration of NMDAR in hippocampus of mice.

(A) Representative immunoblots of proteins extracted from hippocampus of animals infused with patients' CSF (P) or control CSF (C) sacrificed at the indicated time points and probed for expression of GluN1 (NMDAR), PSD95 and β-actin (loading control). Note that there is less visible GluN1 expression on Days 13 and 18. (B, D and E) Quantification of total NMDAR (B), AMPAR (D) or PSD95 (E) protein in animals treated with patients' CSF (filled columns) or control CSF (open columns) sacrificed at the indicated time points (AMPAR Day 18 only, D). Results were normalized to β-actin (loading control). Mean band density of animals treated with control CSF was defined as 100%. Data are presented as mean ± SEM. For each time point six animals infused with patients' CSF and six with control CSF were examined (for Days 26 and 46, only five animals treated with patient's CSF were available). Significance of treatment effect was assessed by two-way ANOVA with an α-error of 0.05 (asterisks) and post hoc testing with Sidak-Holm adjustment ($). $$P < 0.01; ***P < 0.001. See Supplementary Table 2 for detailed statistics. (C) Correlation between concentration of human IgG bound to hippocampus (x-axis, highest hippocampal IgG intensity was defined as 100%) and hippocampal NMDAR protein concentration in mice sacrificed on Day 18 ($R^2 = 0.69$, $P = 0.003$). Filled circles: mice infused with patients' CSF (n = 5), open circles: mice infused with control CSF (n = 5).

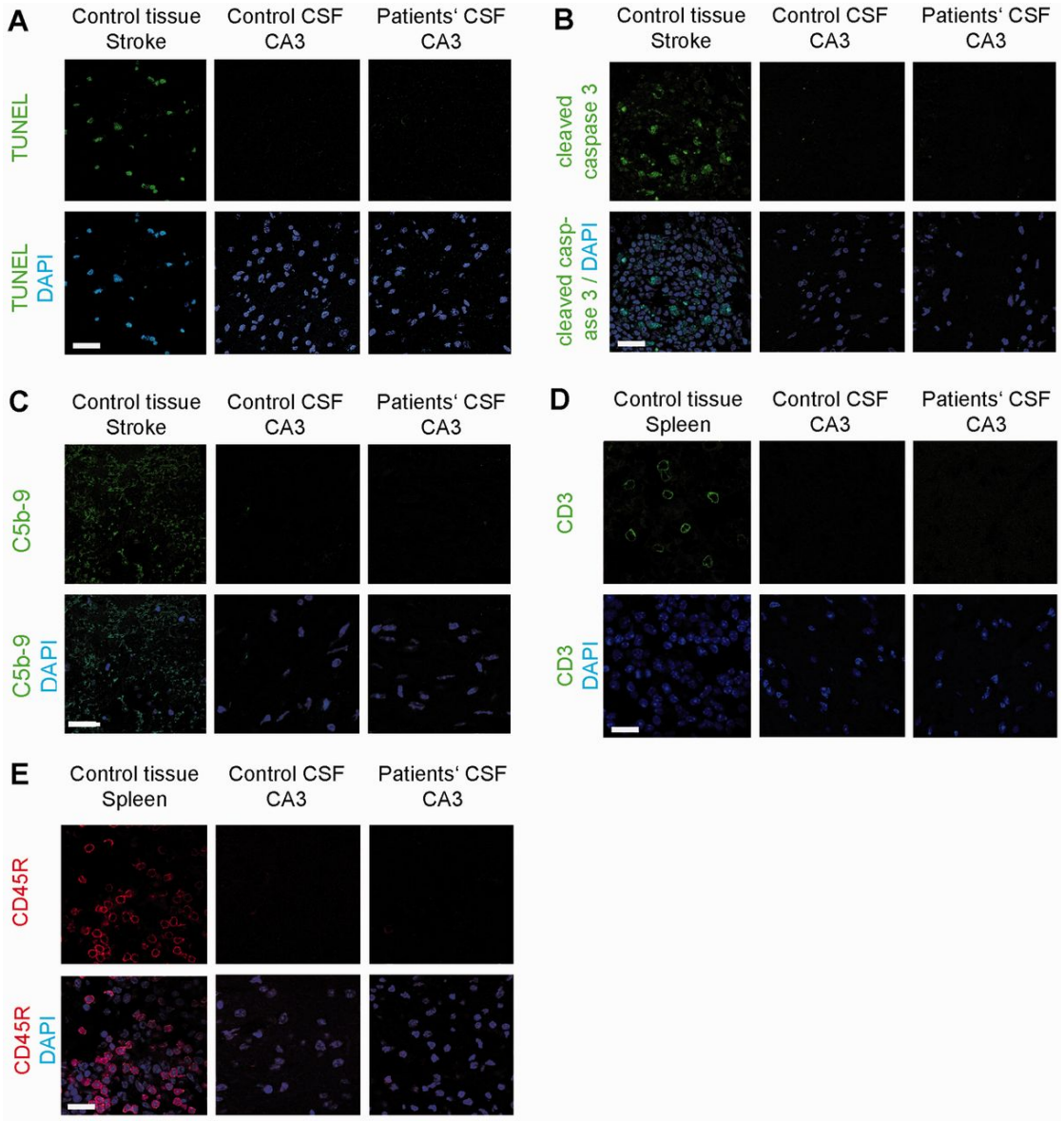

Figure 8. Absence of neuronal apoptosis, deposits of complement, and lymphocytic infiltrates in the hippocampus of mice infused with patients' CSF.

(A and B) TUNEL and cleaved caspase 3 immunolabelling of a representative area of CA3 (area with maximal IgG binding and lower NMDAR concentration) of an animal infused with patients' CSF, showing lack of apoptotic cells. A section of the same region in an animal with transient middle cerebral artery occlusion (stroke model) shows apoptotic cells in the penumbra (left). (C) Same CA3 region as in (A)

immunostained for C5b-9 showing lack of deposit of complement. A section of the same region in the indicated stroke model shows presence of complement in the penumbra (left). (D and E) Same CA3 region as in (A) immunostained for T (CD3) and B (CD45R) lymphocytes showing absence of inflammatory infiltrates. A section of spleen was used as control tissue showing the presence of CD3 (green) and CD45R (red) cells. Scale bar = 10 μm. Total number of animals examined: patients' CSF n = 5; control CSF n = 5. Scale bars = 20 μm.

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CHAPTER 4. Intrapartum anti-NMDA receptor encephalitis

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Anti–N-methyl-D-aspartate Receptor Encephalitis During Pregnancy

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Abstract

Objective: To report 3 patients who developed anti–N-methyl-D-aspartate receptor encephalitis during pregnancy.

Design: Case reports.

Setting: University hospitals.

Patients: Three young women developed at 14, 8, and 17 weeks of gestation acute change of behavior, prominent psychiatric symptoms, progressive decrease of consciousness, seizures, dyskinesias, and autonomic dysfunction.

Main Outcome Measures: Clinical, radiological, and immunological findings.

Results: The 3 patients had cerebrospinal fluid pleocytosis, normal magnetic resonance imaging, and electroencephalogram showing slow activity. All had higher antibody titers in cerebrospinal fluid than in serum and 2 had ovarian teratomas that were removed. The pregnancy was terminated in 1 patient with recurrent bilateral teratomas. All patients had substantial neurological recoveries, and the 2 newborns were normal. Results of extensive antibody testing in 1 of the babies were negative.

Conclusion: The current study shows that anti-NMDAR encephalitis during pregnancy can have a good outcome for the mother and newborn.

Introduction

Anti–N-methyl-D-aspartate receptor (NMDAR) encephalitis is a synaptic autoimmune disorder that is likely mediated by antibodies against the NR1 subunit of the receptor (Dalmau et al., 2008). Despite the severity of the disorder, most patients have substantial recoveries. Because the disease frequently affects women of childbearing age and the antibody subtypes (IgG1, IgG3) can cross the placenta, there is

concern about the effects of the disorder during pregnancy (Tüzün et al., 2009). We report 3 patients who developed the disorder during pregnancy.

Report of cases

Clinical features from the patients are described in this section and summarized in the Table. Antibodies to NMDAR were detected as reported elsewhere (Dalmau et al., 2008) and the titers were determined by serial dilution (starting at 1:10). The baby of patient 1 had antibody studies in the umbilical cord, serum, cerebrospinal fluid (CSF), and amniotic fluid.

Case 1

A 19-year-old woman presented at 14 weeks of gestation with 2 weeks of headache and malaise followed by bizarre behavior and paranoid delusions resulting in hospitalization. Over the course of a week, her mental status worsened until she was minimally responsive. She had a generalized seizure that was treated with fosphenytoin and lorazepam, and she was intubated for airway protection. A bedside electroencephalogram (EEG) revealed generalized slowing but no epileptic activity. On examination, she was minimally responsive to noxious stimuli, had generalized hyperreflexia, and moved all limbs spontaneously. Results of magnetic resonance imaging (MRI) and CSF studies are described in the Table. Treatment with acyclovir was started for presumptive viral encephalitis.

On the third day in the intensive care unit, she developed repetitive pursing of the lips and furrowing of her brow without EEG correlates. These movements became more frequent and the dyskinesias spread to her limbs. By day 8, she developed diaphoresis, tachycardia, mydriasis, and hypertension. These symptoms were difficult to control

despite treatment with fentanyl, lorazepam, propofol, bromocriptine, and β-blockers.

On day 5, a tracheostomy was performed. Treatment with intravenous immunoglobulin did not result in improvement. On day 23, an MRI of the abdomen and pelvis revealed a 2.5 × 3-cm left ovarian simple cyst. On day 43, NMDAR antibodies were identified in CSF. A second course of intravenous immunoglobulin treatment along with 1 g of methylprednisolone was given daily for 5 days. On day 50, a left oophorectomy was performed, revealing an immature teratoma. On day 52, plasmapheresis was initiated, with a total of 7 treatments over 2 weeks. On day 72, she became more alert, responded to voice, and tracked objects. Throughout the hospitalization, the fetus was monitored weekly by Doppler ultrasonography, showing normal heart tones. Obstetric ultrasonography performed at weeks 20 and 26 revealed normal fetal anatomy and appropriate growth for gestational age.

The patient remained in the intensive care unit because of persistence of sympathetic storms. By day 107, she was following simple commands and the sedation was slowly weaned. On day 127, she began mouthing words. A cesarean section and concomitant surgical staging was performed on day 166 (at 37 weeks of gestation, following amniocentesis confirming fetal lung maturity). The infant weighted 6 lb 3 oz and Apgar scores were 3 at 1 minute and 6 at 5 minutes. All pathological specimens (left adnexa, pelvic lymph nodes, and peritoneal samples) were negative for tumor. Over the next 3 days, the patient was weaned from the ventilator and she made steady gains in physical therapy. By day 184, she was able to ambulate with a walker and her speech was fluent, but she was only oriented to self. Her progress was hampered by impulsivity, short-term memory loss, and physical deconditioning. Two months after discharge, she was functioning independently at home, although she was persistently impulsive and

complained of somnolence. The infant has met all developmental milestones to date.

Case 2

A 20-year-old woman developed change of behavior during the eighth week of pregnancy. She became argumentative, refused to talk and eat, and developed stereotyped behaviors, such as walking endlessly around a room or filling and emptying a glass with water. Two days before hospital admission, semirhythmic movements including blinking, licking, and tongue protrusion were noted. One day before admission, she developed hyperthermia, decreased level of consciousness, and a seizure. She had a history of bilateral ovarian teratomas that were removed when she was 16 years of age.

At admission, she had neck stiffness, without fever, and showed repetitive orofacial movements. She was poorly responsive to verbal and painful stimuli and had generalized hyperreflexia. Ancillary test results are described in the Table. Intravenous acyclovir and methylprednisolone administration were started. On day 3, cardiac pauses up to 5 seconds were noted (Figure, A). Over the next few days, she developed hypersalivation and generalized tonic convulsions. On day 13, status epilepticus and respiratory depression led to intubation and mechanical ventilation. On day 15, a pelvic computed tomographic scan revealed bilateral ovarian tumors (Figure, B, arrows); 2 days later, a left salpingo-oophorectomy and removal of both tumors was performed, and the pregnancy was terminated. Pathological studies confirmed bilateral mature teratomas.

From days 23 to 27, she received intravenous immunoglobulin and sedation with midazolam was discontinued. She gradually started tracking objects and following commands but continued having partial seizures that were treated with carbamazepine

and gabapentin. On days 32 to 36, intravenous immunoglobulin administration was repeated, and by day 43, she was able to breathe spontaneously. By day 52, she was able to drink; the last seizure was observed on day 53. On day 64, she was eating regularly, and a few days later, she was able to walk. Her Mini-Mental State Examination score was 27 of 30 on day 85 and she was discharged home with minimal deficits on day 87.

Case 3

A 19-year-old pregnant woman presented at 17 weeks of gestation with acute-onset behavioral change, including increasing nervousness, irritability, and anxiety. She stopped walking and communicating but would say the same word repetitively and was transferred to a psychiatric facility. Her bizarre behavior continued; for example, she was frequently hitting the walls, taking cold baths, and accusing the physicians of “being murderers.” She had fluctuating periods of decreased level of consciousness and agitation. She had a partial tonic seizure involving the left arm without EEG correlate; this showed generalized high-amplitude slow activity (2 Hz). Because of progressive deterioration and the presumptive diagnosis of viral encephalitis, she was transferred 1 week later to a neurology unit. On physical examination, her temperature was 37.1°C, and she had no neck stiffness. She developed hyperhidrosis and repetitive semirhythmic oral movements, like automatisms. Brain computed tomography and MRI were normal. The CSF showed lymphocytic pleocytosis (white blood cell count, 11/μL) with normal protein and glucose concentrations. Results of extensive viral studies and autoimmune and paraneoplastic markers were negative. Anti-NMDAR antibodies were identified in her serum and CSF. A second EEG showed no changes compared with the previous study. Magnetic resonance imaging of the abdomen and pelvis and abdominal ultrasonography were normal.

From day 19, the patient was treated with intravenous methylprednisolone (500 mg/d for 5 days) without clinical improvement, and a similar course of methylprednisolone treatment was started on day 35. After this second treatment, the orofacial dyskinesias subsided, but she continued with a decreased level of consciousness. On day 54, she had a generalized tonic-clonic seizure and treatment with phenobarbital was started. One month later, her level of consciousness started to progressively improve. Throughout the hospitalization, she did not develop hypoventilation. The fetus was monitored regularly by Doppler ultrasonography, showing normal heart tones. At 37 weeks of pregnancy, she spontaneously delivered a healthy 2892-g baby with Apgar scores of 8 at 1 minute and 9 at 5 minutes. Her Mini-Mental State Examination score was 24 of 30, and she was discharged 3 weeks later. At the last follow-up, she was fully functional and had returned to work. The child remains healthy with no obvious adverse effects.

Detection of NMDAR antibodies

All 3 patients had higher NMDAR antibody titers in CSF than serum (Table). No antibodies were identified in the amniotic fluid, umbilical cord blood, serum, or CSF from the baby of patient 1. The baby of the other patient was not examined for antibodies.

Comment

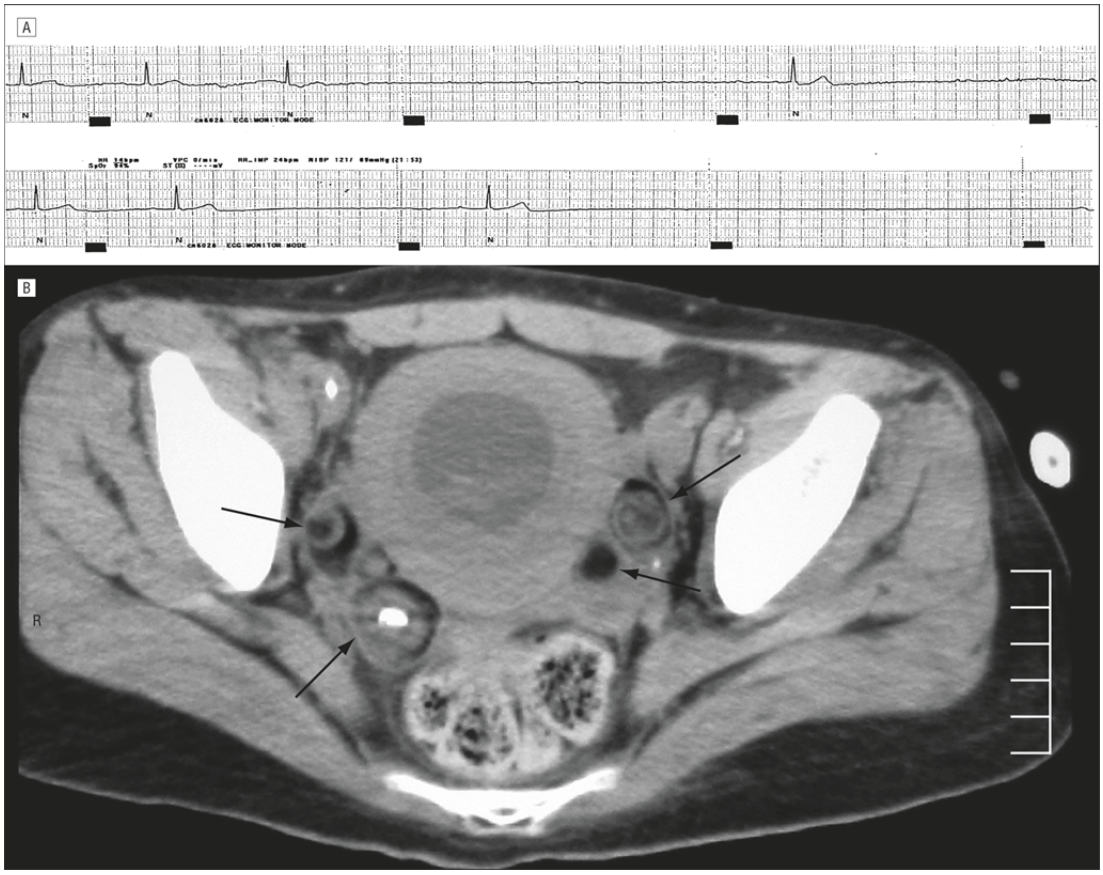
To our knowledge, these are the first reported patients with anti-NMDAR encephalitis diagnosed during pregnancy. The 3 patients had substantial neurological recoveries, although in 1 case the pregnancy was terminated because of the severity of neurological symptoms, presence of recurrent bilateral teratomas, and early stage of pregnancy. The newborns of the other 2 patients were healthy and their physical and cognitive milestones are being closely followed up. Concern for the

fetus and newborns is warranted in this disorder as studies indicate that NR1 antibodies from patients decrease NMDAR clusters in vitro and in animal models (Dalmau et al., 2008; Hughes et al., 2010). Moreover, the antibodies are IgG1 and IgG3, which are the subtypes involved in autoimmune newborn illnesses, such as congenital lupus (Hoftman et al., 2008).

The good outcome of the 2 neonates of our study is likely due to several factors, including the variable effects of autoimmune disorders on the fetus. For example, despite experimental models showing that Ro/SSA antibodies cause congenital heart block, only 2% to 5% of neonates from patients with these antibodies have congenital heart block (Brucato et al., 2002). Two additional factors relate to the levels of serum maternal antibodies and the timing of transplacental transfer of IgG. IgG1 and IgG3 cross the placenta by binding to an Fc neonatal receptor present in syncytiotrophoblasts (Roopenian and Akilesh, 2007). This mechanism of placental transfer develops around weeks 14 to 16, resulting initially in very low levels of fetal blood IgG that gradually increase until the time of delivery. Additionally, the fetal blood-brain barrier becomes functional by the end of the second trimester. Our patients developed symptoms between 8 and 17 weeks of pregnancy when the IgG placental transfer is absent or limited, and assuming the immune response was triggered systemically, the levels of serum NMDAR antibodies decreased rapidly. In fact, 2 patients had negative serum but positive CSF antibody titers (both tested at initial dilution 1:10) by the time they were diagnosed with anti-NMDAR encephalitis, explaining the absence of NMDAR antibodies in the baby who was tested.

With a sharp increase in the number of cases with anti-NMDAR encephalitis, more patients will be identified during pregnancy. This study suggests that these patients and the newborns can do well. The concern should be the search (and

removal) of a teratoma along with supportive care of the mother and fetus. Treatment with corticosteroids, intravenous immunoglobulin, and plasmapheresis was well tolerated but the effects could not be assessed because of the close temporal association with tumor removal in 2 patients. The third patient only received corticosteroids, with questionable improvement of the dyskinesias. The recovery seemed to accelerate after giving birth; this and the predominance of the disorder in young women bring into consideration a possible role of hormonal factors that needs further study.



Figures

Figure 1. Cardiac pauses and bilateral teratomas in patient 2.

A, Cardiac pauses up to 5 seconds were noted on day 3 of hospital admission. B, A pelvic computed tomographic scan revealed bilateral recurrent teratomas (arrows).

Table. Clinical Information and Antibody Titers

Patient	First Symptom of Encephalitis During Pregnancy	Other Symptoms	Teratoma	EEG	CSF WBC Count, /μL[a]	Week of Pregnancy, Antibody Titers in Serum and CSF	Outcome[b]
1	14th wk: Headache, malaise, bizarre behavior	Seizure, orofacial and limb dyskinesias, autonomic instability, minimally responsive	Left immature teratoma	Slow activity	244	14th wk: serum, ND; CSF, 1:80; 21st wk: serum, ND; CSF, 1:10; 32nd week: serum, ND; CSF, ND; baby at birth: serum, CSF, and umbilical cord blood, ND	Cesarean section at week 38; healthy baby; home on day 184; substantial recovery at 2-mo follow-up
2	8th wk: Abnormal, stereotyped behavior	Orofacial dyskinesias, autonomic dysfunction, seizures, minimally responsive, respiratory depression	Bilateral mature teratomas	1-Hz spikes, slow activity predominantly in frontal lobes	57	10th wk: serum, ND; CSF, 1:320; 13th week: serum, ND; CSF, 1:40	Bilateral tumor removal; pregnancy terminated; right ovary left to preserve fertility; home with minimal deficits on day 87
3	17th wk: Affective and behavioral change	Orofacial movements, tonic seizure without EEG correlate, episodes of agitation alternating with minimal response	No tumor or cyst	Generalized high-amplitude slow activity, 2 Hz	11	19th wk: serum, 1:320; CSF, 1:640; 5 mo postdelivery: serum, 1:80; CSF, NA	Spontaneous delivery at 37 wk of pregnancy; healthy baby; home 23 wk after symptom presentation; full recovery at last follow-up

Abbreviations: CSF, cerebrospinal fluid; EEG, electroencephalogram; NA, not applicable; ND, not detectable (<1:10); NMDAR, *N*-methyl-D-aspartate receptor; WBC, white blood cell.

[a] Normal, ≤4/μL. All 3 patients had normal CSF protein and glucose concentrations.

[b] Days relate to duration of stay in the hospital.

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CHAPTER 5. Conclusion

Each of the clinical syndromes under the umbrella of autoimmune encephalitis is an opportunity to not only understand the mechanism by which the human body fails to distinguish self from non-self but also how autoantibodies cause neuropsychiatric disease. The basic science and translational work on the unique aspects on each of the autoimmune encephalitides underscores that despite a failure in immunotolerance, cellular and humoral factors can disrupt their target antigen in distinct ways.

Although the molecular and cellular effects of the antibodies are being rapidly resolved, the effects of the antibody-mediated disruption of synaptic proteins on network and circuit properties are still largely unknown. Extrapolating from the clinical effects of the pathogenic antibodies and from the known functions of the antigenic synaptic components, one can expect that in patients, circuit abnormalities such as altered excitation-inhibition balance, aberrant homeostatic synaptic scaling, loss of synaptic plasticity, reentrant feedback loops result in neuropsychiatric manifestations such as psychotic behavior, impaired memory formation, seizures or movement disorders. Studies in animal models and patients will provide insights into the circuit alterations caused by antibodies that result in aberrant behavior.

A major gap in understanding of CNS autoimmune diseases exists in identification of mechanisms at the interface between the nervous and the immune systems. The current literature strongly supports the model that autoreactive T and B cells extravasate into the parenchymal and perivascular spaces in the CNS where they may survey the inflammatory state of the neuronal tissues that in turn affects the maturation of these lymphocytes. Our understanding of humoral inflammation and cell-

mediated factors and their effects on neurons and glia is burgeoning, but is currently in its infancy.

The mechanism by which $GABA_B$ autoantibodies result in disease is just beginning to be understood. Until this work, all autoimmune encephalitides described were associated with autoantibodies that reduced the levels of cognate receptors. I have shown that $GABA_B$ receptor autoantibodies directly block $GABA_B$ receptor activation by baclofen, suggesting that the clinical manifestations of $GABA_B$ receptor autoantibodies result from diminished $GABA_B$ receptor function. Patient $GABA_B$ receptor autoantibodies do not inactivate $GABA_B$ receptors, however, because the patient antibody mediated baclofen block was circumvented by directly activating the $GABA_{B2}$ subunit with a selective partial allosteric agonist, CGP7930. This provides hope that newly developed brain penetrant $GABA_{B2}$ agonists may be used to treat the intractable seizures that are the primary cause of morbidity and mortality in anti-$GABA_B$ receptor encephalitis patients.

The Sushi domains present in the presynaptically localized $GABA_{B(1a,2)}$ receptors is the immunodominant domain recognized by $GABA_B$ receptor. How the binding to this domain blocks the activation of $GABA_{B1}$ receptors by agonists is unclear. Currently, it is thought that the function of sushi domains is to target $GABA_{B1a}$ to the axonal domain of neurons. While patient autoantibodies are $GABA_B$ receptor antagonists, it is possible that autoantibodies also affect receptor trafficking and presynaptic localization by blocking the two Sushi domains. This can be tested by probing whether patient antibodies can result in a loss of polarity of the distribution a sushi domain containing peptide in cell culture. Our work raises the possibility that sushi domains may have functions other than polar trafficking that are important for the activation of $GABA_{B1a}$ receptors. Specifically, it would be valuable to demonstrate that $GABA_B$ autoantibodies block

$GABA_{B1a}$ containing receptors but not $GABA_{B1b}$ containing receptors. If this is true, the patient $GABA_B$ autoantibodies would be a valuable research tool to dissect the distinct contributions of $GABA_{B1a}$ and $GABA_{B1b}$ in GABA mediated neurotransmission, and be a model for therapeutic strategies that target $GABA_{B1a}$ mediated signaling but not $GABA_{B1b}$ mediated signaling.

The development of the *in vivo* model of anti-NMDA receptor encephalits enables several lines of investigation, with relevance to both clinical management of this disease and our understanding of the role of NMDA receptors in circuits, cognition and behavior. A potential blocking peptide that disrupts the ability of the pathogenic antibody to bind to NMDA receptors can be studied. Also, the hypothesis that the Ephrin receptor interaction is necessary for the pathogenic effect of NMDA receptor autoantibodies can be tested.

Infusion into the cerebral ventricles delivers the pathogenic antibody to nearly every brain structure, and it is unclear what circuits are compromised by the autoantibody to cause a decline in spatial object memory. Focused intraparenchymal infusion of patient CSF with concomitant electrophysiological recording will enable us to pinpoint the defects in circuit function and plasticity that underlies the currently observed spatial memory deficit.

Understanding the mechanisms of recovery after widespread loss of NMDA receptors is of utmost clinical importance. Interestingly, clearance of anti-neuronal antibodies is not a good predictor of amelioration of symptoms. Long after antibodies are undetectable in serum or CSF, patients remain functionally compromised. It can take up to two years for a full return to work and normal routine and sometimes even years after treatment people are left with cognitive deficiencies. In culture, receptor levels return to

baseline 3-4 days after antibody removal and in the animal model, immunoglobulin clearance and recovery of NMDARs is complete within twelve days of cessation of antibody infusion. In patients' brains, which may have been exposed to autoantibodies for months, the loss of NMDA-mediated glutamatergic transmission may have resulted in a cascade of changes, both within and between neurotransmitter systems. As antibody titers fall, even though receptor density returns to normal, the brain may require additional time to reset to its pre-disease baseline. The animal model can be used to probe changes in other neurotransmitter systems and kinetics of these changes that may hopefully shed light on the downstream effects of a global reduction in NMDA receptor functioning.

These diseases offer human models of brain-immune interactions in which the target antigens have critical roles in neuronal synaptic transmission and plasticity. Patient antibodies have proven to be potent tools with no commercial equivalent that enable the roles of target proteins to be addressed at the synaptic, cellular, circuit and behavioral levels. Studies of mechanisms underlying recovery of function as well as lingering effects on behavior as antibody titers are reduced will also be important. Ongoing work may lead to targeted therapeutic strategies for autoimmune encephalitis, and will also provide an unprecedented window into the in vivo role of key synaptic components essential for neuronal communication, circuit function and behavior.