FUNCTIONAL PLASTICITY OF HIPPOCAMPAL GLUTAMATERGIC SYNAPSES IN DEVELOPMENT AND DISEASE

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Abstract
The establishment and maintenance of synaptic connections are critical for the normal function of the central nervous system. The function of mature neural circuits depends critically on the appropriate apposition of pre- and postsynaptic specializations and on the spatial organization of synapses along axon arbors and postsynaptic dendrites. In neurological disease, the integrity of these processes may be compromised or lost, resulting in profound cognitive and behavioral deficits. In my thesis work, I have investigated the spatial distribution of functional properties of presynaptic terminals along axon arbor, and the determinants of these properties across different spatial scales. My results suggest that, for a single axonal arbor, presynaptic strength over short distances is determined by variations in total vesicle pool size, whereas over longer distances presynaptic strength is determined by the spatial modulation of release fraction. Thus the mechanisms that determine synaptic strength differ depending on spatial scale. I have also examined the structural and functional consequences of loss of postsynaptic components, NMDA and AMPA type glutamate receptors, that occur in two forms of human autoimmune encephalitis in which these proteins are specifically targeted, and that result in dramatic deficits in cognition and behavior. My results suggest that patient antibodies against these two types of glutamate receptors selectively result in the internalization of receptor from the neuron surface, decreasing synaptic localization, currents and thus influencing synaptic function. My work extends our understanding of the repertoire of pre- and postsynaptic mechanisms that are required to establish and maintain functional neural circuits during development and in diseases that compromise nervous system function.

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FUNCTIONAL PLASTICITY OF HIPPOCAMPAL GLUTAMATERGIC SYNAPSES IN DEVELOPMENT AND DISEASE

Xiaoyu Peng

A DISSERTATION

in

Biology

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in Partial fulfillment of the Requirements for the Degree of Doctor of Philosophy

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I have also examined the structural and functional consequences of loss of postsynaptic components, NMDA and AMPA type glutamate receptors, that occur in two forms of human autoimmune encephalitis in which these proteins are specifically targeted, and that result in dramatic deficits in cognition and behavior. My results suggest that patient antibodies against these two types of glutamate receptors selectively result in the internalization of receptor from the neuron surface, decreasing synaptic localization, currents and thus influencing synaptic function. My work extends our understanding of the repertoire of pre- and postsynaptic mechanisms that are required to establish and maintain functional neural circuits during development and in diseases that compromise nervous system function.
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Introduction

Part 1: Mechanisms specifying presynaptic strengths in the central nervous system

In the central nervous system (CNS), synapses between pre- and postsynaptic neurons are the essential functional units where information is relayed, integrated and stored. In contrast to peripheral synapses, central synapses, especially the presynaptic terminals, are highly “unreliable” in responding to individual stimuli. Conversely, these “unreliable” synapses are observed to be able to “reliably” respond to diverse and complex spikes among neurons in normal behavior. Mechanisms determining pre- and postsynaptic functional properties in synaptic development and activity-dependent changes have been under investigation for the last century. However, the graphic pictures with spatial information are still missing. While thousands of presynaptic terminals innervate hundreds of different postsynaptic neurons, at every pre-and postsynaptic contact site in every connection, presynaptic strength is regulated to match with the postsynaptic specialization. Recent studies have begun to investigate mechanisms determining presynaptic strengths at different spatial locations along axons and dendrites, yet much remains unknown.

Presynaptic functional properties are heterogeneous

The heterogeneity of functional properties of central presynaptic terminals has long been appreciated. Several studies documented that the initial release probability (Pr)
of central presynaptic terminals is highly heterogeneous even between just one pair of pre- and postsynaptic neurons, ranging from less than 0.05 to close to 1, with the majority of them less than 0.2 (Hessler et al., 1993; Rosenmund et al., 1993; Huang and Stevens, 1997; Murthy et al., 1997). The heterogeneous presynaptic release probabilities also feature broad and/or skewed distributions with large coefficients of variation (Murthy et al., 1997). This means that most central presynaptic terminals are not reliable and will only release less than two times out of ten separate stimuli. In addition to heterogeneity in initial release probabilities, heterogeneity has also been reported for other functional properties. The facilitation ratio of vesicle release during a pair of high frequency stimuli is very different from terminal to terminal (Dobrunz and Stevens, 1997), as is the depletion rate of vesicle release during a train of high frequency stimuli (Dobrunz and Stevens, 1997). These functional properties are related to the initial release probability: the higher Pr terminals have lower facilitation rates and faster depletion rates than the lower Pr terminals. The heterogeneity of presynaptic properties in terminals is critical and is thought to underlie reliable neurotransmission in the presence of highly variable stimuli in normal, behaving animals (Dobrunz and Stevens, 1999). These results together suggest that individual presynaptic terminals, even distinct terminals in the same connection between a pair of pre- and postsynaptic neurons, have their own set of defined properties and are heterogeneous.

Possible presynaptic determinants of vesicle release to specify functional properties
Determinants of presynaptic vesicle release have been studied extensively. Presynaptic terminals in the central nervous system contain a few hundreds of neurotransmitter-filled vesicles and a specialized membrane region for release called the active zone. The cycle of the release of a presynaptic vesicle consists of multiple steps (Sudhof, 2004): 1) a small cluster of vesicles are docked at the active zone and primed for release; 2) an action potential reaches the terminal, causing Ca\(^{2+}\) channels to open in response to the ensuing depolarization; 3) Ca\(^{2+}\) influx and bind to the calcium sensor; 4) the calcium sensor on vesicles mediates the fusion between the plasma membrane and the vesicular membrane; 5) vesicles are endocytosed and recycled for further rounds of release. Every step can be modulated by the current status of the terminal, the history of activity of the terminal, the developmental history of the terminal, postsynaptic signals, etc. Together, these factors specify heterogeneous presynaptic properties from terminal to terminal.

**Ca\(^{2+}\) channels and Ca\(^{2+}\) influx** The spatiotemporal dynamics of Ca\(^{2+}\) influx into presynaptic terminals can be regulated to change presynaptic functional properties. In many regions of the nervous system, a developmentally regulated switch of presynaptic Ca\(^{2+}\) channel types from N-type channels in immature synapses to P/Q-type channel in mature synapses has been observed *in vitro* and *in vivo*, (Iwasaki and Takahashi, 1998; Rosato Siri and Uchitel, 1999; Iwasaki et al., 2000), including cultured hippocampal neurons (Scholz and Miller, 1995). Since the N-type Ca\(^{2+}\) channels are more distant from releasing sites than the P/Q-type (Wu et al., 1999), this developmental switch gives rise
to more synchronous transmitter release (Chuhma and Ohmori, 1998) and thus higher release probability. Other studies using calyx of Held synapses as a model suggest that developmental tightening of the spatial coupling between Ca\(^{2+}\) channels and vesicle release, instead of increasing intrinsic Ca\(^{2+}\) sensitivity of vesicles, enhances presynaptic release probability during synaptic maturation (Fedchyshyn and Wang, 2005; Wang et al., 2008; Kochubey et al., 2009). These results together suggest that modulating the Ca\(^{2+}\) channels and Ca\(^{2+}\) influx can be a common strategy to specify presynaptic functional properties during maturation or, possibly during activity dependent processes.

*Mechanisms regulating active zone components*  
Changing the quantity of active zone components could change presynaptic functional properties. Presynaptic terminals have heterogeneous amounts of active zone components and vesicle associated proteins mediating vesicle docking and fusion. Assembly of new presynaptic terminals from trafficking packets to functional terminals needs a few hours (Ahmari et al., 2000), followed by maturation, which may take a few days *in vitro* (Mozhayeva et al., 2002). During synaptic assembly, two discrete packets containing either synaptic vesicle precursors (Ahmari et al., 2000; Krueger et al., 2003) or active zone components such as Bassoon and Piccolo (Zhai et al., 2001; Shapira et al., 2003) have been shown to be transported along axons. These presynaptic components may pause at sites along an axon (Sabo et al., 2006) or be recruited to pre-clustered postsynaptic specializations (Gerrow et al., 2006). Even after a terminal has been established and reached a static status, its active zone components, and vesicles with associated proteins, are not strictly enclosed
by its own territory. Instead, the neighboring terminals are constantly exchanging vesicles and active zone components (Ahmari et al., 2000; Friedman et al., 2000; Shapira et al., 2003; Bresler et al., 2004; Darcy et al., 2006; Tsuriel et al., 2006; Tsuriel et al., 2009). While mature presynaptic terminals, on average, have a scaling relationship among active zone areas, number of docked vesicles, and total number of vesicles, these components are subject to dynamic changes in development and maturation. The temporal and spatial dynamics of active zones, vesicles, and their associated proteins in presynaptic terminals suggest that these determinants are being regulated constantly to specify functional properties at individual sites.

In addition, adding, eliminating, and/or switching critical molecules associated with active zones and vesicles have been suggested to be another way to modulate presynaptic function. During maturation of the calyx of Held synapse, deletion of the filamentous protein Septin 5 gives rise to features of mature synapses including synaptic vesicles tightly localized to active zones, and increased vesicle fusion coupling with Ca$^{2+}$ influx, leading to increased release probability (Yang et al., 2010). Studies examining the expression profile of synaptotagmin (Syt), the Ca$^{2+}$ sensor of vesicles, during maturation suggests that there is a regulated change of the Syt-isoform expression profile in a single neuron type (Xiao et al., 2010). These data together suggest that some critical molecules participating in vesicle docking and fusion at active zones are regulated during maturation to specify presynaptic functional properties.
**Organization of vesicle pools** Vesicles clustered in presynaptic terminals participate in different pools according to their physiological properties; thus, changing the participation of vesicles in different pools can change presynaptic functional properties. The readily releasable pool (RRP) usually contains between 4 and 8 vesicles, corresponding to the docked vesicles from EM studies (Schikorski and Stevens, 2001), and these are the ones that can be depleted by high frequency stimuli or hypertonic sucrose (Rosenmund and Stevens, 1996). The reserved pool (RP) usually contains 17-20 vesicles (Murthy et al., 1997), which require prolonged stimuli for release. RRP and RP vesicles participate in evoked presynaptic release. The rest of the vesicles, around 170 in number, are in the resting pool. The functions of these vesicles are less clear, but they may participate in spontaneous release (Fredj and Burrone, 2009). Studies suggest that the initial release probability is determined by the number of readily releasable pool vesicles and their average release probability (Dobrunz and Stevens, 1997; Dobrunz, 2002) and sustained release during prolonged stimulation is determined by all recycling vesicles (Dobrunz and Stevens, 1997; Sudhof, 2004). These data together suggest that changing the number of vesicles in each pool can influence different aspect of presynaptic function. For example, a recent study suggests that the fraction of the vesicle pool participating in release can be modulated in chronic inactivity by homeostatic scaling, via CDK5 dependent mechanisms (Kim and Ryan, 2010). In addition, studies examining vesicle pool maturation over time in cultured hippocampal neurons suggest that RRP are preferentially filled during early synapse maturation, followed by reserved
pools (Mozhayeva et al., 2002). Thus, immature terminals may not have the same
number or ratio of RRP and RP vehicles as mature terminals. These results together
suggest that changing the participation of vesicles in different pools in presynaptic
terminals during maturation or activity-dependent plasticity can change presynaptic
functional properties.

Endocytosis machinery  Endocytosis is essential to maintain presynaptic vesicle
release during prolonged stimulation. Since vesicle release ( < 2 ms between action
potential arrival at presynaptic terminals and postsynaptic currents being recorded) is far
more rapid than recycling ( > 3 seconds (Zhu et al., 2009)), efficient endocytosis is
critical in maintaining the defined presynaptic properties over longer terms. However,
the dynamics as well as the molecular mechanisms of synaptic vesicles endocytosis are
still under debate and investigation. The most recent studies using single vesicle imaging
techniques suggest that there is a fast endocytosis pathway (~3 s) as well as a slower
endocytosis pathway (>10 s) (Gandhi and Stevens, 2003; Granseth et al., 2006; Balaji
and Ryan, 2007; Zhu et al., 2009) through clathrin. As stimulation frequency and Ca\(^{2+}\)
influx increase, the slow endocytosis is accelerated and becomes the predominant
pathway (Zhu et al., 2009). Studies showed that presynaptic Ca\(^{2+}\) influx during
stimulation is heterogeneous from terminal to terminal (Brenowitz and Regehr, 2007).
Thus it is possible that the endocytosis rates at different presynaptic terminals are set
differently via fast or slow pathways according to differences in Ca\(^{2+}\) influx, giving rise
to another mechanism to specify presynaptic functional properties in different terminals.
Retrograde mechanisms modulating presynaptic functional properties

Previous work has suggested that postsynaptic targets retrogradely influence presynaptic release probability (Reyes et al., 1998; Koester and Johnston, 2005; Branco et al., 2008; Branco and Staras, 2009). In rat neocortex, presynaptic terminals contacting different postsynaptic neurons have heterogeneous release probability (Reyes et al., 1998; Koester and Johnston, 2005). In cultured rat hippocampal neurons, presynaptic terminals contacting different dendrites of the same postsynaptic neuron also have heterogeneous release probability (Branco et al., 2008). These suggest that there are spatially defined postsynaptic signals from individual neurons/dendrites to specify presynaptic properties.

Several different postsynaptic retrograde mechanisms have been suggested to influence presynaptic functional properties (Tao and Poo, 2001; De Paola et al., 2003; Pratt et al., 2003; Ziv and Garner, 2004; Dalva et al., 2007; Williams et al., 2010). These include cell adhesion molecules such as the neuroligin-neurexin complex (Chih et al., 2005; Levinson et al., 2005; Futai et al., 2007), SynCAM (Biederer et al., 2002), EphBs and ephrin-Bs (Dalva et al., 2000; Kayser et al., 2006), and cadherins (Benson and Tanaka, 1998; Takeichi, 2007; Arikath and Reichardt, 2008), among others, as well as secreted factors such as neurotrophins (Du and Poo, 2004), FGF22, FGF 7 (Terauchi et al., 2010) and Sema3F (Tran et al., 2009), among others.

However, two important aspects are missing. Not much is known about the spatial arrangement of these postsynaptic mechanisms, especially at subcellular levels. In
addition, most studies looking at postsynaptic retrograde signals do not differentiate from mechanisms of being synaptogenic, thus secondarily influencing functional properties, or modulating functional properties directly. The details of functional properties and how different postsynaptic mechanisms interact with different presynaptic functional determinants to specify functional properties are not clear. Recent studies using RNAi knockdown or overexpression of postsynaptic PSD-95-NLG-1 complex suggested that presynaptic release probabilities and facilitation can be altered by changing postsynaptic NLG mediated signals (Futai et al., 2007). However, functional and spatial details are still missing for most studies examining postsynaptic molecular mechanisms. Thus, which of these cell-cell signaling mechanisms contribute to the modulation of presynaptic release properties on different spatial scales across an axon arbor remains to be determined.

**Activity-dependent changes of presynaptic functional properties**

Presynaptic functional properties are constantly adjusted during neuronal network activities. Presynaptic terminals can undergo Hebbian plasticity, which implies the strengthening or weakening of connections upon coordinated activity, such as long-term potentiation (LTP) and long-term depression (LTD). Presynaptic LTP, where release probability increases after potentiation, has been observed in several central synapses, including cerebellar parallel fiber (PF) synapses, hippocampal mossy fiber synapses, and corticothalamic synapses (Zalutsky and Nicoll, 1990; Castro-Alamancos and
Calcagnotto, 1999; Rancillac and Crépel, 2004). Presynaptic activity-dependent plasticity would probably be a more widespread phenomenon if detection technique of presynaptic functions were more sensitive and less dependent on postsynaptic current recording (Becker et al., 2008). The molecular mechanisms of presynaptic plasticity have been investigated. The induction of presynaptic LTP at these synapses is independent of postsynaptic NMDA receptor activation but dependent on presynaptic Ca\(^{2+}\) rise during synaptic transmission (Zalutsky and Nicoll, 1990; Castro-Alamancos and Calcagnotto, 1999; Rancillac and Crépel, 2004). Studies suggest that release machinery proteins such as RIM1α and Rab3a are modified (Castillo et al., 1997; Castillo et al., 2002; Lonart et al., 2003) so that the coupling between Ca\(^{2+}\) and release is enhanced (Lonart et al., 2003). These results suggest that at least some of the pre- and postsynaptic mechanisms that specify presynaptic functional properties are activity-dependent and follow Hebbian rules.

Presynaptic terminals can also undergo homeostatic plasticity, which is another important activity-dependent change in which the synaptic strengths change in the opposite direction to compensate for the loss or increase of network activity, and to maintain gain control. Classic studies with network activity blockade suggested that in addition to upscaling of postsynaptic AMPA receptor function, presynaptic terminal size, active zone area, and readily releasable and total vesicle pool sizes increased, along with increased presynaptic release probabilities (Murthy et al., 2001). A recent study suggested that the fraction of the vesicle pool participating in release can be modulated
by chronic inactivity via homeostatic scaling, by CDK5 dependent mechanisms (Kim and Ryan, 2010). Furthermore, recent studies suggest that homeostatic rules also allow individual synapses to maintain their activity level, at least on postsynaptic sites (Béïque et al., 2010). Thus, it is possible that an individual presynaptic terminal’s functional properties are subjected to Hebbian plasticity as well as homeostatic plasticity to reach an optimum strength.

Spatial distribution

Previous studies mainly focused on assessing presynaptic functional properties and mechanisms modulating these properties without much spatial information. With recent advancement of optical physiology techniques, it is more appreciated that the functional properties of spatially distributed synapses can be regulated differently, and that there is rich information to be explored (Markram et al., 1998; Reyes et al., 1998; Koester and Johnston, 2005; Pelkey and McBain, 2007; Branco et al., 2008). The distribution of synapses along dendrites have been investigated, probably partially due to easier tracking of dendrites (a couple of hundreds microns in length) of individual neurons in vitro and in vivo, comparing to axons (up to thousands of microns or even longer). A study using serial-section electron microscopy to reconstruct individual apical synapses of CA1 pyramidal neurons suggested that synaptic strengths are scaled along dendritic branches in the direction to enhance the contribution of each dendritic branch to neuronal output (Katz et al., 2009). Studies using a genetic synaptic labeling method that
relies on expression of a presynaptic marker, synaptophysin-GFP (Syp-GFP) in individual neurons in vivo observed the distribution of structural properties of presynaptic terminals along cerebellar granule cells’ axons (Li et al., 2010). Another study employed a presynaptic channelrhodopsin-2 (ChR2)-assisted method to map the spatial distribution of presynaptic inputs within the dendritic arborizations of postsynaptic neurons and revealed high specificity in the subcellular organization of excitatory synapses (Petreanu et al., 2009). Together, these studies show that examining the spatial distribution of functional presynaptic terminals along individual axon arbors and dendritic branches, the rules setting synaptic strengths, and how pre- and postsynaptic mechanisms specify these properties, will provide a fundamental understanding of how functional circuits of the nervous system are established and maintained.

Conclusions and future directions

The studies described above have begun to investigate mechanisms specifying presynaptic functional properties in the central nervous system. Investigations into the spatial arrangement of the functional properties of presynaptic terminals and the mechanisms that specify them represent important directions for future research. Examining how functional synaptic connections are established and maintained spatially in the central nervous system will greatly contribute to the understanding and potential treatment of neurological disorders such as epilepsy, autism and mental retardation, in which synapse function is aberrant or reduced.
Thesis rationale and goals

The spatial arrangement of functional presynaptic terminals along axon arbors and the mechanisms specifying functional properties spatially are largely unknown. Understanding this spatial arrangement is critical for investigating the mechanisms of the establishment and maintenance of synaptic connections of the central nervous system.

Using Synaptophysin-pHluorin as an optical physiology tool to measure presynaptic functional properties, I examined the spatial distribution of functional presynaptic terminals across axon arbors and plausible pre-and postsynaptic mechanisms in individual glutamatergic neurons in vitro (Chapter 2). Using optical physiology, immunochemistry, and live imaging techniques, I tested the hypothesis that Determinants of presynaptic strength vary across an axon arbor.

My work demonstrated that over short axon segments, synaptic strength is determined by total vesicle pool size and is related to the density of postsynaptic components such as NMDA receptors and PSD-95. Over the entire axon arbor, synaptic strength is determined by proximal to distal changes in release fraction, independent of total vesicle pool size, that can be accounted for by changes in individual vesicle release probability and/or readily releasable pool size. Our results thus suggest that the mechanisms that establish synaptic strength vary over different spatial scales.
Part 2: Mechanisms underlying autoimmune synaptic encephalitis

Ionotropic receptors are the most critical postsynaptic components in synaptic transmission and activity dependent plasticity in normal circuits. Disruption of Glutamatergic or/and GABAergic receptors functional can lead to severe neurological disease such as epilepsy, schizophrenia and autisms. 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 (Rudnicki and Dalmau, 2000; Phillips, 2003; Tormoehlen and Pascuzzi, 2008; Meriggioli and Sanders, 2009). Patients with these autoantibodies, sometimes associated with teratomas and other neoplasms, present with psychiatric symptoms, seizures, memory deficits, and decreased level of consciousness. These symptoms often improve dramatically after immunotherapy or tumor resection (Rudnicki and Dalmau, 2000; Phillips, 2003; Tormoehlen and Pascuzzi, 2008; Meriggioli and Sanders, 2009). However, the cellular and synaptic mechanisms underlying these autoimmune encephalitides remained to be addressed. 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, studying these mechanisms 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.
Autoimmune synaptic encephalitis

Many encephalitides once considered idiopathic are now thought to be immune mediated. One of these disorders predominantly affects structures of the limbic system, including medial temporal lobes, amygdala, hippocampus and orbitofrontal cortex (Gultekin et al., 2000; Posner and Dalmau, 2000; Graus and Dalmau, 2007). As a result, patients develop short-term memory deficits, emotional and behavioral disturbances such as confusion, irritability, depression, and sleep disturbances, as well as seizures and sometimes dementia (Gultekin et al., 2000; Tüzün and Dalmau, 2007).

Recently, a novel group of disorders associates with autoantigens that are on the cell or synaptic surface has been reported, with or without tumor association and are likely antibody-mediated (Table 1) (Rudnicki and Dalmau, 2000; Phillips, 2003; Tormoehlen and Pascuzzi, 2008; Meriggioli and Sanders, 2009). However in autoimmune synaptic encephalitis the autoantigen is located behind the blood-brain-barrier (BBB) requiring that the antibodies or cells producing antibodies cross this barrier in order to cause neurological dysfunction. In some disorders the patients' cerebrospinal fluid (CSF) show lymphocytic pleocytosis and intrathecal synthesis of antibodies suggesting that after initial systemic immune activation by a tumor or unknown causes, there is an expansion of the immune response within the nervous system (Dalmau and Rosenfeld, 2008). The role of the immune response in the neurological symptoms is further supported by the correlation between antibody titers and symptoms, and the frequent response of the disorders to immunotherapies, including plasmapheresis, IVIg,
corticosteroids, cyclophosphamide, or rituximab, a B-cell depleting monoclonal antibody. These novel autoimmune mediated synaptic encephalitides include:

*Anti-NMDA receptor encephalitis*  A new, severe, potentially lethal, and treatment-responsive disorder, anti-NMDA receptor encephalitis was reported within the last several years by Dalmau and colleagues (Dalmau et al., 2007; Sansing et al., 2007). Patients are usually young women, but also include men, without a past medical history of interest, who, often after prodromic symptoms of mild hyperthermia, headache, or a viral-like process, develop sudden behavioral and personality changes for which they are often seen by psychiatrists (Dalmau et al., 2007; Sansing et al., 2007). This clinical presentation is usually followed by seizures, decreased level of consciousness, abnormal movements (orofacial and limb dyskinesias, dystonia, choreoathetosis), autonomic instability (fluctuating blood pressure, cardiac rhythms, and temperature), and sometimes hypoventilation. MRI is frequently normal, but in about 40% of the patients’ findings suggesting inflammation are transiently identified in hippocampus, cerebral or cerebellar cortex, and subcortical regions (Dalmau et al., 2008).

Patients have serum and CSF antibodies that react with brain antigens predominantly expressed in the hippocampus (Dalmau et al., 2008). In two large cohorts comprising 180 patients, including young adults and children, neurologic improvement was correlated with a decrease in antibody titer (Dalmau et al., 2008). Overall, about 75% of the patients had dramatic or substantial recoveries despite the severity or long duration of symptoms; 19% had partial or limited improvement, and 6% died. Analyses of brain
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, in contrast to other paraneoplastic syndromes in which cytotoxic T-cell infiltrates are prominent (Stein-Wexler et al., 2005).

When patient antibodies are used to stain rodent brain sections, immunoreactivity is observed in the neuropil of the hippocampus, with less staining in cortex, striatum and cerebellum (Dalmau et al., 2008). When used to stain live cultured hippocampal neurons, patient antibodies react with surface antigens localized to synapses. Additional studies, including immunoprecipitation followed by mass spectroscopy, led to the identification of the NR1 subunit of NMDA receptor as the target autoantigen. 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). Domain swapping and other experiments showed that the epitope was located at the N-terminal extracellular domain of NR1 (Dalmau et al., 2008; Gleichman et al., 2009). Since NR1 is ubiquitously expressed in brain as an obligate subunit of functional NMDA receptors (Monyer et al., 1994; Standaert et al., 1994; Waxman and Lynch, 2005), it remains unclear why patient NR1 antibodies preferentially label hippocampus rather than all brain regions. This binding pattern may reflect the relative high density of NMDA
receptors in the hippocampus or a differential posttranslational modification of NR1 in different brain regions (Gleichman et al., 2009).

The underlying cellular and synaptic mechanisms of how patients’ autoantibodies change the structure and function of synapses, neurons and circuits are not well known. It has been recently shown that patients’ antibodies cause a selective but reversible decrease of NMDA receptor surface density, synaptic localization in vitro (Dalmau et al., 2008). Thus whether this change of NMDA receptor localization from postsynaptic specialization underlies the functional changes of synapses and the spectrum of patients syndromes need to be addressed.

Other autoimmune synaptic encephalitides  Currently, ~90% of patients studied by us with limbic encephalitis of non-viral etiology have well-defined immune responses against neuronal antigens (Bataller and Dalmau, 2004; Bataller et al., 2007; Graus et al., 2008). The importance of antibodies to cell surface or synaptic proteins was shown in a recent study in which these antibodies were found to be more prevalent than antibodies to intracellular antigens described in paraneoplastic disorders (54% versus 24%; (Graus et al., 2008)). A study of 1570 patients with diffuse encephalitis by the California Encephalitis Project showed that in only 30% could a final diagnosis be established (viral, bacterial, prion, parasitic, fungal) (Glaser et al., 2006). A pilot study examining a group of cases selected by subphenotype (“encephalitis, psychosis, and dyskinesias”) showed that 50% had NMDA receptor antibodies (Gable et al., 2009). This suggests that other antibodies to currently unknown antigens may occur in the remaining cases.
In the last 2 years, a second form of immune mediated encephalitis in which patients’ serum and CSF antibodies are directed against AMPA receptors was identified by immunoprecipitation followed by mass spectrometry (Lai et al., 2009). Most patients develop a clinical picture of limbic encephalitis including confusion, agitation, seizures, and severe short-term memory deficits. Sometimes patients present with a rapidly progressive abnormal behavior resembling acute psychosis. Patients are usually women older than 50 years, and 70% had an underlying tumor, usually lung or breast cancer or tumors of the thymus that express AMPA receptors. Immunotherapy and treatment of the tumor, if detected, usually results in neurological recovery. The neurological disorder has a tendency to relapse, and for these patients the outcome depends of how well each relapse is controlled.

AMPA receptors mediate most of the fast excitatory synaptic transmission in the brain (Shepherd and Huganir, 2007) and the majority are heterotetramers composed of GluR1, 2, 3 or 4 subunits that 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. Preliminary analyses suggest that the location of the epitope is the N-terminal extracellular domain of GluR1 or/and GluR2 AMPA receptor subunits (Gleichman et al., 2009). None of these patients’ antibodies reacted with GluR3, a subunit identified as an autoantigen in some patients with Rasmussen’s encephalitis (Rogers et al., 1994).
The cellular mechanisms underlying anti-AMAPR encephalitis is not clear. Studies suggest that the treatment of rat hippocampal neurons with patients’ antibodies resulted in a significant decrease in the synaptic localization of AMPA receptor clusters, without a decrease in overall synaptic density or NMDA receptor clusters (Lai et al., 2009). Moreover, these effects were reversible: after 3 days of treatment with patients’ CSF containing GluR1/GluR2 antibodies, followed by 4 days of treatment with control CSF, AMPA receptor cluster density and synaptic localization recovered to levels seen in control cultures (Lai et al., 2009). However, what are the functional consequences of the loss of synaptic localization of AMPA receptors and how these give rise to the severe neurological presentation in patients are to be investigated.

A third subtype of autoimmune encephalitis associated with antibodies against the γ-amino-butyric acid-B (GABA_B) receptor was also recently identified (Lancaster et al., 2009). The median age of a cohort of 15 patients was 62 years (24-75); 8 were men. All presented with early and prominent seizures; other symptoms, as well as MRI and EEG findings, were consistent with predominant limbic dysfunction. Forty-seven percent of patients had small cell lung cancer (SCLC), and 40% showed propensity to autoimmunity. Cancer screening and demographic data indicate the disorder also occurs in patients without cancer. Neurological improvement occurred in 60% of the patients and was correlated with prompt immunotherapy and tumor control. Staining of live neurons showed that all patients’ serum and CSF had antibodies against a cell surface antigen. Immunoprecipitation and mass spectroscopy demonstrated that the autoantigen
was the B1 subunit of the GABA_B receptor, a metabotropic receptor that when disrupted causes seizures and memory dysfunction (Prosser et al., 2001; Schuler et al., 2001). Preliminary studies show that patients’ antibodies do not affect the levels of GABA_B receptor, but block the effects of baclofen, a selective B1 subunit agonist (Lancaster et al. Neurology 2010 abstract, in press). This result suggests that not all anti-synaptic encephalitis have similar underlying cellular mechanisms so that individual diseases need to be investigated. Comparing different mechanisms and syndromes among different type of antoimmune encephalitides may help understanding critical synaptic proteins’ function and dynamics.

While in vitro approaches have been useful to establish the effects of antibodies to NMDA, AMPA, and GABA_B1 receptors on neurons and in particular on synapses, in vivo models will be needed to establish the relationship between the effects of each antibody on synapse and circuit function, and the changes in behavior, memory and cognition that are hallmarks of these disorders.

**Mechanisms underlying antibody pathogenic effects on the target receptors**

Several mechanisms may account for the pathogenicity of autoantibodies in these disorders.

The first possibility is that patient anti-receptor antibodies agonize or antagonize the receptor. NR2 antibodies from patients with SLE cause neuronal death when injected into mouse brain; this effect is attenuated by treatment with the NMDA receptor blocker,
MK-801, suggesting the antibodies mediate cell death by enhancing channel activation (DeGiorgio et al., 2001). Conversely, application of nicotinic acetylcholine receptor (nAChRs) antibodies from myasthenia gravis patients to outside-out patches of mouse myotubes caused an acute block of AChR currents that became irreversible with time (Jahn et al., 2000). The epitope for both NMDA receptor and AMPA receptor antibodies is in the N-terminus raises the possibility that autoantibodies could have direct functional effects. The ligand binding domain for both channels is also in the N-terminus, and conformational changes are thought to couple ligand binding to channel opening (Armstrong and Gouaux, 2000; Sobolevsky et al., 2009). Therefore, patient antibodies could initially sterically hinder ligand binding or enhance its effects. In addition, N-terminal binding sites for channel modulators such as zinc and polyamines may be obscured by patients’ antibodies (Rassendren et al., 1990; Herin and Aizenman, 2004; Paoletti and Neyton, 2007). Whole cell recording experiments during acute application of antibodies will allow this issue to be resolved.

The second possibility is that patient anti-receptor antibodies cause receptor internalization and degradation, resulting in diminished receptor function. AChR antibodies from patients with myasthenia gravis cause a loss of surface AChRs by cross-linking and internalization (Drachman et al., 1978). 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., 1988b; Peers et al., 1993).
Experiments comparing whole patients’ antibody IgG with Fab fragments will help to address this possibility.

The third possibility is that patient anti-receptor antibodies cause complement-mediated neuronal damage or death. 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 later characterized by antibodies to aquaporin-4 (Whitney et al., 1999; Lucchinetti et al., 2002; Waters et al., 2008). IgG1 and IgG3, subclasses of IgG capable of activating complement, are the main IgG types of NMDA and AMPA receptor antibodies. In anti-NMDA receptor encephalitis, we previous have not found evidence of deposits of complement in autopsies of patients. In light of the substantial recoveries made by many of these patients, extensive neuronal damage due to complement activation seems unlikely. Furthermore, it is unclear whether the elements of the complement cascade that are present in the central nervous system are sufficient to induce complement-mediated lysis. This is in contrast with neuromyelitis optica where the autoantigen, aquaporin-4, is expressed by astrocyte foot processes adjacent to the cerebral microvasculature. Further studies are needed to determine the degree of involvement of complement mediated mechanisms in the brain and tumor of patients with synaptic autoimmune encephalitis.

*Pathways that postsynaptic receptors are trafficked and recycled*
NMDA receptors and AMPA receptors are trafficked into and out of postsynaptic sites during physiological processes. Surface NMDA receptors are normally internalized during synapse maturation, in long-term depression (LTD), and in response to ligand-binding (Roche et al., 2001; Vissel et al., 2001; Barria and Malinow, 2002; Montgomery and Madison, 2002; Snyder et al., 2005b). Internalization of NMDA receptors has been shown to be mediated by clathrin via endocytotic signal on C-terminal of NR2A or NR2B subunits (Roche et al., 2001; Lavezzari et al., 2004). NMDA receptors sort into different intracellular pathways after endocytosis, with NR2B containing receptors preferentially trafficking through recycling endosomes and NR2A through late endosomes (Lavezzari et al., 2004). Under physiological conditions, such as during LTD, it has been suggested that AMPA receptors are first moved to extrasynaptic sites followed by endocytosis through clathrin dependent pathways ((Ehlers, 2000; Shi et al., 2001; Park et al., 2004)). The endocytosis of AMPA receptors have at least one GluR2 dependent pathway that requires GluR2 C-terminals interaction with clathrin (Lüthi et al., 1999; Lee et al., 2002) and GluR2 independent pathways (Jia et al., 1996; Meng et al., 2003), which may involve AMPA receptor activity-dependent ubiquitination of GluR1 and subsequent internalization and their trafficking to the lysosome (Ehlers, 2000; Schwarz et al., 2010).

Whether the internalization of the patients’ antibodies in different types of encephalitis is mediated by a pathway that would otherwise be used in normal processes, or by a pathway specific for pathogenic conditions, is a question to be addressed.
Homeostatic compensatory changes in response to antibody-mediated decrease of receptor levels

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 the α, β, δ, and ε subunits of the AChR, as well as increased acetylcholine release upon stimulation (Wilson et al., 1983; Guyon et al., 1994; Plomp et al., 1995; Guyon et al., 1998). Purkinje cells treated with IgG from patients with Lambert-Eaton syndrome show a loss of P/Q-type VGCC currents and a concomitant increase in R-type calcium channel currents (Pinto et al., 1998). Deletion of the α-1a subunit of the P/Q-type channel in mice causes age related ataxia and muscle weakness and results in enhanced L- and N-type calcium channel currents in Purkinje cells (Jun et al., 1999).

These observations raise the possibility that homeostatic mechanisms occur in anti-NMDA receptor and anti-AMPA receptor encephalitis, though this remains to be clearly demonstrated. Support for this idea comes from synaptic changes following pharmacological blockade of glutamate receptors. NMDA receptor or AMPA receptor blockade for 48 hours enhanced mEPSC amplitude (Turrigiano et al., 1998) (Sutton et al., 2006).

Relating the effects of synaptic receptor antibodies to neurological symptoms
Glutamate binding to NMDA receptor and AMPA receptor is crucial for synaptic transmission and plasticity. Pharmacological blockade or genetic reduction of NMDA receptor or AMPA receptors has been shown to alter measures of learning and memory and other behaviors in animal models (Nishikawa et al., 1991; Mohn et al., 1999b; Kapur and Seeman, 2002; Krystal et al., 2002b; Nabeshima et al., 2006; Large, 2007; Schmitt et al., 2007; Labrie et al., 2008).

It’s interesting to consider why patients with anti-NMDA receptor antibodies develop a complex syndrome that includes psychosis, learning and memory dysfunction, abnormal movements, autonomic instability and frequent hypoventilation, while those with AMPA receptor antibodies preferentially develop psychiatric and amnestic symptoms. Studies using genetic deletion of NMDA receptor or AMPA receptor subunits in mouse models provide some insight into this issue. While NR1 knockout mice die shortly after birth of hypoventilation (Li et al., 1994), CA1-specific NR1 knockouts mice 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 (Cui et al., 2004). 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., 1999a; Belforte et al., 2010). Moreover,
subanesthetic doses of NMDA receptor blockers such as phencyclidine and ketamine are psychotomimetic, and they recapitulate many of the positive and negative signs of schizophrenia in both humans and rodents as well as repetitive orofacial movements, autonomic instability and seizures. (Luby et al., 1962; Krystal et al., 1994a; Lahti, 2001; Krystal et al., 2002a). These results together suggest that the hypothetical NMDA receptor hypofunction underlies patients’ syndromes in anti-NMDAR encephalitis as in schizophrenia (Belforte et al., 2010).

The consequences of loss of AMPA receptor expression have also been studied in mouse models. Spatial learning and memory are largely unaffected in GluR1 knockout mice (Zamanillo et al., 1999) and only working memory is diminished (Reisel et al., 2002; Sanderson et al., 2007). GluR2 knockout mice show reduced exploration and impaired motor coordination. While AMPA receptor subunit knockout mice have not provided a satisfying explanation for the role of AMPA receptors in synaptic plasticity related to learning and memory, the fact that patients with AMPA receptor antibodies have short-term learning and memory deficits argues that further studies at the circuit and behavioral levels are warranted.

Conclusions and future directions

We have begun to obtain a better cellular- and synaptic-level understanding of a new and remarkable group of immune-mediated behavioral and memory disorders. On the clinical side, it is important to know the spectrum of the syndromes, especially on
young children and adults. It is also critical to know optimal type of immunotherapy at
different stages of the disease, the duration of treatment as well as whether or how these
treatments modify the effects of antibodies on synapses. On the basic neuroscience side,
the first important question is to ask how the patients’ antibodies from different
encephalitides alter structure and function of synapses and circuits. The ultimate goal
will be to develop and test rodent models in a battery of behavioral tests designed to
assay hippocampal, amygdala, cortical and cerebellar function in each disorder. In this
way, we can begin to relate the cellular, synaptic, and circuit effects of patients’
antibodies to behavioral deficits in learning, memory, and other cognitive and motor
manifestations.

Thesis rationale and goals

While the pathogenesis of autoimmune disorders of the peripheral nervous system
has been well defined, the mechanisms underlying the newly identified disorders, anti-
NMDAR encephalitis and anti-AMPAR encephalitis, remain poorly understood. Whether
this CNS autoimmune disorder utilizes similar mechanisms as in the PNS to cause its
characteristic cognitive and behavioral deficits is unknown. Previous work from our lab
has suggested that autoantibodies against the NR1 subunit of the NMDA receptor for
NMDAR encephalitis, and autoantibodies against the GluR1/GluR2 subunit of the
AMPA receptor for AMPAR encephalitis present in the CSF of patients with the disorder
may mediate the deficits of this disorder (Dalmau et al., 2008; Lai et al., 2009). Based on
these observations, I examined the effects of autoantibodies in patients with anti-
NMDAR encephalitis on NMDA receptors \textit{in vitro} and \textit{in vivo} (Chapters 3, 4), and the
effects of autoantibodies in patients with anti-AMPAR encephalitis (Chapters 5). Using
biochemical, electrophysiology and imaging techniques, I tested the hypothesis that
autoantibodies present in patients with anti-NMDAR encephalitis/anti-AMPAR
encephalitis underlie the cellular mechanism of this disorder of cognition and behavior.

My results suggest that patient antibodies against these two types of glutamate
receptors selectively result in the internalization of receptor from the neuron surface,
decreasing synaptic localization, currents and thus influencing synaptic function.
Moreover, this work has extended our understanding of the cellular mechanisms
underlying anti-NMDAR encephalitis, anti-AMPAR encephalitis and, in the future, may
facilitate a better understanding of the role of glutamate receptors in learning, memory
and behavior.
Determinants of synaptic strength vary across an axon arbor

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Contributions

X.P. designed and carried out the experiments; R.B.-G. and T.D.P helped design experiments; X.P. analyzed the data; T.D.P. developed the model in Fig. 7; X.P. wrote the paper and prepared figures; X.P., R.B.-G. and T.D.P. edited the paper.
Abstract

We used Synaptophysin-pHluorin expressed in hippocampal neurons to ask how functional properties of terminals vary spatially across individual axon arbors. Over short arbor distances (ca. 100 microns), evoked release and total vesicle pool size were spatially heterogeneous when an axon contacted different dendrites. In contrast, evoked release and total vesicle pool size were spatially homogeneous when an axon made contacts along a single dendrite. Regardless of the postsynaptic configuration, the magnitude of evoked release and total vesicle pool size spatially co-varied, suggesting that the fraction of synaptic vesicles available for release was similar over short distances. Evoked release and total vesicle pool size were highly correlated with the amount of NMDA receptors and PSD-95. However, when individual axons were followed over longer distances (several hundred microns), we found greater heterogeneity in the presynaptic strength of terminals in distal rather than proximal segments, as well as a significant increase in evoked release in distal segments. Within proximal or distal axon segments, evoked release was correlated with total vesicle pool size, but release fraction was higher in terminals in distal segments. The increase in distal release fraction was accounted for by changes in individual vesicle probability and or readily releasable pool size. Our results suggest that, for a single axonal arbor, presynaptic strength over short distances is determined by variations in total vesicle pool size, whereas over longer distances presynaptic strength is determined by the spatial modulation of release fraction. Thus the mechanisms that determine synaptic strength differ depending on spatial scale.
Introduction

The ability of hippocampal neurons to differentially regulate presynaptic strength at terminals located along the same axonal branch is thought to underlie reliable neurotransmission in the presence of highly variable stimuli. Several studies documented the heterogeneous functional properties among presynaptic terminals of initial release probability (Hessler et al., 1993; Rosenmund et al., 1993; Huang and Stevens, 1997; Murthy et al., 1997), short term plasticity (Dobrunz and Stevens, 1997) and heterogeneous cellular determinants such as the active zone area, numbers of docked vesicles and total vesicles (Schikorski and Stevens, 1997). Heterogeneity is indicated by broad and/or skewed distributions with large coefficients of variation (Murthy et al., 1997), or large normalized differences in these properties, between any pair of presynaptic terminals (Branco et al., 2008). However, previous studies mainly focused on assessing the functional properties of a small number of terminals without much spatial information, or those in short axon segments (Hessler et al., 1993; Rosenmund et al., 1993; Huang and Stevens, 1997; Murthy et al., 1997; Koester and Johnston, 2005).

Recent studies suggest that postsynaptic neurons (Markram et al., 1998; Reyes et al., 1998; Koester and Johnston, 2005; Pelkey and McBain, 2007), or different dendrites of the same neuron (Branco et al., 2008) can specify different presynaptic functional properties. While CNS axons are usually up to thousands of microns long and making synapses on many targets, how functional properties are distributed along individual long
axon arbors, and how these are associated with postsynaptic specializations, remain unclear.

Here we address three specific questions: what is the spatial distribution of presynaptic functional properties along an axon arbor, specifically the amount of evoked release, total vesicle pool size, and release fraction, as assayed by changes in Synaptophysin-pHluorin fluorescence? How do presynaptic functional properties vary across different postsynaptic neurons and/or dendrites? What is the relationship among presynaptic functional properties and the composition of postsynaptic specializations? The work we report here shows that the answers to these questions are different depending on spatial scale. Over short axon segments, synaptic strength is determined by total vesicle pool size and is related to the density of postsynaptic components such as NMDA receptors and PSD-95. Over the entire axon arbor, synaptic strength is determined by proximal to distal changes in release fraction, independent of total vesicle pool size, that can be accounted for by changes in individual vesicle release probability and/or readily releasable pool size. Our results thus suggest that the mechanisms that establish synaptic strength vary over different spatial scales.
Results

Evoked release and total vesicle pool size are heterogeneous in short axon segments

Previous studies have shown that presynaptic terminals of cultured hippocampal neurons have heterogeneous release properties (Hessler et al., 1993; Rosenmund et al., 1993; Dobrunz and Stevens, 1997; Murthy et al., 1997). However, the spatial arrangement of presynaptic functional properties across long individual axons is poorly understood. To address this question, the strength of presynaptic terminals in ~ 100 µm axon segments of individual glutamatergic hippocampal neurons transfected with Synaptophysin-pHluorin were first assessed by measuring the fluorescence changes (ΔF) evoked by extracellular electrical stimulation, then multiple segments along the entire axon arbor were assessed to map the overall distribution of presynaptic strength.

Axon segments with Synaptophysin-pHluorin labeled presynaptic terminals were selected for imaging based on their presumptive apposition to postsynaptic dendrites, confirmed via post-hoc immunostaining for the dendritic marker MAP2 (Fig. 1A, top). Stimulation at 20 Hz for 3.5 seconds with saturating current (Methods) resulted in an increase in integrated ΔF (ΔF per pixel x area) at discrete sites along a ~ 50 – 100 µm length of axon (Fig. 1A, B). Stimulation evoked ΔF was visible over time at most terminals along an axon segment (Fig. 1A, middle, terminal 1, 2 and 3). As previously reported, ΔF increased montonically for the duration of the stimulus, and then returned to baseline with a slower time course (Fig. 1B), as pHluorin was internalized and reacidified (Sankaranarayanan and Ryan, 2000; Atluri and Ryan, 2006; Burrone et al., 2006).
Evoked release varied among terminals by more than 10 fold (Fig. 1). The local differences in evoked release among terminals along an axon segment did not follow any obvious spatial gradient.

To quantify the extent of presynaptic functional heterogeneity along ~100 µm segments, the random pair ratios of the evoked release between each possible pair of terminals in the field of view along the axon segment were calculated (to reduce random error, the higher value was always divided by a lower value). The higher the ratio, the greater the discrepancy or heterogeneity was between two terminals’ release properties. This measurement enabled us to evaluate a relatively small number of terminals and define a reference value for “homogeneity” using the ratio of evoked release from two consecutive stimulations of the same terminal (at least 5 minutes apart). The average control ratio was significantly less than the average ratio of evoked release between random pairs of presynaptic terminals (Fig. 1C). Thus although ∆F of a given terminal does vary somewhat with repeated stimulation, this source of variation alone does not account for the differences in ∆F between different terminals along short axon segments. This result is consistent with previous reports that presynaptic terminals along an axon segment are functionally heterogeneous. For subsequent analyses, a pair ratio between 1.4 to 2.6 (95% confidence interval of the average of the two repeats ratio) was regarded as indicative of functionally homogenous release properties.

To further explore the functional heterogeneity among terminals along an axon segment, neurons were perfused with an NH₄Cl-containing extracellular solution (Fig.
1A, bottom). This deacidifies synaptic vesicles, yielding a maximum $\Delta F$ that reflects the total pool of vesicles clustered at each presynaptic terminal (Sankaranarayanan et al., 2000; Burrone et al., 2006). Like the evoked $\Delta F$, the $\text{NH}_4\text{Cl}$ induced $\Delta F$ was also heterogeneous among the presynaptic terminals along an axon segment. The average ratio of total vesicle pool size between random pairs of terminals was significantly higher than the average control ratio calculated from two consecutive stimulations of the same terminal (Fig. 1D). As above, homogeneity in total vesicle pool size was defined by the 95% confidence interval of two repeated measurements, which was between 1.3 to 2.3.

To substantiate this measure of total vesicle pool size, we determined whether the surface fraction of pHluorin contributed significantly to the measured total fluorescence signal (Wienisch and Klingauf, 2006). The pHluorin surface fraction was on average 13.7 ± 1.0 % (51 terminals, 4 neurons) and was stable over the time course of experiments. Furthermore, immunostaining showed that pHluorin fluorescence was linearly correlated with immunostaining fluorescence of the endogenous synaptic vesicle markers SV2 and vGlut at each terminal (data not shown). Thus the local heterogeneity in $\Delta F$ after $\text{NH}_4\text{Cl}$ perfusion is consistent with heterogeneity in total vesicle pool size among presynaptic terminals. Taken together, these results show that evoked release as well as vesicle pool size are locally heterogeneous among presynaptic terminals along a ~100 µm axon segment.
Heterogeneity in total vesicle pool size correlates with heterogeneity in evoked release in short axon segments, independent of stimulation frequency

Previous work assessed heterogeneity in exocytosis of presynaptic terminals using low frequency pulse stimulation at 1 Hz or lower (Hessler et al., 1993; Rosenmund et al., 1993; Huang and Stevens, 1997; Murthy et al., 1997; Branco et al., 2008). Because hippocampal neurons firing rates range from < 2Hz to > 100 Hz in awake rodents or during slow wave sleep (Ranck, 1973), we assessed the functional properties of presynaptic terminals across simulation frequency. Terminals were stimulated with 70 action potentials delivered at 4 frequencies between 1 and 100 Hz. A 5 minute interval between the different stimulation trains allowed the internalization and reacidification of surface Synaptophysin-pHluorin. Evoked release decreased as stimulation frequency increased (Fig. 2A, B) when the total number of pulses was held at 70, probably due to an increased number of release failures. Previous work showed that total vesicle pool size correlates with evoked release (Moulder et al., 2007). By plotting the evoked ∆F against ∆F from NH₄Cl, a linear correlation between evoked ∆F and total vesicle pool size was observed at each stimulation frequency (Fig. 2A). Notably, terminals with larger total vesicle pool size showed larger evoked release than smaller terminals across a wide range of stimulation frequency. However, the amount of evoked release from a given total vesicle pool size depended on stimulation frequency. Thus for terminals within short axon segments, the fraction of the total vesicle pool released, i.e. the release fraction, is similar for a particular stimulus frequency.
We next assessed whether heterogeneity is influenced by stimulus frequency. The heterogeneity in evoked release was similar with different stimulation frequencies, as suggested by the evoked $\Delta F$ pair ratios for each stimulation frequency (Fig. 2C). This suggests that, in short segments of axons, the underlying numerical distribution of presynaptic strengths is not influenced by stimulus frequency over a range of 1 to 100 Hz, and likely arises from a heterogeneity in total vesicle pool size.

*Functional properties among presynaptic terminals contacting different dendrites of the same or different postsynaptic neurons are heterogeneous*

Previous studies suggest that different postsynaptic targets – either different neurons or different dendrites of the same neuron - specify presynaptic strengths (Koester and Johnston, 2005; Branco et al., 2008). To verify that the local heterogeneity in evoked release and total vesicle pool size depend on the synaptic configuration of terminals and postsynaptic neurons and/or dendrites, pairs of presynaptic terminals in the same axon segment were classified as contacting the same dendrite (Fig. 3A, terminal 1 and 2), different dendrites from the same postsynaptic neuron (Fig. 3A, terminal 1 and 3, 2 and 3), or different dendrites from different postsynaptic neurons (Fig. 3B, terminal 1 and 2). As predicted, heterogeneity of evoked release and total vesicle pool size from various synaptic configurations were different. The pair ratios of terminals innervating two different dendrites of the same neuron were significantly higher than the control ratios calculated from two consecutive stimulations (Fig. 3A, C). The same was true for
terminals innervating dendrites from two different postsynaptic neurons (Fig. 3B, C). However, evoked release from terminals contacting the same dendrite was homogeneous, because the pair ratios were not significantly different from the control ratios (Fig. 3A, C; 7 neurons, 33 terminal pairs; Mann-Whitney U test, p = 0.15). A similar dependence on synaptic configuration also was observed for the heterogeneity in total vesicle pool size, except that no significant difference was observed for terminals innervating two different dendrites of the same neuron (Fig. 3D). Interestingly, the heterogeneity in evoked release and total vesicle pool size among terminals other than those contacting the same dendrite was not significantly different from the heterogeneity of random pairs of terminals in short axon segments (black bars in Fig. 3C, D).

These results show that the strength of presynaptic terminals contacting different dendrites, whether of the same neuron or different neurons, is heterogeneous. In contrast, the strength of presynaptic terminals innervating the same dendrite is homogeneous. Taken together, these results suggest that synaptic strength is determined by local interactions between presynaptic terminals and postsynaptic target neurons.

*Presynaptic heterogeneity across different synaptic configurations is correlated with the amount of postsynaptic NMDA receptors and PSD-95*

In order to understand how postsynaptic targets may influence presynaptic strength, we asked which of several postsynaptic constituents correlated with presynaptic strength. Post-hoc immunostaining was used to measure the amount several postsynaptic
proteins, including the NMDA receptor subunit NR1, the AMPA receptor subunit GluR1, PSD-95 (colocalized pixel area x fluorescence intensity). MAP2 served as a negative control. Presynaptic terminals that were not colocalized with a postsynaptic dendrite as identified by MAP2 immunostaining, or a postsynaptic protein cluster, were excluded from this analysis.

Presynaptic terminals with higher evoked release (Fig. 4A, left) and larger total vesicle pool size (Fig. 4A, left middle) were colocalized with larger NMDA receptor clusters (Fig. 4A, right middle and right). In the example shown in Fig. 4A, a significant linear relationship was observed between presynaptic evoked release (Fig. 4B) and total vesicle pool size (Fig. 4C), and in most segments (Fig. 4D, E). A similar significant correlation was observed between presynaptic evoked release or total vesicle pool size and the amount of PSD-95 (Fig. 4D, E). In contrast, the amount of GluR1 was not correlated with either evoked release or total vesicle pool size (Fig. 4D, E). As expected, evoked release and total vesicle pool size were not significantly correlated with the amount of postsynaptic MAP2 in the vast majority of axon segments measured. These data show that presynaptic evoked release and total vesicle pool size are correlated with the amount of some, but not all, postsynaptic components.

To determine whether the local heterogeneity observed in presynaptic functional properties varied with the amount of a postsynaptic protein, regardless of whether these were in the same dendrite, or in multiple dendrites from one or multiple postsynaptic neurons, we used MAP2 staining to separate postsynaptic targets. Because it is very
difficult to find an ideal configuration during live imaging without labeling multiple postsynaptic neurons, in this experiment, we estimated presynaptic strength by measuring total pHiouorin fluorescence after fixation, as this mainly reflects total vesicle pool size. As described above, the total vesicle pool size of presynaptic terminals on different dendrites is heterogeneous (Fig. 4F, arrowheads). We found that total vesicle pool size was correlated with the amount of NMDA receptors. On different dendrites from the same postsynaptic neuron, a larger terminal was colocalized with more NMDA receptors (Fig. 4F, G; compare large terminal 5 and small terminal 3). In 7 of 8 axon segments, each with > 5 terminals contacting different dendrites, total vesicle pool size was correlated with the amount of NMDA receptors (as in the examples shown in Fig. 4F, G). Thus, on different dendrites from the same postsynaptic neuron, heterogeneity in the amount of postsynaptic NMDA receptors co-varies with the total vesicle pool size of the apposed presynaptic terminal.

To compare the amount of NMDA receptors beneath presynaptic terminals contacting different postsynaptic neurons, we assessed whether the average total vesicle pool size of two groups of terminals on two adjacent postsynaptic neurons correlated with average NMDA receptor cluster intensity. In the example shown in Fig. 4H, the 6 terminals of one axon on one postsynaptic neuron have, on average, a smaller total vesicle pool size and amount of NMDA receptors (red squares) than the 7 terminals of the same axon on a second nearby neuron (black squares). Four of 6 axon segments showed a similar correlation (Fig. 4I). In the other 2 segments, the terminals contacting
two different neurons had overlapping total vesicle pool size, with similar average amount of NMDA receptors. Notably, the NMDA receptor clusters on individual dendrites of the same or different neurons were heterogeneous with respect to cluster size. Taken together, these data suggest that the correlation between presynaptic total vesicle pool size and the amount of postsynaptic NMDA receptors is specific to a particular pre- and postsynaptic neuron pair, but is not determined by a postsynaptic neuron- or dendrite- autonomous mechanism; if this were the case, all presynaptic terminals on the same postsynaptic dendrite/neuron would have similar functional properties.

While these data suggest that the amount of postsynaptic NMDA receptors and PSD-95 may reflect presynaptic strength, neither protein is required for a presynaptic terminal to be functional. We found that 44 ± 9% of functional presynaptic terminals were not colocalized with NMDA receptors, and 36 ± 7% were not co-localized with PSD-95; 24% were not even colocalized with postsynaptic dendrites. This is consistent with previous work that showed that orphan presynaptic terminals without an apposed postsynaptic specialization were functional (Krueger et al., 2003). Nonetheless, over 7-10 days of maturation in vitro, total vesicle pool size did not increase in the population of presynaptic terminals that were not co-localized with NMDA receptors or PSD-95; after 14 days in vitro, these terminals have significantly smaller total vesicle pool size than terminals co-localized with NMDA receptors or PSD-95 (data not shown). Taken together, these results suggest that NMDA receptor and PSD-95 are dispensable for the
initial functionality of a presynaptic terminal, consistent with work in mice null for these proteins (Elias et al., 2006; Ultanir et al., 2007). However, these proteins accumulate postsynaptically as presynaptic terminals mature, in proportion to evoked release and total vesicle pool size.

**Distal terminals are functionally more heterogeneous than proximal terminals**

We then examined how presynaptic functional properties varied across individual axon arbors by co-transfecting neurons with mCherry to trace axon arbors for > 1000 µm (Fig. 5A) and sampling several 50 – 100 µm segments of axon over as much of the axon arbor as possible, always for more than several hundred microns. The distribution and mean of presynaptic properties was determined in different segments along an axon arbor. The distribution was determined using the heterogeneity pair ratio measurement and the coefficient of variation of individual values, and the mean was determined by averaging the values of a particular functional property across axon segments.

Within an axon segment, there is a wide range of and thus heterogeneity in evoked release (Fig. 5B). For example, the pair ratios of evoked release for 5 axon segments from the neuron shown in Fig. 5A were significantly higher compared to the control ratios calculated from two consecutive stimulations (Fig. 5C). Evoked release was heterogeneous by this criterion in the majority (92%) of segments from each of 6 neurons assessed in this way.
Total vesicle pool size was similarly heterogeneous (Fig. 5D). Within each axon segment, the pair ratios of total vesicle pool size were significantly higher than the control ratios calculated from two consecutive measurements (Fig. 5E). Total vesicle pool size was heterogeneous by this criterion in the majority (75%) of segments from each of 6 neurons assessed in this way.

Previous studies suggest that heterogeneity in presynaptic release probability in short axon segments is not significantly different than the heterogeneity measured among all presynaptic terminals sampled in the same neuron (Branco et al., 2008). Consistent with this, we found that the pair ratios of evoked release of terminals across ca. 1000 μm of an axon arbor were not significantly different from the pair ratios of evoked release for terminals within a short, ~50-100 μm axon segment (e.g., Fig. 5C, compare white bar with colored bars for 1 neuron; for all neurons, average of terminals in all short axon segments, 3.4 ± 0.2; average of all terminals in entire arbor, 3.4 ± 0.2; Mann-Whitney U test, p = 0.41). Comparison of the pair ratios of total vesicle pool size were not significantly different for terminals in short axon segments compared to the entire axon arbor (e.g., Fig. 5E, compare white bar with colored bars for 1 neuron; for all neurons, average of terminals in all short axon segments, 3.2 ± 0.2; average of all terminals in entire arbor, 3.6 ± 0.3; Mann-Whitney U test, p = 0.24).

However, comparing all possible terminal pairs across an entire axon arbor would average out any proximal to distal differences. Thus we also compared the average pair ratio for evoked release (Fig. 5F) and total vesicle pool size (Fig. 5G) in the most distal
segments with those in the most proximal segments. Surprisingly, distal segments had a higher average pair ratio of evoked release (Fig. 5F) and total vesicle pool size (Fig. 5G). These data suggest that presynaptic terminals in distal axon segments are more heterogeneous in evoked release and total vesicle pool size compared to proximal segments.

To further investigate how the heterogeneity in total vesicle pool size was related to the heterogeneity in evoked release proximally and distally, we used the coefficient of variation (CV) which is a size independent measure of heterogeneity. A significant linear correlation was observed between the CV of total vesicle pool size and the CV of evoked release (Fig. 5H). This result suggests that while the distribution of evoked release and total vesicle pool size is similar, the heterogeneity in total vesicle pool size drives the heterogeneity in evoked release, regardless of release fraction. Together, these data suggest that the evoked release of distal terminals is more heterogeneous than proximal terminals, correlated with greater heterogeneity in total vesicle pool size distally compared to proximally, independent of the average total vesicle pool size, because distal terminals did not have larger total vesicle pool size than proximal terminals.

*Evoked release and release fraction are higher in distal segments of individual axon arbors*

We next determined the average value of evoked release and total vesicle pool size in short axon segments, and compared these to values from the most proximal and
most distal axon segments. We found that distal axon segments had a significantly higher evoked release than proximal segments (Fig. 6A; 1.5 fold difference). However, total vesicle pool size was not significantly different (Fig. 6B). Thus, distal axon segments had a significant, 2.1 fold higher release fraction than proximal segments (Fig. 6C).

Since evoked release correlates with total vesicle pool size in short axon segments such that release fraction is constant, how can this observation be reconciled with the observation that terminals in distal axon segments have a higher release fraction? By plotting evoked release against total vesicle pool size, a strong linear relationship was observed between these two properties in most short axon segments, regardless of their location in the axon arbor (Fig. 6D, color coded axon segments shown in Fig. 5A). A similar relationship was observed in the majority of short segments (71%; 17 / 24 segments, 6 neurons; Pearson correlation test, p < 0.05). However, within a neuron, some segments have a similar regression slope, indicative of similar release fraction (Fig. 6D; e.g., segments I and II, III and IV), while release fraction is significantly different among other segments (Fig. 6D; e.g., segments I and V, II and V)). These data suggest that release fraction was not constant across an entire axon arbor. When terminals from the most proximal or distal segments from 13 neurons were pooled, and evoked release plotted against total vesicle pool size, a significant correlation between these two parameters was observed for proximal as well as distal terminals (Fig. 6E), regardless of whether total vesicle pool size was large or small (over a > 10 fold difference). This
observation suggests that the release fraction in proximal segments is small and constant, and that between-neuron differences are smaller than within neuron proximal-to-distal differences. Moreover, distal terminals have a significantly higher release fraction (Fig. 6E). Because terminals in distal axon segments had a wide range of total vesicle pool size, the higher release fraction was not due to a small total vesicle pool size (Fig. 6E).

While most terminals measured were apposed to postsynaptic dendrites, the release fraction of terminals not apposed to dendrites was not significantly different from those that were (5 of 6 neurons; Student’s t-test, p > 0.05; data not shown). This is consistent with the observation that local release fraction is relatively constant regardless of the spatial configuration of postsynaptic targets. These data further support the conclusion that release fraction is unlikely to be retrogradely modulated by postsynaptic targets.

Thus, in contrast to the correlation between evoked release and total vesicle pool size in short axon segments, evoked release and thus terminal release probability is modulated differently across the entire axon arbor. This is because release fraction, but not total vesicle pool size, accounts for higher evoked release distally compared to proximally.
Discussion

Here we report the distribution of the functional properties of presynaptic terminals and their relationship with postsynaptic specializations across two spatial scales. Over short distances (ca. 100 microns), evoked released was spatially heterogeneous for axons contacting dendrites on different postsynaptic neurons and for axons contacting different dendrites on the same neuron. The magnitude of evoked release and total vesicle pool size spatially co-varied, regardless of the spatial configuration of the postsynaptic target(s). This suggests that the fraction of synaptic vesicles released and the average release probability of individual vesicles are similar among neighboring terminals over short axon distances. Interestingly, evoked release and total vesicle pool size were spatially homogeneous when an axon made terminals only along a single dendrite. Evoked release and total vesicle pool size were highly correlated with the amount of two postsynaptic proteins, NMDA receptors and PSD-95, regardless of the spatial configuration of postsynaptic targets. Our results suggest that local presynaptic strength is determined mainly by local interactions with postsynaptic targets, through modulation of total vesicle pool size, associated with the amount of postsynaptic NMDA receptors and PSD-95.

We report for the first time that over long distances (ca. 1000 µm) across the arbor of single axons, a proximal to distal difference exists in the heterogeneity of both evoked release and total vesicle pool size. In contrast to short axon segments, distal terminals have higher evoked release, but not total vesicle pool size, than proximal
terminals. Thus release fraction is increased in distal terminals, likely due to greater release probability of individual vesicles and/or readily releasable pool size distally compared to proximally. Our results suggest that, across the arbor of a single axon the mechanisms that determine synaptic strength are different depending on spatial scale.

*Retrograde cellular and molecular determinants of heterogeneous presynaptic release properties in short axon segments*

Previous work has suggested that postsynaptic targets retrogradely influence presynaptic release probability (Reyes et al., 1998; Koester and Johnston, 2005; Branco et al., 2008; Branco and Staras, 2009). In rat neocortex, presynaptic terminals contacting different postsynaptic neurons have heterogeneous release probability (Reyes et al., 1998; Koester and Johnston, 2005). In cultured rat hippocampal neurons, presynaptic terminals contacting different dendrites of the same postsynaptic neuron also have heterogeneous release probability (Branco et al., 2008). Our results suggest that the heterogeneity among terminals contacting the same neuron, but on different dendrites, are as heterogeneous as the terminals contacting different postsynaptic neurons, extending previous studies by comparing the different spatial configurations in individual axons. Our results suggest that regardless of different postsynaptic configurations, and possibly different postsynaptic retrograde signals, the dynamic range of presynaptic strength of terminals in short axon segments is similar across different spatial configurations of postsynaptic targets. This may be due to local, dynamic sharing of presynaptic vesicles.
and active zone components among many terminals (Ahmari et al., 2000; Friedman et al., 2000; Shapira et al., 2003; Bresler et al., 2004; Darcy et al., 2006; Tsuriel et al., 2006; Tsuriel et al., 2009).

Several different postsynaptic retrograde mechanisms have been suggested to influence presynaptic functional properties (Tao and Poo, 2001; De Paola et al., 2003; Pratt et al., 2003; Ziv and Garner, 2004; Dalva et al., 2007; Williams et al., 2010). These include cell adhesion molecules such as the neuroligin-neurexin complex (Chih et al., 2005; Levinson et al., 2005; Futai et al., 2007), SynCAM (Biederer et al., 2002), EphBs and ephrin-Bs (Dalva et al., 2000; Kayser et al., 2006) and cadherins (Benson and Tanaka, 1998; Takeichi, 2007; Arikkath and Reichardt, 2008), among others; as well as secreted factors such as neurotrophins (Du and Poo, 2004), FGF22, FGF7 (Terauchi et al., 2010) and Sema3F (Tran et al., 2009), among others. Which of these cell-cell signaling mechanisms contribute to the modulation of presynaptic release properties on different spatial scales across an axon arbor remains to be determined.

Here we show that the amount of synaptic NMDA receptors and PSD-95 is highly correlated with presynaptic strength, regardless of the postsynaptic neuron target(s). However, it is unlikely that NMDA receptors or PSD-95 directly specify presynaptic strength. Synapse formation and function are largely normal in the absence of PSD-95, probably due to functional redundancy (Elias et al., 2006). However, neuroligins which bind to the PDZ domain of PSD-95 has been shown to retrogradely modulate presynaptic release probability (Futai et al., 2007; Wittenmayer et al., 2009). This suggests that PSD-
95 correlates with presynaptic strength indirectly, possibly via neuroligin-1 signaling. While blocking NMDA receptor currents doesn’t change the distribution of other pre- and postsynaptic molecules, except for a homeostatic increase in NMDA receptors (Rao and Craig, 1997), deletion of NMDA receptors in cortical pyramidal neurons resulted in fewer but larger spines and presynaptic terminals (Ultanir et al., 2007). The correlation between NMDA receptors and presynaptic strength we observed may be because NMDA receptors bind the first PDZ domain of PSD-95 (Irie et al., 1997), which through its interactions with neuroligins retrogradely modulates release probability, resulting in a correlation between the amount of PSD-95 and presynaptic strength. Other adhesion and signaling molecules associated with NMDA receptors or PSD-95, such as EphBs (Dalva et al., 2000; Kayser et al., 2006), may also retrogradely modulate synaptic strength. The observation that the amount of GluR1 doesn’t correlate with presynaptic strength, while the amount of NMDA receptors and PSD-95 does, suggests that not all postsynaptic proteins are affected by retrograde signaling that matches pre- and postsynaptic properties. Recent studies showed that the amount of AMPA receptors doesn’t correlate with presynaptic strength with baseline activity (Tokuoka and Goda, 2008) and AMPA receptor subunits retrogradely stabilize presynaptic terminals when neuroligin-1 is present (Ripley et al., 2010). Our work suggests that the amount of two postsynaptic proteins, NMDA receptors and PSD95, is an indirect indicator of presynaptic strength, regardless of the spatial configuration or the identity of postsynaptic neuron targets.
**Spatial distribution of heterogeneous presynaptic terminals across axon arbors**

Different spatial patterns of presynaptic strength have been documented for axons and terminals in several different model systems. Heterogeneity in the strength of presynaptic evoked release has been observed along CNS axons segments and within individual mouse motor nerve terminals, while proximal-to-distal gradients of exocytosis have been observed in motor nerve terminals of fly, toad and crayfish (Atwood, 1967; Bennett et al., 1986; Guerrero et al., 2005). Whether large scale spatial patterns of presynaptic strength also exist across the arbor of CNS axons has not been well studied prior to the work we present here.

Consistent with previous work, we found that in short axon segments, presynaptic terminals were heterogeneous with respect to evoked release and total vesicle pool size, over a ca. 3 fold range of values (Hessler et al., 1993; Rosenmund et al., 1993; Murthy et al., 1997; Moulder et al., 2007; Branco et al., 2008). In short axon segments, evoked release was correlated with total vesicle pool size, similar to other studies using pHluorin (Moulder et al., 2007) and electron microscopy studies that showed that large presynaptic terminals contain more vesicles (Rosenmund and Stevens, 1996; Dobrunz and Stevens, 1997; Schikorski and Stevens, 2001). The magnitude of evoked release can be predicted by a simple model that takes into account the size of the readily releasable pool size, the release probability of individual vesicles and number of times the terminal is stimulated (Fig. 7A). For short axon segments, this model also mimics the dependence of the heterogeneity in evoked release on total vesicle pool size, if the readily releasable pool
size is constrained to scale in proportion to the total vesicle pool size (Fig. 7B; note linear relationship for either proximal or distal segments). Thus in short axon segments, the heterogeneity in evoked release is determined by the heterogeneity in total vesicle pool size, independent of stimulation frequency, and this may be important for maintaining the relative strength of terminals across a range of action potential firing rates similar to those present in vivo.

We report here for the first time that evoked release was significantly higher, by 1.5 fold, in terminals located in proximal compared to distal axon segments. In contrast to the heterogeneity in short axon segments, in which terminals with larger total vesicle pool size have higher evoked release, the proximal low / distal high evoked release was not correlated with total vesicle pool size, because the distal segments which had higher evoked release had the same, or smaller, average total vesicle pool size. The distal increase in evoked release and release fraction is also predicted by the simple model, given either an increase in individual vesicle release probability and or an increase in readily releasable pool size, if the readily releasable pool is increased independent of total vesicle pool size (Fig. 7B; compare change in slope of linear relationship for proximal versus distal segments). The postulated presynaptic loci for the distal increase in release properties is consistent with our observation that these differences are not correlated with the spatial configuration of postsynaptic targets or the amount of postsynaptic components. A previously reported mechanism that could be attributed to increasing distal release by increasing individual vesicle release probability is found in *Drosophila*
motor nerve terminals, where higher distal release is coupled to higher Ca\textsuperscript{2+} influx (Guerrero et al., 2005). Conversely, in crayfish, higher distal release is correlated with boutons that have denser T-bars within active zones (Atwood, 1967), suggestive of a larger readily releasable pool. Future studies employing ultrastructural studies of the number of docked vesicles in distal compared to proximal terminals, and measurements of Ca\textsuperscript{2+} influx in individual presynaptic terminals to assay the sensitivity of the release fraction to external Ca\textsuperscript{2+}, would provide further insights into the prominent proximal to distal difference in evoked release across an individual axon arbor.

Evoked release and total vesicle pool size were observed to be significantly more heterogeneous in distal compared to proximal axon segments, evidenced by a higher coefficient of variance and assessment of random pair ratios of these values. This heterogeneity is independent of the mean values of evoked release or total vesicle pool size distally, and instead is determined by the distribution of these values, which is greater distally than proximally. What might drive the proximal to distal difference in the heterogeneity of these presynaptic properties? One possibility is a difference in spontaneous action potential activity, which may be lower in distal segments due to branch point failures along an axon arbor. However, activity blockade results in a shift to higher readily releasable pool size, but the coefficient of variation in the distribution of these values is not different (Murthy et al., 2001), suggesting that activity-dependent, homeostatic scaling doesn’t change the heterogeneity of release probabilities. During development, proximal segments were once distal segments, and could be less
heterogeneous than later added, distal segments if the heterogeneity in the functional properties of terminals decreases over time. Studies employing pHluorin and imaging over time as axons grow in vitro and in vivo will be useful to address the spatial dynamism in the functional properties of presynaptic terminals as circuits are established.
Materials and Methods

Cell Culture

Primary neurons co-cultured with astrocytes were prepared as described previously (Elmariah et al., 2005), with minor modifications. Briefly, hippocampi were dissected from embryonic day (E) 18 rats, dissociated in Hanks’ Balanced Salt Solution (Invitrogen, Grand Island, NY; with MgCl₂ and HEPES) containing 1% papain for 20 min, triturated in Basal Medium Eagle (Invitrogen), and plated at 75000 cells/ml on a poly-L-lysine (Sigma, at a concentration of 1mg/ml) coated coverslips with grids (Bellco Biotechnology) in 24-well plates. Cells were grown at 37°C, 5% CO₂, 95% humidity in Neurobasal medium (Invitrogen) plus B27 (Invitrogen) and Fetal Bovine Serum (Hyclone, to sustain astrocytes in the first week) that was changed weekly. Neurons co-cultured with astrocytes instead of those treated with astrocyte conditioned medium were used, because contact with astrocytes has been shown to increase the number of functional presynaptic terminals in vitro (Elmariah et al., 2004; Hama et al., 2004).

Constructs and transfection

The Synaptophysin-pHluorin construct, in which 1 copy of the pHluorin sequence was inserted into the lumenal loop of the mouse Synaptophysin sequence, was obtained from Dr. Y. Zhu (Zhu et al., 2009). Primary hippocampus neuron cultures were transfected with this construct (0.03 / 0.06 µg DNA) using Lipofectamine 2000™ (0.06 / 0.12 µg per coverslip Invitrogen) at 7 DIV at very low efficiency (< 5-10 Synaptophysin-
pHluorin+ neurons per coverslip) and were imaged 7-10 days after transfection. In some experiments, neurons were co-transfected with mCherry (obtained from Dr. R.Y. Tsien), to identify axons from individual neurons. Synaptophysin-pHluorin was localized primarily to axons, accumulated at presynaptic terminals. Immunostaining showed that total pHluorin fluorescence was linearly correlated with immunostaining fluorescence of the endogenous synaptic vesicle markers SV2 and vGlut at each terminal (data not shown).

*Live imaging and extracellular stimulation*

At 14-17 DIV, coverslips were mounted in a customized imaging chamber (modified RC26 chamber, Warner Instruments). Coverslips were constantly perfused with physiological saline solution containing (in mM) 119 NaCl, 2.5 KCl, 2 CaCl₂, 1.3 MgCl₂, 25 HEPES (pH = 7.4) and 30 glucose. NH₄Cl saline solution (pH = 7.4) was prepared by substituting 50 mM NaCl in normal saline with NH₄Cl. In experiments to measure evoked release during 1 Hz 70 seconds stimulation, Bafilomycin A1 to block the re-acidification (Calbiochem, 0.25 uM) was included in the bath. An in-line heater (Warner Instruments) was used to maintain the solution in the chamber at ca. 35 °C. The chamber was mounted onto an inverted microscope (Leica DMI3000B) and neurons were imaged with a 63x, 1.2 numerical aperture objective using a Hamamatsu cooled CCD camera (C9100), appropriate fluorescence excitation and emission filters (fN2.1 filter cube for mCherry, GFP filter cube for pHluorin; Leica), and the incident light was
attenuated so that photobleaching was minimal or absent during the imaging session. Only glutamatergic neurons that had complex dendritic arbors, short dendritic segment lengths and relatively more round cell bodies under DIC illumination (c.f. (Benson et al., 1994)) were imaged. Axon segments with Synaptophysin-pHluorin labeled presynaptic terminals were selected for imaging based on their presumptive apposition to postsynaptic dendrites, confirmed via post-hoc immunostaining for the dendritic marker MAP2 (Fig. 1A). Axon segments were stimulated with a glass pipette (0.5 – 0.9 MΩ) placed near an axon of interest at 20 Hz using 1 ms, 400 – 800 µA square pulses in trains of 3.5 sec. duration if not stated otherwise. Pulses were delivered through Iso-Flex isolator controlled by a Master-8 (AMPI, Inc.).

Stimulation parameters were chosen to maximize the presynaptic response and also to saturate the image field. Over a range of stimulation amplitudes, from 50 µA to 1200 µA, we found that presynaptic responses began to saturate at about 600 µA (data not shown). When the stimulation exceeded 1000 µA, damage to axons was noted (i.e., fluorescence did not return to baseline after 5 min.). Thus 400 - 800 µA was used for experiments. To determine whether a presynaptic terminal’s response varied significantly with electrode location, the electrode was placed at two positions within a field and terminal responses compared in two trials at least 5 min. apart. The ratio of evoked release between trials 1 and 2 was ~ 1 with a correlation coefficient r = 0.9. These data suggest that presynaptic responses are replicable and are independent of electrode placement.
When multiple segments of axon arbors were measured, segments were at least 300 µm away from each other. The most proximal axons segments were those closest to the cell body with Synaptophysin-pHluorin clusters. Axon arbors were sampled and measured until reaching the growth cone or a point where ambiguously tracking an axon was no longer possible. The stimulation was either moving from proximal to distal or vice versa in different neurons.

Coverslips were perfused with NH₄Cl saline to deacidify synaptic vesicles, allowing measurement of maximum ∆F that reflects the total pool of vesicles clustered at each presynaptic terminal (Sankaranarayanan et al., 2000; Burrone et al., 2006). This was followed by acidic saline to quench surface fluorescence. The surface fluorescence fraction was measured as surface fluorescence / total fluorescence (Wienisch and Kingauf, 2006).

Time lapse images were acquired using Metamorph (Molecular Devices, Inc.) controlling a Lambda SC Smart shutter (Sutter Inst.) at 2Hz for evoked release, and at 0.5 Hz for NH₄Cl saline perfusion, to visualize all Synaptophysin-pHluorin+ clusters and total vesicle pool size. At the end of the experiment, a bright field image was taken to document the field of interest, and coverslips fixed and processed for subsequent immunostaining.

Immunostaining

60
Coverslips were fixed in 4% paraformaldehyde and 4% sucrose at room temperature for 15 minutes and rinsed in PBS with 0.25% Triton X-100. For anti-NR1 immunostaining, coverslips were fixed in 4% paraformaldehyde and 4% sucrose at room temperature for 1 minute, followed by MeOH at -20 °C for 4 minutes. Coverslips were rinsed in PBS and blocked in PBS containing 5% normal goat serum. Coverslips were then incubated in one or more of the following primary antibodies: anti-PSD-95 (mouse monoclonal; Affinity BioReagents, CO), anti-MAP2 (mouse polyclonal; gift from Dr. V. Lee), anti-NR1 (rabbit polyclonal; Sigma, MO), anti-GluR1 (rabbit polyclonal; Chemicon, MO), anti-vGlut (guinea pig polyclonal, Chemicon, MO), anti-SV2 (mouse monoclonal, Dev. Studies Hybridoma Bank, IA). Antibodies were visualized after staining with the appropriate FITC-, TRITC- or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch, PA). After immunostaining, the region of interest was located and imaged using a confocal microscope (Leica TCS 4D). Field size and pixel resolution were adjusted so that confocal images were in register with live images pixel by pixel. In each image, laser light levels and detector gain and offset were adjusted so that no pixel values were saturated in the regions analyzed.

**Analysis**

An averaged image was made from all time lapse images obtained in NH₄Cl, in which all Synaptophysin-pHluorin+ clusters were visualized (Fig. 1). This process averaged out small, mobile Synaptophysin-pHluorin+ clusters within axons, while
retaining stable clusters that were likely to be bona fide presynaptic terminals. Regions of interest (ROIs) were then made from the averaged image, using a customized algorithm in ImageJ software (adapted from (Bergsman et al., 2006)). The major advantage of this algorithm is that a wide range of presynaptic terminal area, from 0.3 to 8 µm², was more accurately identified compared to simple thresholding. ROI were defined using the cluster size range and a thresholded intensity, determined by measuring presynaptic terminals defined by eye in 3 representative images from the same set of images. These parameters were then used in all subsequent analyses.

Evoked release was measured by subtracting the total fluorescence in ROIs averaged from 3 consecutive frames right before the stimulation from the total fluorescence in ROIs in the 1 frame right after the stimulation was stopped. The total fluorescence in each ROI was determined by summing pixel intensity in each ROI. Among all ROIs, the fluorescence intensity of ROIs was plotted versus time, some fluorescence traces increased linearly during stimulation, while others were flat (Fig. 1). The R² of the regression slope was calculated for each trace of fluorescence changes during stimulation for each ROI. The R² was also calculated for baseline fluorescence over 3.5 sec. prior to stimulation, assuming that during this period of time, there is no evoked fluorescence change and any R² value is due to random fluorescence fluctuation. The distribution of R² (n = 1387 terminals) values in the absence of stimulation was compared to the distribution of R² values during stimulation. As expected, during stimulation, the frequency of large R² values increases. To keep the rate of false
positives low (≤ 5%), 0.72 was used as a threshold value. Thus a fluorescence trace with a positive slope during stimulation and with an $R^2$ value > 0.72 was characterized as a releasing terminal. Those with an $R^2$ value < 0.72 were regarded as non-releasing terminals. If not stated otherwise, only presynaptic terminals that were characterized as releasing during 20 Hz stimulation were used in subsequent analyses.

Total vesicle pool size was estimated by subtracting the baseline fluorescence from the total fluorescence in NH$_4$Cl. Thus total vesicle pool size was measured as the maximum change of fluorescence in NH$_4$Cl, as if all the vesicles clustered within a terminal were exocytosed. The release fraction of individual terminals was determined as $\Delta F / \text{total vesicle pool size}$ and reflected the percentage of vesicles exocytosed upon stimulation.

Co-localization between pHluorin+ terminals and a pre- or postsynaptic marker after post-hoc immunostaining was evaluated in time lapse or immunostaining images taken at the same pixel resolution. The orientation of images after immunostaining was adjusted using free rotation in Metamorph, and the ROIs defined from time lapse images were superimposed. The ROIs were moved together to align them with pHluorin+ terminals. Individual ROIs were then moved by a few pixels for final alignment. If there was no pHluorin+ terminal within 5 pixels of an ROI defined from time lapse imaging, the ROI was not analyzed further. Different fluorescence channels were separated and segmented with the ImageJ algorithm as described in segmenting pHluorin+ live images. An overlap mask of Synaptophysin-pHluorin and an immunostained marker was made,
ROIs superimposed, and any pair of clusters with more than 1 pixel overlap within the ROI was defined as co-localized.

The Komolgorov-Smirnoff test was used to determine whether values were parametrically distributed. Student’s t test was used for parametrically distributed values; the Mann-Whitney U test or Wilcoxon matched-pairs test was used for non-parametrically distributed values; the Pearson correlation test was used to compare correlated values; and the F test was used to compare linear regressions (GraphPad Prism).
Figure 1. **Evoked release and total vesicle pool size are heterogeneous in short axon segments.**

**A. Top:** Representative images of Synaptophysin-pHluorin+ synaptic vesicle clusters (green) along an local axon segment from a 14 DIV neuron after live imaging followed by fixation and immunostaining for MAP2 (red). All of the terminals analyzed in this segment were apposed to neuronal dendrites from multiple postsynaptic neurons. Arrows and arrowheads indicate all presynaptic terminals in this axon segment (defined using criteria described in Materials and Methods). Arrows indicate releasing terminals, arrowheads indicate non-releasing terminals. The numbers 1-4 indicate the releasing or
non-releasing presynaptic terminals shown in the middle pseudo-colored time lapse image series. The cluster labeled with an asterisk was trafficked to that location in the interval between when the NH$_4$Cl image was taken and fixation and was excluded in the analysis. Scale bar = 10 µm.

**Middle:** Pseudo-colored time lapse images of presynaptic terminals 1-4 taken before, during (black line, 3.5 sec. duration) and after electrical stimulation. The ΔF of evoked vesicle exocytosis (evoked release) at terminals 1-4 are measured as fluorescence by the end of stimulation – baseline fluorescence (also refer to Methods and Materials). The color scale is in arbitrary fluorescence units (AFU).

**Bottom:** The maximal ΔF of Synaptophysin-pHluorin+ synaptic vesicle cultures is assessed in NH$_4$Cl saline at the end of stimulation. The total vesicle pool size of presynaptic terminals 1-4 is estimated by measuring maximal ΔF within each terminal.

**B.** Total fluorescence of individual presynaptic terminals plotted against time to show changes in fluorescence (ΔF) during stimulation. The stimulation duration is indicated with the black line below the x axis from 3.5 to 7 sec. The ΔFs of presynaptic terminals 1-4 are heterogeneous, varying over a more than 10 fold range; e.g., the ΔF for terminal 2 was 30.9 x 10$^4$ AFU, whereas ΔF for terminal 3 was 3.4 x 10$^4$ AFU. Fluorescence returned to baseline within 1-2 minutes after stimulation ceased, as Synaptophysin-pHluorin+ vesicles were endocytosed and reacidified (τ = 16.1 ± 0.3 sec., 11 presynaptic terminals, comparable to (Sankaranarayanan and Ryan, 2000)).
C. The average pair ratio of evoked release between two consecutive trials of stimulation at the same terminals was significantly less than the average pair ratio of two random presynaptic terminals in a short axon segment (49 terminals, 6 neurons; Mann-Whitney U test, p = 0.004). Error bars indicate SEM.

D. The average pair ratios of ΔF in NH₄Cl, reflecting total vesicle pool size, between two consecutive trials of the same terminals was significantly lower than the average pair ratio between two random presynaptic terminals in a short axon segment (49 terminals, 6 neurons; Mann-Whitney U test, p = 0.004). Error bars indicate SEM.
Figure 2. Heterogeneity in total vesicle pool size correlates with heterogeneity in evoked release in short axon segments, independent of stimulation frequency.

Local axon segments are stimulated with 70 pulses delivered at 1 Hz, 5 Hz, 20 Hz, 100 Hz for 70 sec., 12 sec., 3.5 sec., and 0.7 sec., respectively. Baflomycin was added for the 1 Hz measurement (see Methods). Evoked release was measured at each stimulation frequency; at the end of the experiment, total vesicle pool size was measured.

A. Plots of scaling between total vesicle pool sizes and evoked release of the same set terminals across different simulation frequencies (correlation coefficient for 1,
5, 20, 100 Hz: r = 0.92, 0.95, 0.84, 0.83, respectively; Pearson correlation test, p = 0.001, <0.001, 0.008, 0.01, respectively; similar for 4 of 4 neurons). Each symbol indicates individual terminal across different stimulation frequencies.

B. Plot of average evoked release of the representative terminals as 70 pulses delivered at different frequencies. The average is fitted by a one exponential decay ($R^2 = 0.99$; one exponential decay regression analysis, p = 0.0004).

C. Random pair ratios of evoked release of terminals within short axon segments at different stimulation frequencies were similarly heterogeneous (28 terminals, 4 neurons; one-way ANOVA, p = 0.13).
Figure 3. Functional properties among presynaptic terminals contacting different dendrites of the same or different postsynaptic neurons are heterogeneous.

Local axon segments are stimulated with 70 pulses delivered at 20 Hz to measure evoked release, followed by NH$_4$Cl saline perfusion to measure total vesicle pool size. Posthoc immunostaining was performed with anti-MAP2 to reveal postsynaptic dendrites/neurons.

A. Representative presynaptic terminals’ evoked release and total vesicle pool sizes. On the left image panel, the top image is posthoc immunostaining with MAP2 showing that terminal 1 and 2 overlap with the same dendrite, terminal 3 overlaps with a different dendrite from the same neuron. The middle image is a pseudocolor image
showing evoked $\Delta F$ by the end of the 20 Hz stimulation train. The bottom image is a pseudocolor image showing $\Delta F$ in NH$_4$Cl. The right panel traces are showing evoked $\Delta F$ over time of terminals 1, 2, 3. Scale bar = 10 µm.

**B.** On the left image panel, the top image shows terminal 1 and 2 overlap with different dendrites from different neuron. The middle image is a pseudocolor image showing evoked $\Delta F$ at the end of the 20 Hz stimulation train. The bottom image is a pseudocolor image showing $\Delta F$ in NH4Cl. Right panel, evoked $\Delta F$ over time of terminal 1, 2. Scale bar = 10 µm.

**C.** The random pair ratios of evoked release were significantly higher than the two repeats control ratios for terminals innervating two different dendrites of the same neuron (8 neurons, 40 terminal pairs; Mann-Whitney U test, $p = 0.04$), for terminals innervating dendrites from two different postsynaptic neurons (5 neurons, 29 terminal pairs; $p = 0.002$), and for random pairs of terminals (8 neurons, 40 terminal pairs; $p = 0.001$), but not for terminals innervating the same dendrite (7 neurons, 33 terminal pairs; $p = 0.15$).

**D.** The random pair ratios of total vesicle pool size were significantly higher than the two repeats control ratios for terminals innervating dendrites from two different neurons ($p = 0.02$) and for random pairs of terminals ($p = 0.004$), but not for terminals innervating the same dendrite ($p = 0.14$) or two different dendrites of the same neuron ($p = 0.25$).
Figure 4. Presynaptic heterogeneity across different synaptic configurations is correlated with the amount of postsynaptic NMDA receptors and PSD-95.

Local axon segments were stimulated with 70 pulses delivered at 20 Hz to measure evoked release, followed by NH₄Cl saline perfusion to measure total vesicle pool sizes. Posthoc immunostaining was performed with anti-PSD95, NR1, GluR1 or MAP2 antibodies to reveal postsynaptic specializations or dendrites.

A. Representative images of evoked ΔF (left), total vesicle pool size (left middle), posthoc immunostaining of NR1 clusters (right middle, green channel is
pHluorin, red channel is NR1), and colocalized pixels between NR1 and Synaptophysin-
pHluorin (right). Arrowhead indicates the terminals plotted in B and C. Scale bar = 5 µm.

**B.** Correlation plot of NR1 intensity and evoked ∆F of terminals in Fig. 4A (correlation coefficient r = 0.99, Pearson correlation test, p = 0.006).

**C.** Correlation plot of NR1 intensity and total vesicle pool size of terminals in Fig. 4A (correlation coefficient r = 0.97, Pearson correlation test, p = 0.03).

**D.** The average correlation coefficient between evoked release and NR1 (9 segments; r = 0.8 ± 0.04) or PSD-95 (11 segments; r = 0.80 ± 0.04) was significantly different from the MAP2 correlation coefficient (asterisk; Mann-Whitney U test, NR1 p = 0.003, PSD-95, p = 0.003), but not that of GluR1 (r = 0.6 ± 0.1; p = 0.27). Evoked release was not significantly correlated with the amount of MAP2 for terminals in 7 / 8 axon segments measured (r = 0.4 ± 0.1).

**E.** The average correlation coefficient between total vesicle pool size and NR1 (r = 0.8 ± 0.03) or PSD-95 (r = 0.80 ± 0.04) were significantly different from the MAP2 correlation coefficient (asterisk; Mann-Whitney U test, NR1 p = 0.008, PSD95 p = 0.01), but not that of GluR1 (r = 0.7 ± 0.1; p= 0.17). Total vesicle pool size was not significantly correlated with the amount of MAP2 for terminals in 6 / 8 segments measured (r = 0.5 ± 0.1).

**F.** Left: Posthoc immunostaining images of MAP2 (blue) and NR1 (red) of several terminals from a segment of axon contacting different dendrites of one single
postsynaptic neuron. Arrow heads indicate presynaptic terminals that are overlapping with MAP2. **Middle:** Higher magnification images of numbered presynaptic terminals 1-5. **Right:** Masks of Synaptophysin-pHluorin (green) and NR1 (red) and their overlapped pixels (yellow). Scale bar = 10 µm.

**G.** Linear correlation between total vesicle pool size and amount of NR1 for terminals 1-5 shown in 4F (correlation coefficient r = 0.83; Pearson correlation test, p = 0.08).

**H.** Correlation between total vesicle pool size and amount of NR1 among terminals from an axon contacting two different postsynaptic neurons (red squares, black squares; (correlation coefficient r = 0.75; Pearson correlation test, p = 0.003).

**I.** Average values ± SEM of total vesicle pool size and amount of NR1 from 6 axons contacting two different neurons (red and black symbols represent two postsynaptic neurons contacted by the same axon). Some axons only made one or two terminals on a postsynaptic neuron, thus there are no error bars. The squares represent the average values of the terminals shown in Fig. 4H.
Figure 5. Distal terminals are functionally more heterogeneous than proximal terminals.

A. Neurolucida tracing of a neuron transfected with Synaptophysin-pHluorin and mCherry (not shown) to enable long distance axon tracking. Colors indicate axon segments that were stimulated and imaged. Images taken after stimulation show the distribution of releasing presynaptic terminals (arrows). Scale bar = 100 µm.

B. Evoked release of individual presynaptic terminals, color coded by segment.

C. The average random pair ratios of evoked release for terminals in color coded axon segments in A (range 2.9 ± 0.5 to 4.4 ± 0.5; average of all 5 segments from this
neuron, white bar), as well as 22 / 24 axon segments from 6 neurons (average of terminals in all segments, 3.4 ± 0.2), is significantly higher than the two repeats control (indicated by dashed line, 1.9 ± 0.2; Mann-Whitney U test, p < 0.05). The random pair ratios of evoked release for terminals in color coded segments and all segments from all axons are not significantly different (one-way ANOVA, p = 0.66).

D. Total vesicle pool size of individual presynaptic terminals, color coded by segment.

E. The average random pair ratios of total vesicle pool size for terminals in short axon segments in A (range 3.3 ± 0.6 to 5.0 ± 0.9; average of all 5 segments from this neuron, white bar), as well as 18 / 24 axon segments from 6 neurons (average of terminals in all segments, 3.2 ± 0.2), is significantly higher than the two repeats control (indicated by dashed line, 1.9 ± 0.1; Mann-Whitney U test, p < 0.05). The random pair ratios of total vesicle pool size for terminals terminals in color coded segments and all segments from all axons are not significantly different (one-way ANOVA, p = 0.92).

F. Heterogeneity in evoked release, assessed by random pair ratios, was significantly greater distally compared to proximally (distal, 3.7± 0.5; proximal, 2.6 ± 0.3; 6 neurons; paired Student’s t-test, p = 0.04).

G. Heterogeneity in total vesicle pool size, assessed by random pair rations, was significantly greater distally compared to proximally (distal, 3.7± 0.5; proximal, 2.5 ± 0.2; paired Student’s t-test, p = 0.02).
H. Relationship between coefficient of variance (CV) of evoked release and total vesicle pool size of presynaptic terminals from the most proximal (white circles; correlation coefficient, $r = 0.65$, Pearson correlation test, $p < 0.05$) and the most distal (black circles; correlation coefficient, $r = 0.82$; Pearson correlation test, $p < 0.05$) segments from 13 neurons (slope = $0.96 \pm 0.05$; Pearson correlation test, $p < 0.0001$).
Figure 6. Evoked release and release fraction are higher in distal segments of individual axon arbors.

A. Average evoked release in proximal axon segments (black dots; $1.3 \pm 0.2 \times 10^4$ AFU) is slightly but significantly smaller than distal segments ($1.9 \pm 0.3 \times 10^4$ AFU; 16 neurons; Wilcoxon matched-pairs test, $p < 0.001$). The average evoked release in proximal compared to distal segments is indicated by red dots.

B. Average total vesicle pool size in proximal axon segments (black dots; $15.2 \pm 3.9 \times 10^4$ AFU) of individual neurons is not significantly different from that in distal segments ($10.1 \pm 2.0 \times 10^4$ AFU; 16 neurons; Wilcoxon matched-pairs test, $p = 0.17$). The average of total vesicle pool size in proximal compared to distal segments is indicated by red dots.
C. Average release fraction in proximal axon segments (black dots; 0.15 ± 0.02) is significantly smaller than in distal segments (0.30 ± 0.04; 16 neurons; Wilcoxon matched-pairs test, p = 0.003). The average release fraction of proximal compared to distal segments is indicated by red dots.

D. Relationship between evoked release and total vesicle pool size of presynaptic terminals from axon segments 1-5 from the representative neuron shown in Fig. 5A, color coded as in 5A, C, E. For each segment, correlation coefficient and Pearson correlation test: Segment I, r = 0.67, p = 0.15; II, r = 0.86, p = 0.006; III, r = 0.81, p = 0.002; IV, N/A; V, r = 0.88, p = 0.004. In this example, as for all axons measured (24 segments, 6 neurons), some segments have a similar regression slope, indicative of similar release fraction (segments I and II, segments III and IV), while release fraction is significantly different among other segments (one way ANOVA, p = 0.007).

E. Relationship between evoked release and total vesicle pool size of all terminals in the most proximal segments (gray) and the most distal (black) segments from 13 neurons (evoked release, correlation coefficient r = 0.90, Spearman correlation test p < 0.0001; total vesicle pool size, correlation coefficient r = 0.85; Spearman correlation test p < 0.0001). The linear regression lines are significantly different (average proximal slope = 0.05 ± 0.005, distal slope = 0.14 ± 0.009; F test, p < 0.001), indicating that distal terminals have a significantly higher release fraction compared to proximal terminals.
A simple model of vesicle exocytosis in hippocampal nerve terminals was capable of recapitulating the experimental data and provides some insight in the possible mechanisms underlying spatial differences in release properties.

A. The number of vesicles releasing neurotransmitter (ER, evoked release) was given by the number of vesicles in the readily releasable pool (RRP), the release probability of individual vesicles (Pv) and the number of times the axon was stimulated (N). The size of the RRP was proportional to total vesicle pool size (TVP), defined as the sum of the RRP, the reserve pool, and the resting pool. The fraction of vesicles released (RF) was defined as the ratio of ER to TVP. Thus, \( ER = RRP \times Pv \times N; \) \( RRP = k \times TVP; \) and \( RF = ER / TVP, \) where \( k = RRP / TVP. \) Based on an anatomical model of...
vesicle distribution in terminals (Sudhof, 2000), TVP was set at 200 vesicles and the RRP to 5 vesicles which yields an initial value of k of 0.025. Following substitution and rearrangement, individual vesicle release probability is \( P_v = \frac{RF}{k \times N} \); the measured value of RF, 0.15 for proximal segments, can be used to determine an initial value for \( P_v \) in the model.

**B.** TVP was systematically varied over a 10-fold range to generate heterogeneity in TVP and yield values of ER consistent with the experimental data from proximal segments (black line). The measured increase in RF for distal segments from 0.15 to 0.30 could be achieved in the model by either doubling the individual release probability (\( P_v \), red line) or increasing the size of the RRP independent of the size of the TVP, i.e. doubling k (blue line). This holds true for any combination of \( P_v \) and RRP such that \( P_v \times k = \frac{RF}{N} \). These model findings are consistent with the notion that differences in ER within short axon segments (either proximal or distal) can be attributed to the changes in terminal size, or at least TVP size, whereas proximal / distal differences arise from either changes in RRP size and or \( P_v \).
Cellular and synaptic mechanisms of anti-NMDA receptor encephalitis

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Contributions

E.G.H and X.P. designed and carried out most of the experiments, contributing equally; J.D. and R.B.-G. helped design experiments; A.J.G. and D.R.L. provided the Western blot analyses in part of Fig.1 and Fig.3; M.L. prepared IgGs and Fab fragments; L.Z. implanted Alzet mini-pump in rats and carried out IgG infusion; R.T. helped with the experiments in Fig. 4; T.D.P. advised on the electrophysiology analyses; J.D. provided patients’ reagents; E.G.H. and X.P. analyzed the data, wrote the paper and prepared figures; E.G.H, X.P., J.D. and R.B.-G. edited the paper.
Abstract

We recently described a severe, potentially lethal, but treatment responsive encephalitis that associates with autoantibodies to the N-methyl-D-aspartate receptor (NMDAR) and results in behavioral symptoms similar to those obtained with models of genetic or pharmacologic attenuation of NMDAR function. Here we demonstrate that patients’ NMDAR antibodies cause a selective and reversible decrease in NMDAR surface density and synaptic localization that correlates with patients’ antibody titers. The mechanism of this decrease is selective antibody mediated capping and internalization of surface NMDARs, as Fab fragments prepared from patients’ antibodies did not decrease surface receptor density, but subsequent crosslinking with anti-Fab antibodies recapitulated the decrease caused by intact patient NMDAR antibodies. Moreover, whole-cell patch clamp recordings of miniature excitatory postsynaptic currents in cultured rat hippocampal neurons showed that patients’ antibodies specifically decreased synaptic NMDAR-mediated currents, without affecting AMPA receptor-mediated currents. In contrast to these profound effects on NMDARs, patients’ antibodies did not alter the localization or expression of other glutamate receptors or synaptic proteins, number of synapses, dendritic spines, dendritic complexity, or cell survival. In addition, NMDAR density was dramatically reduced in the hippocampus of female Lewis rats infused with patients’ antibodies, similar to the decrease observed in the hippocampus of autopsied patients. These studies establish the cellular mechanisms through which antibodies of patients with anti-NMDAR encephalitis cause a specific,
titer-dependent, and reversible loss of NMDARs. The loss of this subtype of glutamate receptors eliminates NMDAR-mediated synaptic function resulting in the learning, memory and other behavioral deficits observed in patients with anti-NMDAR encephalitis.
Introduction

Synaptic plasticity is thought to underlie mechanisms of memory, learning, and cognition. Central to these neurological functions is the proper synaptic localization and trafficking of the excitatory glutamate NMDA and AMPA receptors (Lau and Zukin, 2007; Shepherd and Huganir, 2007). The roles of these receptors at the synaptic and cellular levels have been established through animal models in which the receptors have been genetically or pharmacologically altered (Jentsch and Roth, 1999; Mouri et al., 2007). In humans the role of these receptors in memory, learning, cognition and psychosis comes from more indirect approaches, such as pharmacological trials (e.g., NMDAR antagonists causing psychosis) (Gunduz-Bruce, 2009), and analysis of brain tissue from patients with Alzheimer’s disease or schizophrenia in which several molecular pathways causing a downstream alteration of glutamate receptors are affected (Snyder et al., 2005a; Hahn et al., 2006). We recently identified a disorder in which the extracellular domain of the NR1 subunit of the NMDAR is directly targeted by autoantibodies (Dalmau et al., 2007; Dalmau et al., 2008). Patients develop prominent psychiatric and behavioral symptoms, rapid memory loss, seizures, abnormal movements (dyskinesias), hypoventilation, and autonomic instability (Dalmau et al., 2007; Dalmau et al., 2008; Iizuka et al., 2008). In two series comprising 181 cases (Dalmau et al., 2008; Florance, 2009), there was a strong female predominance (ratio 8.5:1.5) and the median age of the patients was 19 years (23 months-75 years; 40% children). In 55% of the adults (less frequently in children), the disorder appears to be triggered by the presence of
a tumor, mostly an ovarian teratoma that contains nervous system tissue and expresses NMDARs. Despite the severity of the symptoms, 75% of patients recover after receiving immunotherapy and, when appropriate, tumor removal, and 25% are left with memory, cognitive and motor deficits, or, rarely, die of the disorder. The autoantibodies are present in patients’ serum and cerebrospinal fluid (CSF), the latter usually showing intrathecal synthesis and high antibody concentration (Dalmau et al., 2008; Florance, 2009). All patients’ antibodies recognize the N-terminal extracellular domain of NR1 (amino-acid residues 25-380), suggesting an antibody-mediated pathogenesis (Dalmau et al., 2008). While patients’ antibodies can cause a decrease in NMDAR cluster density, the underlying mechanisms remain poorly understood (Dalmau et al., 2008). Here we report in vitro and in vivo studies that indicate the cellular mechanisms by which patients’ antibodies lead to a reduction in surface and synaptic NMDAR density and function, likely underlying the learning, memory and other behavioral deficits observed in patients with anti-NMDAR encephalitis.
Results

Patients’ antibodies reduce surface NMDA receptor clusters and protein in a titer dependent fashion

Hippocampal neurons were cultured for 1 day with CSF or purified IgG containing anti-NR1 antibodies from patients with anti-NMDAR encephalitis (see Supplemental Table 1), followed by immunohistochemical and Western blot analyses of surface and total NR1 protein. Patients’ antibodies significantly decreased NR1 or NMDAR surface and total cluster density in a titer dependent fashion, compared to CSF or IgG from control patients (Fig. 1a, c). Similar findings were obtained after treating the neurons for 3 or 7 days with patients’ antibodies (Supplemental Fig. 3).

A significant titer-dependent decrease in surface and total NR1 protein was also observed with Western blot analyses (Fig. 1b, d). Moreover, Western blot analyses of the effect of patients’ antibodies on NR2 subunits (which assemble with NR1 to form NMDARs) showed that patients’ antibodies significantly decreased surface and total NR2A and NR2B proteins in a titer dependent fashion (Supplemental Fig. 4).

To determine whether the effects of patients’ antibodies correlate with the change of titers during the course of the disease, hippocampal neurons were cultured with CSF samples obtained at two different time points of the disease of two patients. The initial CSF was obtained at the time of symptom presentation and the second sample during symptom improvement in one patient and during symptom worsening in the other. The CSF obtained at symptom presentation had a higher NR1 antibody titer than the CSF
obtained during symptom improvement of the first patient; in contrast, the CSF obtained during symptom worsening had a higher antibody titer than the CSF obtained at symptom presentation of the second patient. In both cases the CSF with higher NR1 antibody titer decreased NMDAR surface and total cluster density (or total NMDAR protein measured by Western blot) (Fig. 1e, f) to a greater extent than the CSF with the lower titer. Together, these results show that NR1 antibodies from patients with anti-NMDAR encephalitis decrease NMDAR surface cluster density and protein in a titer-dependent manner and that the effects of the antibodies vary with the change of titers during the course of the disease.

Patients’ antibodies reversibly reduce synaptic NMDA receptor clusters without affecting the number of synapses and other synaptic components

Because patient antibodies decreased overall NMDAR surface cluster density and protein, we determined whether the antibodies also affected NMDAR synaptic localization, the number of synapses, and other synaptic components. Hippocampal neurons were cultured with CSF or purified IgG for 3 or 7 days, followed by immunostaining or Western blot analysis of NR1 and synaptic components such as presynaptic VGlut, postsynaptic PSD-95, AMPA receptor subunits GluR1 and GluR2, and GABA receptors.

While the overall structural integrity of excitatory neurons and synapses was not affected (see below), patients’ antibodies dramatically reduced the synaptic localization
of NMDAR clusters in a titer-dependent fashion compared to controls (Fig. 2a, c; see also Supplemental Fig. 5), consistent with the overall decrease in surface NMDAR cluster density (Fig. 1). To determine whether the antibody-mediated decrease in NMDAR synaptic localization is reversible, patient antibodies were removed from the culture medium after 3 days of treatment and neurons were cultured for 4 additional days. The density of synaptically localized NMDAR clusters returned to baseline levels 4 days after patient antibodies were removed (Fig. 2a, c). These results show that patients’ antibodies cause a specific loss of NMDARs from excitatory synapses and that this loss is reversed after antibody removal.

Patients’ antibodies did not affect the number of excitatory synapses compared to controls (Fig. 2a, b). Moreover, patients’ antibodies did not affect the density of postsynaptic PSD-95, GluR1, GluR2 receptor clusters, or the surface or total amount of these proteins or the amount of surface GABA receptor protein (Fig. 3), dendritic branching, dendritic spine density, or Bassoon cluster density (Fig. 4). In addition, patients’ antibodies did not affect cell survival (Fig. 4h,i). The effects of patients’ antibodies on NMDAR cluster density were not mediated by complement, because purified patient IgG from serum or heat-inactivated patient CSF decreased NMDAR cluster density and localization to a similar extent as non-heat inactivated patient CSF (Supplemental Fig. 1).
These results show that patients’ antibodies specifically affect NMDAR without any demonstrable effect on AMPA or GABA receptors, other synaptic proteins, the number of excitatory synapses, and neuronal morphology or viability.

**Patients’ antibodies selectively decrease synaptic NMDAR currents**

We next assessed the effects of patient antibodies on NMDAR function using whole-cell patch recordings of miniature excitatory postsynaptic currents (mEPSCs), which consist of a fast AMPA receptor-mediated current and a slow NMDAR current. Neurons were treated for 1 day with patient or control CSF and spontaneous mEPSCs were recorded at -70 mV in a 0 Mg$^{2+}$ extracellular solution to unmask the synaptic NMDAR-mediated component. TTX was used to block action potentials, CNQX was used to block AMPA receptor mediated mEPSCs, APV was used to block NMDAR-mediated mEPSCs, and picrotoxin was used to block GABA receptor-mediated miniature inhibitory postsynaptic currents (Fig. 5a).

In neurons treated for 1 day with CSF from control patients, CNQX blocked large, fast AMPA receptor-mediated currents, revealing small, slower NMDAR-mediated currents that were completely blocked by APV (Fig. 5a, left). In contrast, in neurons treated for 1 day with patient CSF, CNQX blocked all mEPSCs, and no further reduction was observed after APV (Fig. 5a, right). This result shows that patient antibody treatment decreases NMDAR-mediated current.
To quantify the reduction in synaptic NMDAR-mediated currents, currents were examined before and after APV application. In neurons treated for 1 day with CSF from control patients, APV reduced or abolished the late, slow NMDAR-mediated component of the mEPSC (Fig. 5b, left; 5c, left). In contrast, in neurons treated for 1 day with patient CSF, APV application did not further reduce the NMDAR-mediated component of the mEPSC (Fig. 5b, middle; 5c, left). The difference between the 0 Mg$^{2+}$ and the 0 Mg$^{2+}$ + APV traces shows that neurons treated for 1 day with patient CSF have less NMDAR-mediated synaptic current than neurons treated with control CSF (Fig. 5b, right; 5c, left). No difference was observed in the peak AMPA receptor-mediated component of the mEPSC (Fig. 5c, right). Patient antibody treatment did not affect mEPSC frequency or amplitude (Supplemental Fig. 6), suggesting that presynaptic release probability is unaltered. These data are also consistent with structural analyses that showed that patients’ antibodies do not affect the number of excitatory synapses or the number of postsynaptic sites containing AMPA receptors. These results show that patients’ antibodies specifically decrease synaptic NMDAR-mediated currents and do not affect AMPA receptor mediated currents, consistent with the specific loss of surface, synaptically localized NMDAR clusters.

**Patients’ antibodies crosslink and internalize NMDA receptors**

We next determined the mechanism by which patients’ antibodies decrease surface NMDAR cluster density and protein. The Fc IgG domain was enzymatically removed from patients’ antibodies to generate Fab fragments. These Fab fragments, like
intact patient IgG, bound to surface NR1 clusters identified with commercial anti-NR1 immunostaining (Supplemental Fig. 2). Neurons treated for 1 day with patients’ Fab fragments had the same NMDAR cluster density and surface protein as neurons treated with control IgG (Fig. 6a, b). In contrast, neurons treated for 1 day with patients’ Fab fragments and anti-Fab secondary antibodies (linking two Fab fragments in a conformation similar to unmodified patients’ antibodies) had significantly lower NMDAR cluster density and surface protein as compared to neurons treated with control IgG (Fig. 6a, b). These results show that patients’ antibodies mediate the loss of surface NMDARs in part by binding to, capping and crosslinking NMDARs, resulting in their internalization (Fig. 6c).

**Patients’ antibodies decrease NMDA receptor cluster density and protein in rodent and human hippocampus *in vivo***

Our results show that, *in vitro*, patients’ anti-NR1 antibodies lead to a selective loss of surface NMDAR clusters and their function, without loss of other synaptic components or neuron viability. To determine the effects of patients’ antibodies *in vivo*, CSF from patients with high titers of NR1 antibodies, or control CSF from individuals without NR1 antibodies, was infused directly into the hippocampus of adult rats for two weeks, followed by immunostaining for human IgG to examine the diffusion and deposition of patients’ antibodies, immunostaining and Western blot analysis of NMDARs and other synaptic components to assess the effects of patients antibodies, and
analysis of cell death using the TUNEL assay. Patients’ antibodies colocalized with NMDAR clusters in vivo as in vitro (Supplemental Fig. 7 a). Moreover, IgG from infused patient CSF, but not from control CSF, was found bound to rat hippocampus in a predictable pattern that was dependent on NMDAR density (e.g., high density in proximal dendrites of dentate gyrus, Supplemental Fig. 7 b). This pattern was similar to the direct immunostaining of bound IgG reported in the autopsy of two patients with anti-NMDAR encephalitis (Dalmau et al., 2007). Moreover, in regions where human IgG was deposited, there was a significant decrease in NMDAR cluster density and intensity of NR1 immunostaining without affecting the number of synapses, the density of other synaptic components (Fig. 7a-e; Supplemental Fig. 7 b) or cell death (Supplemental Fig. 7 c). The magnitude of the effects of each patient’s CSF was significantly correlated with the titer of NR1 antibodies infused into rat brains (Fig. 7b), as in in vitro studies (Fig. 1). Furthermore, the total amount of NR1 protein was reduced in rodent hippocampus infused with patients’ CSF compared to the contralateral, uninfused hippocampus (Fig. 7c).

To investigate whether NMDAR cluster density is reduced in the brains of patients with anti-NMDAR antibodies, paraffin-embedded sections of the hippocampus of two patients with anti-NMDAR encephalitis and the hippocampus of three age-matched, anti-NR1 negative, neurologically normal individuals were immunostained with commercial anti-NR1 antibodies. The intensity of NMDAR immunostaining was significantly decreased in patients’ hippocampus compared to controls (Fig. 7f-h).
Moreover, deposits of human IgG, but not complement, were identified in some of the regions with reduced NMDAR clusters (data not shown). These data show that patient anti-NMDAR antibodies reduce NMDAR clusters in rodent neurons *in vitro* and *in vivo* as well as in the brain of patients with the disorder.
Discussion

Anti-NMDAR encephalitis is a recently described disorder that is associated with antibodies against the NR1 subunit of the NMDAR and results in a well defined set of symptoms. Our previous studies noted that the resulting syndrome resembled the phenotype of animals in which the NMDAR function had been attenuated pharmacologically or genetically, suggesting that patients’ antibodies decreased NMDAR levels (Dalmau et al., 2008). We now demonstrate using *in vitro* and *in vivo* studies that patients’ antibodies decrease the surface density and synaptic localization of NMDAR clusters via antibody mediated capping and internalization, independent of the presence of complement, and without affecting other synaptic proteins, AMPA receptors or synapse density. The magnitude of these changes depends on antibody titer, and the effects are reversible when the antibody titer is reduced. Moreover, patients’ NR1 antibodies decrease NMDAR, but not AMPA receptor mediated synaptic currents. Thus the selective loss of surface clusters abolishes NMDAR mediated synaptic currents. These findings indicate that NR1 antibodies from patients with anti-NMDAR encephalitis decrease glutamatergic synaptic function without a substantial loss of synapses.

This reversible loss of NMDARs, and the resulting synaptic dysfunction, may underlie the deficits of memory, behavior and cognition that are hallmarks of anti-NMDAR encephalitis (Sansing et al., 2007; Dalmau et al., 2008; Iizuka et al., 2008). Indeed, a remarkable feature of this disorder is the frequent reversibility of symptoms, even when these are severe and protracted (Iizuka et al., 2008; Ishiura et al., 2008).
Previous studies with 100 patients showed a correlation between clinical outcome and antibody titers, which are often higher in CSF than serum due to intrathecal antibody synthesis (Dalmau et al., 2008; Seki et al., 2008). The work we present here demonstrates that the effect of patients’ CSF on surface NMDARs correlates with the antibody titers and is coupled to changes in antibody titers and symptom severity during the course of the disease. Analysis of the hippocampus of two patients who died of this disorder showed a substantial decrease of NMDAR levels compared with the hippocampus of three age-matched, neurologically normal individuals. This decrease of NMDARs was comparable to that observed in rats infused with patients’ antibodies. Moreover, we previously reported that patients’ hippocampus showed deposits of IgG and absence of complement (Dalmau et al., 2007), consistent with the complement-independent antibody effects demonstrated in *in vitro* studies.

In the peripheral nervous system, immune-mediated disruption of synaptic structure and function results in well known disorders of neuromuscular transmission such as myasthenia gravis and the Lambert-Eaton syndrome (Sanders, 2002; Conti-Fine et al., 2006). Anti-NMDAR encephalitis provides a new model of central nervous system synaptic autoimmunity, antigenically different but mechanistically similar to the Lambert-Eaton syndrome in which autoantibodies, but not monovalent Fab fragments, crosslink and internalize voltage-gated calcium channels, without complement activation (Nagel et al., 1988a). Both disorders may occur as paraneoplastic manifestation of a tumor that expresses neuronal proteins (e.g., small-cell lung cancer in Lambert-Eaton
syndrome)(Titulaer et al., 2008) or contains ectopic nervous tissue (e.g., teratoma in anti-NMDAR encephalitis)(Dalmau et al., 2007). Moreover, in both disorders the immunological trigger of cases without tumor association is unknown, although a genetic predisposition to autoimmunity has been demonstrated or suggested(Wirtz et al., 2004; Wirtz et al., 2005; Florance, 2009). Although both disorders respond to immunotherapy and when appropriate tumor removal, the response of anti-NMDAR encephalitis is slower and less predictable, particularly in cases with delayed diagnosis or without a detectable tumor(Dalmau et al., 2008; Florance, 2009). These patients usually have persistently high CSF antibody titers, despite the effectiveness of plasma exchange or IVIg in reducing serum antibody titers. In these cases, symptoms frequently respond to cyclophosphamide, which crosses the blood-brain barrier, or rituximab, which depletes memory B-cells(Sansing et al., 2007; Dalmau et al., 2008; Ishiura et al., 2008; Florance, 2009). As postulated in other disorders, these cells are able to cross the blood-brain-barrier, and are believed to undergo re-stimulation, antigen-driven affinity maturation, clonal expansion, and differentiation into antibody-secreting plasma cells(Hauser et al., 2008).

NMDAR dysfunction has been implicated in several other cognitive disorders, including schizophrenia (Olney and Farber, 1995; Gunduz-Bruce, 2009). Studies investigating the effects of phencyclidine and ketamine (noncompetitive antagonists of NMDARs) in human subjects show these drugs induce behaviors similar to the positive and negative symptoms of schizophrenia, along with repetitive orofacial and limb
movements, autonomic instability, and seizures (Luby et al., 1959; Bailey, 1978; Castellani et al., 1982; Krystal et al., 1994b; Weiner et al., 2000). In rodents, drugs that antagonize NMDAR function induce cataleptic freeze, and locomotor and stereotype behaviors, consistent with schizophrenia-like manifestations (Haggerty et al., 1984; Jentsch and Roth, 1999; Chartoff et al., 2005; Mouri et al., 2007). Furthermore, mice with decreased expression of NR1 have similar behavioral deficits, while mice lacking NR1 develop breathing problems and die in the perinatal period (Mohn et al., 1999b). Interestingly, most patients with anti-NMDAR encephalitis present with acute schizophrenia-like symptoms and are admitted to psychiatric institutions before they develop catatonia, catalepsy, stereotyped movement disorders, and frequent autonomic instability and hypoventilation. The striking similarity between these phenotypes, the effect of patients’ antibodies resulting in a dramatic decrease of surface NMDAR clusters and function, and the reduced levels of NMDARs in autopsied patients, support an antibody-mediated pathogenesis of anti-NMDAR encephalitis. The psychosis and cognitive and behavioral deficits in patients with anti-NMDAR encephalitis most likely result from NMDAR hypofunction, directly and indirectly affecting synapse and circuit structure and function in regions that bind NR1 autoantibodies. Thus the findings we report here also support the hypothesis that NMDAR hypofunction underlies many manifestations of schizophrenia. Future studies will focus on the circuit-level dysfunction caused by patients’ antibodies in hippocampus and other brain regions in order to begin
to connect synaptic and circuit dysfunction with the behavioral abnormalities that are hallmarks of this disorder.
Materials and Methods

Patients, NR1 antibodies, titers, and controls

Cerebrospinal fluid and serum were obtained from randomly selected patients with anti-NMDAR encephalitis (Supplemental Table 1) among a series of 320 cases. All patients had well characterized clinical manifestations of anti-NMDAR encephalitis, including at least 4 of the following features: prominent psychiatric symptoms, decreased level of consciousness, seizures, dyskinesias, autonomic instability, or hypoventilation. Antibodies to extracellular epitopes of the NR1 subunit of the NMDAR were demonstrated using three different assays, as reported (Dalmau et al., 2008): immunohistochemistry with rat and human brain, immunostaining of live, non-permeabilized cultures of rat hippocampal neurons, and immunolabeling of HEK293 cells transfected with NR1 or NR1 and NR2 (forming NR1/2 heteromers). We previously reported that patients’ NMDA receptor antibodies are IgG1 and IgG3, but not IgM (Tuzun et al., 2009); therefore we will refer to purified antibodies from patients’ serum as purified IgG. CSF from patients with high antibody titer were diluted so that the final titer used in experiments was within the range of undiluted CSF of many patients with this disorder (Dalmau et al., 2008).

Control serum or CSF samples were obtained from normal individuals and patients undergoing CSF analysis for a variety of disorders not associated with antibodies to the NMDAR; samples were randomly selected from 1,500 cases negative for NR1 antibodies applying similar test and criteria as above.
Antibody titers from patients and controls were determined by ELISA (Dalmau et al., 2008).

*Preparation of patient and control CSF and IgG*

Patient or control cerebrospinal fluid (CSF) and serum were collected, filtered, and kept frozen until use. CSF from individual patients with high NMDAR antibody titer was diluted 1:15-60 to treat neurons *in vitro*, and used undiluted for *in vivo* experiments. In some experiments, patient IgG antibodies were purified from serum with protein A/G sepharose columns and used to treat neurons. To prepare patient and control IgG, 2 ml of serum were incubated with a 1 ml bio-spin chromatography column (Bio-Rad) of protein A/G sepharose beads (50:50) for 30 min. on an orbital shaker at 4 °C. After 3 washes with phosphate buffered saline (PBS), eluted with 100 mM glycine, pH = 2.5 and neutralized with Tris-HCl, pH = 8.0, dialyzed against PBS, concentrated in stock solutions of 20 mg/ml, and stored at -80 °C. IgG concentration (~1mg/ml) and pH (7.4) was adjusted prior to use. Each IgG preparation was tested for antibody reactivity by staining human or rat brain sections or HEK cells expressing NR1/NR2 heteromers of the NMDAR as previously described (Dalmau et al., 2007; Dalmau et al., 2008). Both patients’ CSF and IgG decreased surface and total NMDARs to the same extent (Supplemental Fig. 1).

*Cell culture and patient antibody treatment*
Briefly, isolated rat hippocampi were placed in Ca^{2+} free HBSS (Hanks balanced salt solution, Life Technology) containing 1% papain for 20 min., triturated in Basal Media Eagle (BME; Invitrogen) supplemented with B-27 (Life Technology) and plated at 100,000 or 400,000 (for biotinylation) cells per ml in Neural Basal (NB; Life Technologies) supplemented with 10% FBS (Hyclone), B-27, 1% Penicillin and Streptomycin (Life Technologies), and 1% L-Glutamine (Life Technologies) on poly-L-lysine coated (Sigma) coverslips in 24-well plates. Culture media was changed to NB supplemented with B27 at 4 div. Cells were maintained at 37 °C, 5% CO₂, 95% humidity; medium was changed weekly. Neurons were treated with CSF or IgG from individual patients or controls for 1 day beginning at 14 days in vitro; in some experiments, neurons were treated for 3 or 7 days beginning at 14 days in vitro.

**Immunostaining for pre- and postsynaptic components, confocal imaging, and image analysis**

To stain surface NMDAR clusters, control or treated neurons were washed in Neurobasal plus B27 and incubated with patient CSF containing anti-NR1 antibodies for 30 min., washed and incubated with fluorescently conjugated anti-human secondary antibodies for 30 min., and washed in PBS. Neurons were then fixed in 4% paraformaldehyde, 4% sucrose in PBS, pH = 7.4 for 15 min., permeabilized with cold 0.25% Triton X-100 for 5 min., and blocked in 5% normal goat serum (Invitrogen) for 1 hour at RT. Additional immunostaining was performed with various combinations of
primary antibodies: to label glutamate receptors, anti-NR1 against the intracellular C-terminus (1:1000; Chemicon), anti-GluR1 (1:10; CalBioChem) or anti-GluR2 (1:100; Chemicon); to label postsynaptic densities, PSD-95 (1:500; Bioaffinity Reagents); to label dendrites, mouse anti-MAP2 (1:1000; gift from Dr. V. Lee); to label presynaptic terminals, mouse anti-SV2 (1:200; DHSB), guinea pig anti-VGLUT 1 (1:1000; Chemicon), or mouse anti-Bassoon (1:400; Stressgen Bioreagents). Antibodies were visualized after staining with the appropriate fluorescently conjugated secondary antibodies (1:200; Jackson ImmunoResearch).

Images were obtained using a confocal microscope (Leica TCS SP2). Images were thresholded automatically using iterative segmentation (Bergsman et al., 2006), and the number and area of individual immunostained pre- or postsynaptic clusters were determined using interactive software (custom-written ImageJ macros). Clusters with pixel overlap of pre- and postsynaptic markers were considered colocalized and thus synaptic (Krivosheya et al., 2008).

**Biotinylation of surface proteins and analysis by Western blot**

Neurons were treated with 1 μg – 1 mg/ml IgG for 1 day, washed with PBS supplemented with 0.1 mM CaCl₂ and 1 mM MgCl₂ (rinsing buffer) and incubated for 30 min. at 4 °C with 1 mg/ml Sulfo-NHS-Biotin (Thermo Scientific) in rinsing buffer. Neurons were then washed with rinsing buffer + 100 mM glycine (quenching buffer), incubated in quenching buffer for 30 minutes at 4°C to quench excess biotin, then 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 cocktail III, Calbiochem) at 4 °C for 1 hour. Lysates were cleared of debris by centrifugation at 12,400 x g for 20 min. An aliquot of the remaining supernatant was taken for the lysate fraction, and a second aliquot was incubated with avidin-linked agarose beads (Immobilized Monomeric Avidin, Thermo Scientific) overnight at 4 °C. After centrifugation, the supernatant was removed and the beads (surface fraction) were washed 1X RIPA buffer, 2X high-salt wash buffer (500 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.1% Triton X-100, pH 7.5), and 1X no-salt wash buffer (50 mM Tris, pH 7.5). The surface fraction was eluted from the beads with 2X sample buffer and proteins separated on an 8% gel using SDS-PAGE. Samples were transferred to nitrocellulose membranes and probed for antibodies against NR1 (1:1000, 556308, BD Pharmingen), NR2A (1:1000, AB1555, Millipore; 1:500, MAB5216, Millipore; 1:500, A6473, Invitrogen), NR2B (1:1000, AGC-003, Alomone; 1:500, 06-600, Upstate), GABA<sub>A</sub>α<sub>1</sub> (1:1000, 06-868, Upstate), GABA<sub>A</sub>α<sub>2</sub> (1:500, AB5984, Chemicon), GluR 2/3 (1:1000, 07-598, Upstate), PSD-95 (1:1000, 610496, BD Pharmingen), and actin (1:2000, A2066, Sigma). Actin and GABA<sub>A</sub>Rs were used as loading controls for total and surface fractions, respectively. Blots were incubated with HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (1:3000, Cell Signaling), and signals were visualized using chemiluminescence (SuperSignal Chemiluminescent Substrate, Thermo Scientific). All
quantified films were in the linear range of exposure, were digitally scanned and signals quantified using NIH ImageJ.

_Whole cell electrophysiological recordings of synaptic NMDA and AMPA receptor mediated currents_

Whole cell voltage clamp recordings were performed as previously described (Elmariah et al., 2004; Elmariah et al., 2005) from 14 – 21 div pyramidal neurons treated for 24 hours with patient CSF containing anti-NR1 antibodies, control CSF or left untreated. Briefly, neurons were incubated in an extracellular physiological solution without Mg$^{2+}$ (in mM: 119 NaCl, 5 KCl, 2 CaCl$_2$, 30 Glucose, 10 HEPES, pH = 7.4). Voltage-clamp recordings were made at RT (22-25 °C) using glass microelectrodes (resistance 4-6 MΩ) filled with a Cesium substituted intracellular solution (in mM: 100 Cesium gluconate, 0.2 EGTA, 5 MgCl$_2$, 2 ATP, 0.3 GTP, 40 HEPES, pH = 7.2). Pipette voltage offset was neutralized before the formation of a gigaohm seal. 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, using the whole cell application of PatchMaster software (HEKA Elektronik). Criteria for cell inclusion in the data set included a series resistance ≤ 30 MΩ and stability throughout the recording period. Currents were amplified, low-pass filtered at 2.5 kHz, and sampled at 5 Hz using PatchMaster software. Spontaneous miniature excitatory postsynaptic currents (mEPSCs) were recorded at -70mV in the presence of TTX (1 µM) and picrotoxin (10
μM). APV (50 μM) and CNQX (10 μM) were bath applied to block NMDAR and AMPAR mediated currents respectively. mEPSC events were detected and analyzed using MiniAnalysis (Synaptosoft, Leonia, NY), which employs a threshold-based event-detection algorithm. NMDAR and AMPAR components of mEPSCs were separated temporally by their distinct kinetics (Hestrin et al., 1990; Watt et al., 2000; Yang et al., 2003). The amplitude of the NMDAR mediated current was determined in a window between 15 and 25 ms after the peak of the AMPAR mediated component, which has a fast, <1 ms rise time. All values are presented as mean ± s.e.m.

**Fab fragment preparation and treatment**

Fab fragments were prepared from serum IgG using a kit according to the manufacturer’s directions (Fab preparation kit, Pierce Protein Research Products, Thermo Scientific). Briefly, serum IgG was digested for 2-4 hours at 37 °C with 1% (w/w) papain pH= 7.0 with 0.01 M cysteine, resulting in cleavage into Fab and Fc fragments. Fab fragments were isolated by chromatography and concentration determined by absorption at 280 nm, and then used to treat neurons at a concentration of 4 μg/ml. Control experiments showed that incubating neurons with patient Fab fragments for 30 min. resulted in surface staining of NR1 clusters (Supplemental Fig. 2).

**Alzet mini-pump placement, IgG infusion, and analysis of effects on NMDA receptors**
7-8 week old female Lewis rats were anesthetized and a cannula was placed into the left hippocampus using predetermined coordinates (-3.2 mm posterior to bregma, 2 mm lateral, and 3 mm deep to the dura mater). The cannula was secured to a head probe mounted to the skull, and attached with sterile tubing to an Alzet minipump (Alzet brain infusion kit #3, pump model 2002) implanted subcutaneously on the back. Patient or control CSF was then delivered at a rate of 0.5 µl / hr for 2 weeks. Rats were then euthanized, brain tissue harvested, immersion fixed in 4% paraformaldehyde in PBS, pH = 7.4 for 15 min., cryoprotected in 30% sucrose in PBS, pH = 7.4 overnight at 4 °C, and snap frozen in isopentane cooled in dry ice. Frozen 10 µm sections from infused hippocampus (where the track of the cannula was visible) and contralateral matched area of the non-infused hippocampus were immunostained in parallel to determine the presence of human IgG and the levels of NR1 using the primary and secondary antibodies described above. The degree of cell death was assayed with TUNEL. Sections were imaged and thresholded with the same parameters, and confocally imaged and analyzed as described above.

Additionally, protein extracts from 20 µm sections of the infused and contralateral hippocampus were separated electrophoretically, transferred to nitrocellulose, incubated with anti-NR1 antibody (Chemicon), and the amount of NR1 protein quantified as described above, using Tubulin as a loading control.

*Immunostaining, imaging, and image analysis of human tissue*
Hippocampal sections of human tissue were immunostained in parallel as described above. Control and patients’ tissue sections were imaged with a Zeiss Axioskop 2 plus (software AxioVision 4.5) with identical optical settings and exposure times. For analysis of high magnification regions 7-10 images were collected from the CA1 region of the hippocampus. These images were inverted and a cumulative histogram of pixel intensity was calculated for each image. The average cumulative histogram of pixel intensity was generated for each sample and the cumulative probability of pixel intensity for each sample was determined, plotted and compared using a paired Komolgorov-Smirnov test (see below).

Statistical analysis

Titer dependence was assessed with a linear regression analysis. In experiments involving two conditions, the data was analyzed with a two-tailed unpaired Student’s t test. In experiments involving three or more conditions, the normality of the data was analyzed with the D’Agostino and Pearson omnibus normality test, before using a one-way ANOVA test followed by Bonferroni’s multiple comparison test. Differences in distributions of NR1 intensity were assessed with a paired Komolgorov-Smirnov test. All values are presented as mean ± s.e.m.
Figures and Legends

Figure 1: Patients’ antibodies reduce surface NMDA receptor clusters and protein in a titer dependent fashion

(a) Hippocampal neurons immunostained for surface and total NMDAR clusters and presynaptic Bassoon. Surface NMDARs are defined as the colocalization between non-permeabilized patient CSF staining (extracellular NR1 epitope) and commercial NMDAR staining (intracellular epitope; left). Patient CSF treatment for 1 day reduces surface and total NMDAR cluster density without affecting Bassoon cluster density. Scale bar = 10 µm. (b) Western blot analyses of surface biotinylated and total lysate NMDAR protein. Patient IgG treatment for 1 day reduces surface as well as total NMDAR protein; GABA<sub>A</sub>α1, actin are loading controls. Control NR1 levels have been overexposed in this image to visualize patient treated NR1 bands. (c) Surface (left) and
total (right) NMDAR cluster density after treatment with CSF from several patients with
different antibody titer, showing a titer-dependent decrease in NMDA cluster density
(linear regression analysis; surface $R^2 = 0.59$, $p < 0.001$; total $R^2 = 0.44$, $p < 0.008$). All
values are mean ± s.e.m. (n = 18 cells, 3 independent expts.; 12 patient, 2 control
samples; see Supplemental Table 1). (d) Surface (left) and total (right) NMDAR protein
after treatment with IgG from several patients with different antibody titer (n = 3-5
Western blots; 10 patient, 2 control samples), showing a titer-dependent decrease in
NMDA protein (linear regression analysis; surface $R^2 = 0.53$, $p < 0.0001$; lysate $R^2 =
0.30$, $p < 0.002$). (e) Surface (left) and total (right) NMDAR cluster density after
treatment with CSF from the same patient at two timepoints with different antibody titer;
a higher antibody titer decreases surface and total NMDAR clusters to a greater extent.
(f) Surface (left) and total (right) NMDAR protein after treatment with IgG from two
patients at two time points with different antibody titer; a higher antibody titer decreased
surface and total NMDAR protein to a greater extent than IgG isolated from the same
patient when a lower antibody titer was present.
Figure 2: Patients’ antibodies reversibly reduce synaptic NMDA receptor clusters without affecting the number of synapses

(a) Hippocampal neurons immunostained for total NMDAR clusters, a presynaptic glutamatergic terminal protein, VGlut, and a postsynaptic protein localized to glutamatergic synapses, PSD-95. Treatment with patient CSF for 3 days reduces the density of synaptic NMDAR clusters without affecting the number of excitatory synapses. After removal of patient CSF, the proportion of NMDAR clusters localized to synapses returns to baseline. Scale bar = 10 μm. (b) Colocalization of pre- and postsynaptic proteins at excitatory synapses (n = 36 cells, 3 independent experiments; 2 patient, 1 control samples). (c) NMDAR cluster density at excitatory synapses (synaptic NR1). Asterisk indicates significant difference (One-way ANOVA test followed by Bonferroni’s multiple comparison test, p < 0.001).
Figure 3: Patients’ CSF and IgG treatment does not affect other synaptic components
(a) Hippocampal neurons immunostained for an excitatory postsynaptic protein, PSD-95, excitatory presynaptic protein, VGlut, and surface clusters of AMPA receptor subunits GluR1 or GluR2. Scale bar = 5 µm. (b) Quantification of excitatory synaptic protein density. Treatment with patient CSF did not affect the density of these excitatory synaptic proteins. All values are shown as mean ± s.e.m. (n = 18 cells, 3 independent expts.; 1 patient, 1 control sample; Student’s t test, p > 0.09). (c) Western blots of excitatory postsynaptic proteins, AMPA receptor subunits GluR2/3, excitatory postsynaptic protein PSD-95 and GABA<sub>A</sub> receptors. Treatment with patient IgG did not affect surface or total neurotransmitter receptor or excitatory synapse protein levels. (d) Quantification of surface (left) and total (right) protein after treatment with IgG from several patients with different antibody titer. Patient IgG resulted in a decrease in surface and total NMDAR NR1 protein but did not affect the levels of other synaptic proteins. All values are shown as mean ± s.e.m. (n = 3-5 Western blots; 10 patient, 2 control samples, One-way ANOVA test followed by Bonferroni’s multiple comparison test, p < 0.001).
Figure 4: Patient CSF treatment does not affect dendritic branching, spines, Bassoon cluster density or cell survival

(a) Hippocampal neurons transfected with the fluorescent protein, Tomato-td, imaged before and after one day of treatment with control or patient CSF, and traced with NeuronJ. Control (top) or patient (bottom) CSF treatment did not affect dendritic
branching or complexity. Scale bar = 100 μm. (b) Quantification of primary dendrite number. (c) Quantification of primary dendrite length. All values are shown as mean ± s.e.m. (n = 9 cells, 3 independent expts.; 1 patient, 1 control sample; Student’s t test, p > 0.6). (d) Sholl analysis of dendrite complexity before (white) and after (black) one day of control (left) or patient CSF (middle) treatment. Comparison of the difference before and after control and patient CSF treatment (right). (e) Hippocampal neurons transfected with fluorescent protein, Tomato-td, and treated for one day with control or patient CSF. Control (top) or patient (bottom) CSF treatment did not affect dendritic protrusion density. Scale bar = 5 μm. (f) Quantification of the density of dendritic protrusions (Student’s t test, p > 0.3). (g) Patient CSF treatment for 1 day does not affect Bassoon cluster density (linear regression analysis; R² = 0.005, p = 0.79). All values are mean ± s.e.m. (n = 18 cells, 3 independent expts.; 12 patient, 2 control samples; see Supplemental Table 1). (h) Quantification of the density of dissociated hippocampal cells in vitro after 1 day treatment of control or patient CSF. (i) Quantification of the percent of TUNEL positive neurons in vitro (apoptotic cells). These measures were not significantly different between control or patient CSF treatment (n = 30 fields (750 μm²), 4 independent expts.; 1 patient, 1 control sample; Student’s t test, p > 0.6).
Figure 5: Patients’ antibodies selectively decrease synaptic NMDA currents.

(a) mEPSCs recorded in physiological saline with TTX, picrotoxin, and $0 \text{Mg}^{2+}$ to isolate synaptic NMDAR mediated currents (left upper trace). APV, an NMDAR antagonist, blocks the slow decay of mEPSCs leaving only AMPA receptor mediated currents which account for the fast rise of mEPSCs (left upper middle trace). CNQX, an AMPA receptor antagonist, blocks the fast rise of mEPSCs, allowing NMDAR mediated currents to be isolated (left lower middle trace). Both AMPA and NMDAR mediated synaptic currents are blocked by CNQX plus APV (left bottom trace). Note that, under the same recording conditions, treatment of hippocampal neurons with patient CSF for 1
day dramatically reduces synaptic NMDAR mediated currents (right traces). (b) Representative average mEPSCs from neurons treated for 1 day with control CSF (left) or patient CSF (middle). The difference between the 0 Mg $^{2+}$ and the 0 Mg $^{2+}$ + APV traces, plotted at right, shows the NMDAR current. Neurons treated for 1 day with patient CSF have less NMDAR-mediated synaptic current than neurons treated with control CSF. (c) Effect of patient antibodies on NMDA (left) and AMPA (right) receptor mediated synaptic currents (n = 13 cells, 7 control CSF, 6 patient CSF, 4 independent expts.; 2 patient, 2 control samples). Asterisk indicates significant difference (Student’s t test, p < 0.001).
**Figure 6: Patients’ antibodies bind, crosslink and internalize NMDA receptors**

(a) Hippocampal neurons immunostained for surface and total NMDAR clusters. Treatment with patient IgG decreases surface and total NMDAR cluster density (middle left). Treatment with patient Fab fragments does not affect surface or total NMDAR cluster density (middle right), while treatment with divalent patient Fab fragments (Fab fragments + anti-Fab secondary antibodies) decreases surface and total NMDAR cluster density (right). Scale bar = 10 \( \mu \text{m} \).

(b) Effects of patient IgG, Fab fragments, and divalent Fab fragments on surface and total NMDAR cluster density. (n=30 cells, 4 independent expts.; 2 patient, 2 control samples). Asterisk indicates significant difference (One-way ANOVA test followed by Bonferroni’s multiple comparison test, p < 0.001).

(c) Cartoon that outlines the effect of each treatment on surface receptor clusters.
Figure 7: Patients’ antibodies decrease NMDA receptor cluster density and protein in rodent and human hippocampus in vivo

(a) Brain sections from rats infused with control CSF (top left) contain many NMDAR clusters in CA1, while brain sections from rats infused with patient CSF (top
right) contain significantly reduced NMDAR clusters. Presynaptic synapsin immunostaining is similar between groups (bottom left, right). Scale bar = 2 μm. (b) Effect of infusion of patient CSF with varying antibody titer on NMDAR cluster density in CA1. Each point represents the mean NMDAR cluster density from 3-5 images from an infused rat. Patient CSF with higher antibody titers reduce NMDAR cluster density to a greater extent than low titer samples. Infusion with patients’ CSF for 2 weeks results in a titer-dependent decrease in NMDA cluster density (linear regression analysis; R² = 0.32, p < 0.03). All values are mean ± s.e.m. (n = 9 animals sacrificed after 14 days of infusion; 5 patient, 4 control samples). (c) Western blot analyses of NR1 protein in patients’ CSF infused rat hippocampus (top); ipsilateral (I) and contralateral (C) to infusion; Tubulin protein is a loading control (below). NR1 protein is reduced in ipsilateral patient CSF infused hippocampus compared to the contralateral, uninfused hippocampus. F indicates NR1 protein in the frontal cortex ipsilateral to the hippocampus infused with patients’ CSF. (d) Synapsin cluster density (Student’s t test, p > 0.6). (e) Excitatory synapse density (colocalization between synapsin and AMPAR clusters; Student’s t test, p > 0.5). (f) Hippocampal section from a control patient (left) and from a patient with anti-NMDAR encephalitis (right) immunostained with a commercial anti-NR1 antibody. (g) Boxed areas in f shown at higher magnification. Scale bars = 1 mm (top); 25 μm (bottom). (h) Intensity of NR1 immunostaining is dramatically reduced in the hippocampi of anti-NMDAR encephalitis patients (n = 2) compared to hippocampi of control patients (n = 3). The distribution of both patient
values for NR1 intensity is significantly different from the distribution of control values (paired Komolgorov-Smirnov test, p < 0.05).
Supplemental Table 1: Summary of patients whose CSF or IgG were used in all studies

<table>
<thead>
<tr>
<th>Gender, age</th>
<th>Tumor(^a)</th>
<th>CSF(^b)</th>
<th>Brain MRI</th>
<th>CSF NR1 antibody titer (rfu)(^c)</th>
<th>Treatment</th>
<th>Outcome(^d)</th>
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<td>Increased FLAIR signal in medial temporal lobes</td>
<td>376408</td>
<td>C, IVIg, PLEX, CTX</td>
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<td>Increased FLAIR signal in medial temporal lobes</td>
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<td>Tumor removal; C, IVIg, RTX</td>
<td>Partial improvement</td>
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<tr>
<td>F, 12</td>
<td>No tumor</td>
<td>Normal</td>
<td>Normal</td>
<td>103336</td>
<td>C, IVIg</td>
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<td>F, 25</td>
<td>Ovarian teratoma (Im)</td>
<td>14 WBC</td>
<td>Normal</td>
<td>95207</td>
<td>Tumor removal; C, IVIg, PLEX</td>
<td>Full recovery</td>
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<tr>
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<td>Ovarian teratoma (M)</td>
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<td>Normal</td>
<td>61744</td>
<td>Tumor removal; PLEX</td>
<td>Full recovery</td>
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<td>26088</td>
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<td>Full recovery</td>
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<tr>
<td>F, 24</td>
<td>Ovarian teratoma (M) (autopsy)</td>
<td>219, OB</td>
<td>Increased FLAIR signal in parietal cortex; mild leptomeningeal enhancement</td>
<td>1843616</td>
<td>Supportive care</td>
<td>Died(^f)</td>
</tr>
</tbody>
</table>

\(^a\) All tumors contained nervous tissue; 5/5 tumors examined had expression of NMDARs (Dalmau et al., 2007).
(b) Normal: ≤ 4/ microliter.

(c) rfu: reference fluorescence units. Values in 100 randomly selected negative control CSF: <5000 rfu.

(d) Full recovery: able to return to all activities; Partial recovery: patient living at home, independent for most daily activities, but unable to return to work at the time of this report.

(e) Hippocampal sections from this patient were used for anti-NR1 immunostaining; see Fig. 5.

(f) Hippocampal sections from this patient were used for anti-NRI immunostaining; see Fig. 5; the limited amount of CSF and/or IgG from this patient precluded their use in other experiments.

CSF from the first 12 patients was used in the experiments in Fig. 1a, b. IgG from 10 of these patients was used in the experiments in Fig. 1c, d and Supplemental Fig. 2. Individual patient CSF and/or IgG were used for all other experiments.

Abbreviations: M, mature; Im, immature; WBC, white blood cells; OB, oligoclonal bands; n.a., not available; C, corticosteroids; RTX, Rituximab; PLEX, plasma exchange; CTX, Cyclophosphamide; IVIg, intravenous immunoglobulin; FLAIR: fluid-attenuated inversion recovery.
Supplemental Figure 1: Patient IgG and CSF treatment have similar effects and these effects are not mediated via the complement pathway

(a) Quantification of hippocampal neurons immunostained for surface NMDAR clusters treated with Control IgG, CSF, patient IgG, CSF, and heat inactivated patient CSF. Treatment with patient IgG and CSF for one day decrease surface NMDARs to a similar extent. Heat inactivated patient CSF also decrease surface NMDARs to a similar extent as patient IgG and CSF, suggesting that these effects are not mediated by complement-mediated pathways (n = 18 cells, 3 independent expts.; 1 patient, 1 control sample; One-way ANOVA test followed by Bonferroni’s multiple comparison test, p < 0.001). (b) Quantification of dissociated hippocampal neurons immunostained for total NMDARs treated with Control IgG, CSF, patient IgG, CSF, and heat inactivated patient CSF.
Supplemental Figure 2: Patient antibody Fab fragments colocalize with NMDA receptor clusters

Hippocampal neurons immunostained for NMDAR clusters in neurons treated for 1 day with control IgG (top row), patient IgG (middle top), patient IgG clustered with anti-IgG secondary antibodies (middle), patient Fab fragments (middle bottom), patient Fab fragments reclustered with anti-Fab secondary antibodies (bottom). Color overlays of NR1 (red) and human IgG (green) are shown at right. While patient IgG and patient IgG + anti-IgG stain neurons more intensely, patient Fab fragments and patient Fab fragments + anti-Fab colocalize with NMDARs to a similar extent (n = 18 cells, 3 independent expts.; 1 patient, 1 control sample). Scale bar = 10 µm. Abbreviations: anti-hIgG 2°, anti-human IgG secondary antibody; anti-hFab 2°, anti-human Fab secondary antibody.
Supplemental Figure 3: Treatment with Patient CSF for 1, 3, or 7 days decreases total NMDAR cluster density.

(a) Total NMDAR cluster density after treatment with CSF for 1, 3, or 7 days, each of which decreased NMDAR cluster density to a similar extent. All values are mean ± s.e.m. (n = 18 cells, 3 independent expts.; 1 patient, 1 control sample, One-way ANOVA test followed by Bonferroni’s multiple comparison test, p < 0.05).
Supplemental Figure 4: Patient IgG treatment decreases surface and protein of NMDA receptor NR2A/B subunits in a titer dependent fashion

(a) Western blots of surface and total NMDAR, NR2A and NR2B protein. Treatment with patient IgG reduced surface as well as total NMDAR, NR2A and NR2B protein. GABA<sub>α</sub>1 and actin are loading controls for surface and total protein, respectively. This blot was reprobed with antibodies against NR1 and displayed in Fig 1b and is representative of the data set. (b) Quantification of surface (left) and total (right) NMDAR NR2A protein after treatment with IgG from several patients with different antibody titer. IgG from patients with higher titer resulted in a greater decrease in surface and total NMDAR NR2A protein than patients with a lower titer. Thus treatment with patients’ CSF for 1 day results in a titer-dependent decrease in NR2A protein (linear regression analysis; surface $R^2 = 0.35$, $p < 0.005$; lysate $R^2 = 0.33$, $p < 0.002$). (c) Quantification of surface (left) and total (right) NMDAR NR2B protein after treatment with IgG from several patients with different antibody titer. IgG from patients with higher titer resulted in a greater decrease in surface and total NMDAR NR2B protein than patients with a lower titer. Thus treatment with patients’ CSF for 1 day results in a titer-dependent decrease in NR2B protein (linear regression analysis; surface $R^2 = 0.23$, $p < 0.02$; lysate $R^2 = 0.29$, $p < 0.002$). All values are shown as mean ± s.e.m. (n = 3-5 Western blots, 10 patient, 2 control samples, from independent expts. with individual patients’ IgG (Supplemental Table 1)).
Supplemental Figure 5: Patient IgG treatment decreases synaptic localization of NMDA receptors in a titer dependent fashion

Synaptic NMDAR cluster density after treatment with CSF from several patients with different antibody titer for 1 day, showing a titer-dependent decrease in synaptic NMDAR cluster density. Synaptic NMDARs are defined as the colocalization between commercial NMDAR staining (intracellular NR1 epitope) and presynaptic marker Bassoon (linear regression analysis; $R^2 = 0.43, p < 0.008$). This data was obtained from the data set displayed in Fig. 1. See also Figure 2.
Supplemental Figure 6: Patient CSF treatment does not affect mEPSC frequency or amplitude

(a) Quantification of mEPSC amplitude in neurons treated with control CSF, patient CSF, with and without APV. The total mEPSC amplitude (which represents the amount of functional postsynaptic AMPA receptors) is not significantly different among control, patient CSF or APV conditions. All values are shown as mean ± s.e.m. (n = 13 cells, 7 control CSF, 6 patient CSF, 4 independent expts.; 1 patient, 1 control sample; pairwise comparison, Student’s t test, p > 0.4). (b) Quantification of mEPSC frequency in neurons treated with and without control, patient CSF, and APV. The frequency of mEPSCs (which represents the number of excitatory synapses) is not significantly different between control, patient CSF or APV though the trend of lower frequency of patient CSF and APV treated conditions could be the result of blockade of silent synapses.
which have been shown to contribute to mEPSC frequency (Liao et al., 1995; Liao et al., 2001). All values are shown as mean ± s.e.m. (n = 6 cells, 4 independent expts.; 1 patient, 1 control sample; pairwise comparison, Student’s t test, p > 0.2).
Supplemental Figure 7: Patient CSF recognizes NMDA receptor clusters in vivo and infusion into rat hippocampus results in deposition of human IgG without increasing cell death

(a) Rat brain sections immunostained with patient CSF (top left), NMDARs (top middle), and a postsynaptic protein, PSD-95 (top right). Clusters immunostained with patient CSF are highly colocalized with NMDARs (yellow puncta, bottom left). Clusters immunostained with patient CSF colocalize with PSD-95 to a similar extent as NMDARs (compare bottom middle to bottom right). (n = 9 images, 3 independent expts.; 1 patient, 1 control sample).

(b) Brain sections from rats infused with control (left) or patient CSF (right) into one hippocampus, and immunostained with human IgG, NR1, and TO-PRO to label nuclei. The deposition of human IgG was seen in the hippocampus of rats infused with patient CSF but not control CSF. Below, higher magnification views of the CA1 region of the hippocampus show that areas with human IgG deposits have reduced NMDAR clusters (see Fig. 5) and decreased overall staining intensity (n=6 animals; 3 infused with patient CSF, 3 with control samples).

(c) Brain sections from rats infused with control or patient CSF and immunostained with TO-PRO to label nuclei and TUNEL to label apoptotic cells. Infusion with control (left) or patient CSF (middle) did not cause significant cell death. While several apoptotic cells were found along the cannula tract (right), the total number and distribution did not differ between rats infused with control or patient CSF.
Acute mechanisms underlying anti-NMDA receptor encephalitis

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Contributions

X.P., E.H.M. and E.G.H. designed and executed the experiments, with help from R.B.-G.; T.D.P. advised on the electrophysiological analyses; J.D. provided patients’ reagents; X.P. wrote the chapter; X.P. and R.B.-G. edited the chapter.
Abstract

Autoimmunity to NMDA receptor subunit 1 (NR1) is the cause of a severe but treatment responsive limbic encephalitis in which patients develop psychosis, seizures and profound deficits in memory, movement and other behaviors. Previous studies showed that patients’ antibodies decreased the synaptic localization, and currents mediated by NMDA receptors, due to antibody mediated receptor cross-linking and internalization. Here we show that patient antibodies rapidly increased the internalization rate of NMDA receptor clusters over the time course of a few hours. The effects of patient antibodies are independent of the activity of NMDA receptors themselves. Furthermore, this internalization accounts for much if not all of the decrease in NMDA receptor mediated currents without an antibody mediated acute receptor blockade. These suggest that patient antibodies decrease synaptic and circuit function due to the rapid, activity independent loss of NMDA receptors from the cell surface.
Introduction

The proper localization and trafficking of synaptic proteins is critical for neurological functions such as memory, learning and cognition. There are several recently identified autoimmune disorders in which patients develop autoantibodies against cell surface and synaptic proteins, including NMDA receptors (Dalmau et al., 2007; Dalmau et al., 2008) and AMPA receptors (Lai et al., 2009), among others (Moscato et al., 2010). Patients with anti-NMDA receptor encephalitis develop prominent psychiatric and behavioral symptoms, including psychosis, short term memory loss, seizures and abnormal movements (dyskinesias), that if untreated can progress to catatonia, hypoventilation, and autonomic instability (Dalmau et al., 2007; Dalmau et al., 2008). However, patients can recover if antibody titer is reduced (Dalmau et al., 2007; Dalmau et al., 2008). Using in vitro studies, we previously showed that patients’ antibodies selectively and reversibly cause a decrease in the surface density and synaptic localization of NMDA receptor clusters via antibody mediated capping and internalization (Dalmau et al., 2008; Hughes et al., 2010). Moreover, patient antibodies decrease NMDA, but not AMPA, receptor mediated synaptic currents, consistent with a selective loss of surface NMDA receptor clusters (Hughes et al., 2010). Using in vivo studies, we have also demonstrated that NMDA receptor cluster density is dramatically reduced in the hippocampus of rats infused with patients’ antibodies, as well as in the brain of autopsied patients with this disorder (Hughes et al., 2010). These studies established some of the cellular mechanisms by which patient anti-NMDA receptor
antibodies alter NMDA receptor density, localization and function, underlying the spectrum of neuropsychiatric and neurological dysfunction. The onset of this disease is very rapid: there are only a few days to a couple of weeks from prodromal symptoms to prominent dysfunction requiring hospital admission and intensive care (Dalmau et al., 2007; Dalmau and Rosenfeld, 2008). Thus, it was of interest to investigate whether patient antibodies also have acute effects on NMDA receptors, including whether antibodies block NMDA receptor mediated currents prior to internalization. When we examined the effects of patients’ antibodies on hippocampal neurons in vitro on a time scale of hours, we found that rapid, activity independent NMDA receptor internalization accounts for loss of receptor function.
Results

Patients’ antibodies rapidly internalize surface NMDA receptors

Previous studies in rodent hippocampal neurons in vitro suggested that antibodies from patients with anti-NMDA receptor encephalitis decreased the density of surface and synaptic NMDA receptor clusters and decreased NMDA receptor mediated currents. We asked how rapid these effects were, and whether the loss of receptor mediated currents was due to internalization or acute receptor blockade, or both.

Neurons at 14 div were treated with either Fab fragments or whole IgG prepared from patient serum for 1 to 24 hours. Since Fab fragments bind with NMDA receptors without leading to cross-linking and internalization (Hughes et al., 2010), Fab fragments serve as a control for steady state turnover of NMDA receptors. After treatment, surface Fab or IgG bound NMDA receptors were labeled with FITC conjugated secondary antibodies in live, unpermeabilized neurons (Fig. 1A, B). After fixation and permeabilization, internalized Fab or IgG bound NMDA receptor clusters, as well as remaining surface NMDA receptor clusters, were labeled with TRITC conjugated secondary antibodies (Fig. 1A, C). Internalized NMDA receptors are red-only clusters without colocalization with green surface clusters. After 1 hour of patient IgG treatment (Fig. 1A, C), internalized NMDA receptor clusters begin to accumulate. By 2 hours, the density of internalized NMDA receptors clusters is significantly higher than at 15 minutes and surface NMDA receptor cluster density is significantly decreased (Fig. 1A-C). Over 24 hours, internalized NMDA receptor clusters continue to accumulate and the
surface clusters continue to decrease (Fig. 1A-C). These data show that the loss of decreased surface NMDA receptor clusters is due to internalization of antibody bound clusters. Neurons treated for 24 hours with Fab have a small number of internalized NMDA receptor clusters, without a significant decrease in the density of surface NMDA receptors, probably due to the rapid turnover and/or recycling of NMDA receptors to the cell surface. However, by 24 hours, whole IgG treated neurons have a significantly higher density of internalized NMDA receptors, suggesting that the internalization rate of NMDA receptors is increased compared to constitutive NMDA receptor internalization. These results suggest that the rapid internalization of NMDA receptor account for the rapid decrease in NMDA receptor cluster density in the presence of anti-NMDA receptor antibodies from patients.

**Patient antibody induced internalization of NMDA receptor clusters is independent of receptor activity**

To determine whether the rapid internalization of surface and synaptic NMDA receptors induced by patient antibodies is modulated by NMDA receptor activity, we used APV ((2R)-amino-5-phosphonovaleric acid) to block NMDA receptor activity during patient antibody treatment. If NMDA receptor internalization were increased by inactivity, then APV would accentuate the effect of patient antibodies on the loss of surface and synaptic NMDA receptor clusters. A wide range of patient antibody concentration was used to explore the effect of activity over a wide range of antibody
titer and thus NMDA receptor internalization (c.f. Hughes et al., 2010). We found that APV treatment did not affect the extent of patient antibody induced loss of surface or synaptic NMDA receptors (Fig. 2A, B). These data suggest that patient antibody induced receptor internalization is not activity dependent.

**Patient antibody mediated loss of NMDA receptor currents is due to internalization, not acute receptor blockade**

Since the decrease in surface NMDA receptor cluster density is detectable 1-2 hours after patient antibody treatment, we next asked whether patient antibodies acutely block receptor function and thus rapidly diminish NMDA receptor mediated synaptic currents. Neurons were incubated with control or patient CSF for 10 minutes, followed by whole cell patch clamp recording to assess NMDA receptor mediated miniature excitatory postsynaptic currents (mEPSCs) (Fig. 3A). Within 10 to 30 minutes after treatment with patient antibodies, the antibody mediated internalization of NMDA receptors is not yet significant (Fig. 1). During this time window, there is no significant change in NMDA receptor mediated mEPSC amplitude or frequency (Fig. 3B, C). These data suggest that patient antibodies don’t acutely block NMDA receptor function. On the other hand, NMDA receptor mediated currents are significantly reduced after 24 hours of treatment with patient antibodies, confirming that over this time frame, there is a significant decrease in surface and synaptic NMDA receptor clusters (Fig. 3C).
We found that, while the NMDA receptor antagonist APV decreases both the amplitude and frequency of NMDA receptor mediated currents (Fig. 3B, C), the amplitude but not the frequency of NMDA receptor mediated currents was reduced after treatment with patient antibodies. This is not likely to be due to a decrease in presynaptic release probability, since our previous studies suggested that the frequency of AMPA receptor mediated currents was unaffected (Hughes, et al., 2010). These data suggest that the number of postsynaptic sites with functional NMDA receptors is decreased by patient antibodies, consistent with the observation that surface NMDA receptor cluster density is decreased.

If patient antibodies led to an acute increase or potentiation of NMDA receptor currents, this might be rapidly offset by NMDA receptor internalization, within 30 minutes of treatment. To investigate this possibility, neurons were exposed to patient or control antibodies and NMDA receptor mediated mEPSCs were recorded over time before and after treatment (Fig. 3D-F). No significant difference in NMDA receptor mediated mEPSC amplitude or frequency was observed before and 10 minutes after antibody treatment (Fig. 3D-F). These data suggest that patient antibodies are unlikely to have robust, acute effects on NMDA receptor function on a time scale of minutes.
Discussion

We have studied short term effects of autoantibodies from anti-NMDA receptor encephalitis patients on cultured hippocampal neurons. We showed that patients’ antibodies rapidly and significantly increased the internalization of NMDA receptor clusters, in just a few hours. While the patient antibodies bind to surface NMDA receptors, they do not interfere with the activity of NMDA receptor currents. Furthermore, our data suggests that the NMDA receptors activity is not interacting with required for patient’s antibody’s effects on decreasing antibody mediated internalization of synaptic NMDA receptor clusters. Together, our data suggest cellular mechanism of short term effects of antibodies from anti-NMDA receptor patients which cause a rapid, activity independent internalization independent of NMDA receptor activity, without blocking NMDA receptors.

Surface NMDA receptors are normally internalized during synapse maturation, in long-term depression (LTD), and in response to ligand-binding (Roche et al., 2001; Vissel et al., 2001; Barria and Malinow, 2002; Montgomery and Madison, 2002; Snyder et al., 2005b). Internalization of NMDA receptors has been shown to be mediated by clathrin via endocytotic signal on C-terminal of NR2A or NR2B subunits (Roche et al., 2001; Lavezzari et al., 2004). NMDA receptors sort into different intracellular pathways after endocytosis, with NR2B containing receptors preferentially trafficking through recycling endosomes and NR2A through late endosomes (Lavezzari et al., 2004). We
showed here that the patient’s antibody associated with NMDA receptor clusters are internalized after a few hours of incubation, and that the net accumulation rate of internalized NMDA receptors are much higher than normal internalization. Whether the patients’ antibodies induced internalization is mediated by the clathrin dependent pathway as observed in normal conditions but at a higher rate, or other pathways for pathologicagenicel conditions is a question to be addressed in the future.

In addition to causing antibody mediated NMDA receptors internalization by patients’ antibodies capping and cross-linking NMDA receptors, as our previous studies suggested (Hughes et al., 2010), another mechanism to compromise NMDA receptor’s receptor function is that patient anti-receptor antibodies agonize or antagonize the receptor. This has been suggested in other autoimmune diseases. NR2 antibodies from patients with SLE cause neuronal death when injected into mouse brain; this effect is attenuated by treatment with the NMDA receptor blocker, MK-801, suggesting the antibodies mediate cell death by enhancing channel activation (DeGiorgio et al., 2001). Conversely, application of nicotonic acetylcholine receptor (nAChRs) antibodies from myasthenia gravis patients to outside-out patches of mouse myotubes caused an acute block of AChR currents that became irreversible with time (Jahn et al., 2000). However, in our studies, whole cell recording experiments during acute application of antibodies in our studies showed that there are no significant changes of NMDA receptor mediated miniature amplitude and frequency, suggesting that neither agonist nor antagonist effects. It is possible that the whole cell NMDA receptor mediated miniature recording is not sensitive
enough to detect very small changes in NMDA receptor currents, but since this technique can detect the changes of current resulted from internalization and reduction of synaptic NMDA receptors, at least our results suggest that possible agonist or antagonist effects are minimal, if any, compared to the later reduction of NMDA receptor currents induced by internalization. Further more, if there is any direct interference of NMDA receptor currents by patients’ antibodies, it does not induce internalization of receptors, since in presence of NMDA receptor blocker APV, the patients’ antibodies’ effect in reducing synaptic NMDA receptor clusters are not changed. Together, these together data suggest that antibody mediated cross-linking and capping is the major mechanism to that triggers the internalization and reduction of surface NMDA receptor clusters and thus their function.
Methods

Preparation of patient and control CSF and IgG

Patient or control cerebrospinal fluid (CSF) was collected and filtered using protein A/G sepharose columns. CSF was diluted 1:15-100 to treat neurons in vitro. In some experiments, IgG purified from serum was used to treat neurons (Fig. 1; Fig. 2A, E). Briefly, 10 ml of patient or control serum were incubated with a 5 ml column of protein A/G Sepharose beads (50:50) for 30 min. on an orbital shaker at 4 °C. After elution IgG was added to a bio-spin chromatography column (Bio-Rad) followed by 3 washes with PBS, eluted with 100 mM glycine, pH = 2.5 and neutralized with Tris-HCl, pH = 8.0, dialyzed against PBS and concentrated to stock solutions of 20 mg/ml and stored at -80 °C. IgG concentration (~1mg / ml) and pH (7.4) were adjusted prior to use. Each CSF or IgG preparation was tested for antibody reactivity by staining mouse or rat brain sections or HEK cells expressing NR1/NR2 heteromers of the NMDAR as previously described (Dalmau et al., 2007; Dalmau et al., 2008).

Cell culture and patient antibody treatment

Primary rat hippocampal neuron and astrocyte cultures were prepared from embryonic day 18-19 as previously described (Goslin et al., 1988). Briefly, hippocampi were in Ca²⁺ free HBSS containing 1% papain for 20 min., trituated in Basal Media Eagle (BME; Invitrogen) supplemented with B-27 (Life Technology) and plated at 100,000 or 400,000 (for biotinylation) cells per ml in Neural Basal (NB) supplemented
with 10% FBS (Hyclone), B-27, 1% Penicillin and Streptomycin (Life Technologies) and 1% L-Glutamine (Life Technologies) on poly-L-lysine coated (Sigma) coverslips in 24-well plates. Culture media was changed to Neural basal (Life Technologies) supplemented with B27 at 4 div. Cells were maintained at 37 °C, 5% CO₂, 95% humidity; medium was changed weekly. CSF was diluted 1:15-100 to treat neurons in vitro. In some experiments, IgG purified from serum was used to treat neurons (10 µg to 1 mg/ml).

Immuno staining for pre- and postsynaptic components

To label surface NMDAR clusters and measure the subsequent internalization, neurons were incubated with patient IgG/CSF containing anti-NR1 antibodies or Fab fragments for 1 to 24 hours. Since Fab fragments can bind with NMDA receptors but not cross link and cause decrease in receptors clusters (Hughes et al., 2010) and do not induce significant reduction of surface NMDA receptors, they can serve as a control of static status of NMDA receptor removal and reinsertion of surface and synaptic sites. After treatment, surface Fab or IgG bound NMDA receptor can be labeled by applying FITC conjugated secondary antibodies while the neurons are alive and without permeabilization. After fixation and permeabilization, internalized Fab or IgG bound NMDA receptor clusters as well as remaining surface NMDA receptor clusters can be visualized by applying TRITC conjugated secondary antibodies.
To stain surface NMDAR clusters, control or treated neurons were washed in NB plus B27 and were incubated with patient CSF containing anti-NR1 antibodies for 30 min., washed and incubated with fluorescently conjugated anti-human secondary antibodies for 30 min., and washed in PBS. Neurons were then fixed in 4% paraformaldehyde, 4% sucrose in PBS, pH = 7.4 for 15 min., permeabilized with cold 0.25% Triton X-100 for 5 min., and blocked in 5% normal goat serum (Invitrogen) for 1 hour at RT. Additional immunostaining was performed with various combinations of primary antibodies: to label glutamate receptors, anti-NR1 (1:1000; Chemicon), to label presynaptic terminals, mouse anti-Bassoon (1:400; Stressgen Bioreagents). Antibodies were visualized after staining with the appropriate fluorescently conjugated secondary antibodies (1:200, Jackson ImmunoResearch).

Confocal imaging, image analysis and statistical analysis

For all experiments, 6-12 randomly selected pyramidal neurons, identified by morphology (Elmariah et al., 2004; Elmariah et al., 2005) in each condition were confocally imaged (Leica TCS 4D system) on each of 2-3 coverslips in 3-5 independent experiments. Images were thresholded automatically using an iterative thresholding technique (Bergsman et al., 2006), and the number and area of individual immunostained pre- or postsynaptic clusters were determined using interactive software (custom-written ImageJ macros). Clusters with pixel overlap of pre- and postsynaptic markers were considered colocalized and thus synaptic. Cluster density was compared among
conditions using the Kruskal-Wallis nonparametric ANOVA test followed by Dunn’s pairwise multiple comparison test, unless otherwise indicated. All values are presented as mean ± s.e.m.

Whole cell electrophysiological recordings of synaptic NMDA receptor mediated currents

Whole cell voltage clamp recordings were performed as previously described (Hughes et al., 2010) from 14 – 21 div pyramidal neurons treated for indicated amount of time with patient CSF/IgG containing anti-NR1 antibodies, control CSF/IgG or left untreated. Briefly, neurons were incubated in extracellular physiology solution without Mg\(^2+\) and with Glycine to increase detectable NMDA receptor mediated minis (in mM: 119 NaCl, 5 KCl, 2 CaCl\(_2\), 30 Glucose, 10 HEPES, 0.001 Glycine, pH = 7.4). Voltage-clamp recordings were made at RT (22-25 °C) using glass microelectrodes (resistance 4-6 MΩ) filled with a Cesium substituted intracellular solution (in mM: 100 Cesium gluconate, 0.2 EGTA, 5 MgCl\(_2\), 2 ATP, 0.3 GTP, 40 HEPES, pH = 7.2). Pipette voltage offset was neutralized before the formation of a gigaohm seal. 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, using the whole cell application of HEKA software. Criteria for cell inclusion in the data set included a series resistance ≤ 30 MΩ and stability throughout the recording period. Currents were amplified, low-pass filtered at 2.5 kHz, and sampled at 5 Hz using pCLAMP. Miniature
excitatory spontaneous currents (mEPSCs) were recorded at -70mV in the presence of TTX (1 µM), Picrotoxin (10 µM) and CNQX (10 µM). APV (50 µM) were bath applied to block NMDAR mediated currents respectively. To test whether patient antibodies rapidly decreased NMDAR mediated currents, consistent with a receptor blocking effect, neurons were recorded for 10 min. then treated with bath applied patient or control CSF/IgG for 10 min. mEPSC events were detected and analyzed using MiniAnalysis (Synaptosoft, Leonia, NY), which employs a threshold-based event-detection algorithm. NMDAR and AMPAR components of mEPSCs were separated temporally by their distinct kinetics (Hestrin et al., 1990; Watt et al., 2000; Yang et al., 2003). All values are presented as mean ± s.e.m.

*Fab fragments preparation and treatment*

Fab fragments were prepared from serum IgG using a kit according to the manufacturer’s directions (Fab preparation kit, Pierce Protein Research Products, Thermo Scientific). Briefly, serum IgG was digested for 2-4 hours at 37 °C with 1% (w/w) papain pH= 7.0 with 0.01 M cysteine, resulting in cleavage into Fab and Fc fragments. Fab fragments were then isolated by chromatography and concentration determined by absorption at 280 nm. Fab fragments were used to treat neurons at a concentration of 4 µg/ml.
Figures and Legends

Figure 1. Patient antibodies rapidly internalize surface and synaptic NMDA receptor clusters.

A. Neurons were labeled and treated with patient or control IgG for 15 minutes – 24 hours, or Fab fragments from patient or control IgG for 24 hours. At the end of treatment, the remaining surface NMDA receptors associated with IgG were labeled with FITC conjugated secondary antibodies in live, unpermeabilized neurons. After fixation and permeabilization, internalized receptors were labeled with TRIC conjugated secondary. Over time, treatment with patient IgG decreased surface IgG-NMDA receptor cluster density and increased the density of internalized IgG-NMDA receptor clusters.
Treatment with Fab fragments from patient for 24 hours had no effect. (N = 6 - 12 neurons from each of 2 independent experiments for each condition.

**B.** Quantification of surface NR1 cluster density over time and type of treatment. Asterisk indicates significant difference compared to 15 minutes treatment (ANOVA, Kruskal-Wallis test, followed by Dunn’s multiple comparison test, p < 0.0001).

**C.** Quantification of internalized NR1 clusters density over time and type of treatment. Asterisk indicates significant difference compared to 15 minutes (ANOVA, Kruskal-Wallis test, followed by Dunn’s multiple comparison test, p < 0.0001).
Figure 2. Patient antibody induced internalization of NMDA receptor clusters is independent of receptor activity.

A. Effects of control or patient CSF with or without APV on the density of synaptic NMDA receptor clusters. 1:20 dilution patient CSF, but not a 1:100 dilution, significantly decreased the density of synaptic NMDA receptor clusters compared to control (Mann-Whitney U test, p = 0.002). However, the presence of APV did not affect the density of synaptic NMDA receptor clusters (p = 0.08 to 0.2; N = 3-17 neurons from each of 2 experiments.

B. Control or patient CSF with or without APV had no effect on the density of presynaptic Bassoon clusters (Kruskal-Wallis test, p = 0.04).
Figure 3. Patient antibody mediated loss of NMDA receptor currents is due to internalization, not acute receptor blockade.

A. mEPSCs recorded in physiological saline with TTX, picrotoxin and CNQX to isolate synaptic NMDAR mediated currents. Under the same recording conditions, treatment of hippocampal neurons with patient CSF for 1 day dramatically reduced synaptic NMDAR mediated currents (second trace). Treatment with patient CSF for 30 minutes didn’t significantly change NMDAR mediated current amplitude or frequency (N = 3 neurons treated with control IgG for 1 day; 3 neurons treated with patient IgG for 1 day; 2 neurons treated with control CSF for 30 minutes; 2 neurons treated with patient’s CSF for 30 minutes; 3 neurons recorded in presence of APV).

B. Quantification of patient antibody effects on NMDA receptor mediated synaptic current amplitude (control 24 h = 16.7 ± 1.3 pA; patient’s IgG 24 h = 15.4 ± 1.0; control 30 min = 16.1 ± 0.4; patient’s CSF 30 min = 16.1 ± 1.1; APV = 10.0 ± 0.4; asterisk indicates significant difference, Mann-Whitney U test, p = 0.05).

C. Quantification of patient antibody effects on NMDA receptor mediated synaptic current frequency (control 24 h = 4.2 ± 1.5 Hz; patient’s IgG 24 h = 1.9 ± 1.1; control 30 min = 3.8 ± 0.4; patient’s CSF 30 min = 3.7 ± 0.8; APV = 0.7 ± 0.3; asterisk indicates significant difference, Mann-Whitney U test, p = 0.05).

D. mEPSCs recorded in physiological saline with TTX, picrotoxin and CNQX to isolate synaptic NMDAR mediated currents. Baseline NMDA receptor mediated currents were recorded for 10-20 minutes, then control or patient IgG was added to the recording
bath and recordings continued over time (N = 5 control IgG treated neurons; 4 patient
IgG treated neurons).

E. Quantification of patient antibody effects on NMDA receptor mediated
synaptic current amplitude (control before = 16.3 ± 1.6; control IgG 5 min = 14.4 ± 1.1;
patient before = 13.5 ± 0.7, patient IgG 5 min = 13.4 ± 1.3; Wilcoxon matched-pairs test,
p > 0.05).

F. Quantification of patient antibody effects on NMDA receptor mediated
synaptic current frequency (control before = 5.1 ± 0.5; control IgG 5 min = 5.5 ± 0.3;
patient before = 4.3 ± 0.2; patient’s IgG 5 min = 6.2 ± 0.2; Wilcoxon matched-pairs test,
p > 0.05).
Cellular and synaptic mechanisms underlying anti-AMPA receptor encephalitis

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Contributions

X.P., E.H.M. and E.G.H. designed and executed the experiments, with help from R.B.-G.; T.D.P. advised on the electrophysiological analyses; J.D. provided patients’ reagents; X.P. wrote the chapter; X.P. and R.B.-G. edited the chapter.
Abstract

We recently described a severe but treatment responsive type of limbic encephalitis that is associated with autoantibodies to the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR). Anti-AMPAR encephalitis results in a severe short-term memory loss and seizures. Here we demonstrate that in cultured hippocampal neurons, patients’ anti-AMPAR antibodies cause a selective decrease in the surface density and synaptic localization of AMPARs and their association with the postsynaptic protein Stargazin, by increasing the internalization of surface AMPAR clusters. Patient antibodies also specifically decreased synaptic AMPAR mediated miniature excitatory postsynaptic currents, while NMDAR mediated currents are not affected. While commercially available anti-AMPAR antibodies directed against extracellular epitopes can compete with patient antibodies for binding to surface AMPA receptors, not all commercial antibodies result in loss of surface and synaptic receptor clusters due to internalization. These results suggest that in anti-AMPAR encephalitis, patient antibodies result in a selective loss of surface and synaptic receptor clusters that is specific to patient antibodies.
Introduction

There are several recently identified paraneoplastic autoimmune disorders in which patients develop autoantibodies against cell surface and synaptic proteins including NMDA receptors (Dalmau et al., 2007; Dalmau et al., 2008) and AMPA receptors (Lai et al., 2009). Patients with anti-AMPA encephalitis mainly present with confusion, agitation, seizures, and severe short-term memory deficits, which recover with appropriate intervention to reduce antibody titer (Lai et al., 2009). Patients with anti-AMPA encephalitis have a significant tendency to relapse, and for these patients the outcome depends on how well each relapse is controlled. In contrast, patients with anti-NMDA receptor encephalitis develop prominent psychiatric and behavioral symptoms, including psychosis, short-term memory loss, seizures, and abnormal movements (dyskinesias), that if untreated can progress to catatonia, hypoventilation, and autonomic instability (Dalmau et al., 2007; Dalmau et al., 2008). The distinct roles of NMDA receptors and AMPA receptors in basic synaptic transmission, circuit function, and activity-dependent plasticity may underlie the distinct syndromes of these two encephalitides (Nakazawa et al., 2004; Shepherd and Huganir, 2007).

AMPA receptors mediate most of the fast excitatory synaptic transmission in the brain (Shepherd and Huganir, 2007) and the majority are heterotetramers composed of GluR1, 2, 3 or 4 subunits that 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.
(Lai et al., 2009). Preliminary studies suggest that the location of the epitope is the N-terminal extracellular domain of these AMPA receptor subunits (Gleichman et al., 2009).

Our previous studies showed that the treatment of rat hippocampal neurons with patients’ antibodies resulted in a decrease in the surface and synaptic localization of AMPAR clusters that was reversible (Lai et al., 2009). Thus as for anti-NMDA receptor encephalitis, patient antibodies likely reduce surface AMPAR receptors due to antibody mediated capping and internalization (Hughes et al., 2010).

In this study, we demonstrate that patients’ anti-AMPAR antibodies caused a selective decrease in the surface density and synaptic localization of AMPARs and an associated protein, Stargazin, due to increased internalization of AMPAR clusters. Patient antibodies also specifically decreased synaptic AMPAR, but not NMDAR, mediated miniature excitatory postsynaptic currents. In contrast, while commercially available anti-AMPAR antibodies directed against extracellular epitopes can compete with patient antibodies for binding to surface AMPA receptors, not all commercial antibodies result in loss of surface and synaptic receptor clusters due to internalization. These results suggest that in anti-AMPAR encephalitis, patient antibodies result in a selective loss of surface and synaptic receptor clusters that is specific to patient antibodies, likely due to the specific extracellular epitope that is targeted.
Results

Patients’ anti-GluR1 or GluR2 antibodies selectively decrease synaptic and surface AMPA receptor cluster density

Previous work showed that the antibodies from a single patient with anti-AMPAR encephalitis selectively and reversibly decreased synaptic AMPA receptor clusters (Lai et al., 2009). To determine whether different patient antibodies had similar effects, and to further determine whether patient anti-GluR1 or anti-GluR2 antibodies have similar effects on localization of AMPA receptor subunits, hippocampal neurons were cultured for 1-3 days with patient anti-GluR1 or anti-GluR2 antibodies, followed by immunohistochemical and Western blot analyses of synaptic and surface GluR1 or GluR2/3 protein. Patient anti-GluR1 and anti-GluR2 antibodies significantly decreased both GluR1 and GluR2/3 containing AMPAR synaptic cluster density (Fig. 1A, B, C) and surface cluster density (Fig. 1E, F), compared to CSF from control patients. There is no significant compensatory increase of GluR2/3 subunits to synapses, or compensative increase of surface expression of GluR2/3 subunits when treated with anti-GluR1 patient’s CSF or vice versa, suggesting that antibodies from both type of anti-AMPAR encephalitis patients have similar effects on decreasing GluR1 and GluR2 containing AMPA receptors.

Patients’ antibodies also reduce the density of synaptic Stargazin clusters but not other synaptic proteins
We next determined whether the antibodies also affected synapse density and/or the density of other postsynaptic components. Hippocampal neurons were cultured with CSF containing anti-GluR1/anti-GluR2 antibodies or purified IgG for 1-3 days, followed by immunostaining or Western blot analyses of synaptic proteins, including presynaptic VGlut, postsynaptic PSD-95, the AMPA receptor interacting protein Stargazin, NMDA receptor subunits NR1 and GluR2, and GABAb receptors.

Patients’ antibodies did not affect the number of excitatory synapses compared to controls (Fig. 1D). Moreover, patients’ antibodies did not affect the density of presynaptic Bassoon, vGlut and postsynaptic PSD-95, NR1 clusters (Fig. 2A – E), or the amount of surface NR1 and GABAb receptor protein (Fig. 2 F, G). However, patient’s CSF treatment for 1 day reduced synaptic localization of Stargazin, an AMPA receptor interacting protein (Fig. 2E), suggesting that proteins that directly interact with AMPA receptor subunits may also be affected by reduction of surface and synaptic AMPA receptors induced by patient’s antibodies.

These results show that patients’ antibodies reduce synaptic localization of AMPA receptor interacting protein Stargazin without significant effect on NMDA or GABAb receptors, other synaptic proteins, or the number of excitatory synapses.

**Patients’ antibodies decrease AMPA receptor but not NMDA receptor mediated synaptic transmission**
In order to examine the functional outcome of patients’ antibody treatment, AMPA receptor mediated miniature excitatory postsynaptic currents (mEPSCs) were measured using whole cell voltage patch recording at -70 mV from neurons treated with patient’s of control’s CSF for 1 day. The recording was carried out in presence of TTX, picrotoxin and APV to block action potentials, GABA receptor mediated inhibitory currents and NMDA receptor mediated currents, respectively.

In neurons treated with control CSF, there are frequent AMPA receptor mediated miniature current events with an average amplitude of 17.6 ± 3.7 pA and average frequency of 3.2 ± 0.4 Hz (Fig. 3A, upper trace, B, left average trace, C,D). In contrast, the AMPA receptor mEPSCs are significantly smaller with amplitude of 10.9 ± 0.7 pA (Fig. 3A, bottom trace, 3B, right average trace, 3C; Student’s t-test, p = 0.04 ). The frequency of AMPA receptor mEPSCs in neurons treated with the patient’s CSF is 2.3 ± 1.4 Hz, not significantly different from the control treated neurons.(Fig. 3D, student’s t-test, p = 0.48). This result suggests that patient antibodies decrease AMPA receptor mediated currents.

We also examined NMDA receptor mediated currents. To measure NMDA receptor minis independently from AMPA minis, recordings were carried out in presence of TTX, picrotoxin, CNQX (an AMPA receptor blocker) and Glycine to reveal all functional NMDA receptors (Wilcox et al., 1996). The amplitude and frequency of NMDA receptor mediated currents, which can be blocked by APV (Fig. 5E, bottom trace), are not significantly different in neurons treated with control or patient CSF (Fig.
This result suggests that the patient antibodies specifically affect the function of AMPA receptors. In addition, the observation that the frequency of NMDA receptor mediated currents was not affected also suggests that the presynaptic release probability is not affected by patient antibodies.

Together, these results show that patients’ antibodies specifically decrease synaptic AMPAR-mediated currents and do not affect NMDA receptor mediated currents, consistent with the specific loss of surface, synaptically localized AMPAR clusters.

Patients’ antibodies increase the internalization of AMPA receptor clusters

Next, we asked whether the reduction of synaptic AMPA receptors is due to increased internalization induced by patient’s antibodies. The surface AMPA receptors were labeled by a commercial anti-GluR1 antibody against extracellular epitope (Calbiochem) for two hours. This commercial antibody labeled surface AMPA receptors without decreasing its synaptic localization (see below). After removal of extra unbound commercial antibodies, neurons were treated with control or patient CSF containing anti-GluR1 antibodies for 1 day. Remaining surface AMPA receptor clusters were assayed by FITC conjugated secondary antibody, in a saturating amount, against the commercial GluR1 in live neurons, i.e., in the absence of fixation and permeabilization. Internalized AMPA receptor clusters were revealed by TRITC conjugated secondary antibody after fixation and permeabilization.
One day of treatment with patient CSF significantly increased the density of internalized AMPA receptor clusters (Fig. 4A, C). However, the density of pre-labeled surface AMPA receptor was not significantly decreased with patient’s CSF treatment (Fig. 4A, B). This is likely due to pre-incubation of the commercial antibody that may have sterically hindered binding of patient antibody, thus reducing the effect of patient CSF. Together, these results suggest that the patient anti-AMPAR antibodies increased the internalization of AMPA receptors.

**Commercial antibodies compete with patients’ antibodies**

To further understand the different effects of patient’s versus commercial antibodies against extracellular epitopes of AMPA receptors, we examined whether pre-incubation of patients’ CSFs can interfere with commercial antibodies staining. After fixation, neurons were pre-incubated with control, anti-GluR1 or anti-GluR2 patient’s CSF for 1 hour (Fig. 5A, left row of images), followed by standard immunostaining for commercial anti-GluR1. Both staining intensity and the density of surface GluR1 clusters decreased significantly (Fig. 5B). Interestingly, both anti-GluR1 and anti-GluR2 patient’s CSF interfered with commercial anti-GluR1 antibody binding, suggesting that this interference is not subunit specific. Similar observations were obtained for a commercial anti-GluR2 antibody after pre-incubation of patients’ CSF (Fig. 5C). Furthermore, anti-NMDA receptor encephalitis patient’s CSF didn’t interfere with commercial anti-GluR2 antibody’s staining, suggesting that when the competing antibody
is physically further away from AMPA receptors, no interference occurred. Together, these results suggest that patient anti-GluR1/2 antibodies compete with commercial antibodies for extracellular space for their own epitopes.

**Effects of commercial anti-AMPAR antibodies on surface AMPA receptor density**

Next, we asked whether the commercial antibodies against extracellular AMPA receptor epitopes have similar effects as the patients’ antibodies in decreasing the synaptic localization of AMPA receptor clusters. After 1 day treatment with commercial anti-GluR1 antibody (Calbiochem; immunogen is a synthetic peptide (RTSRSRDHTRVDWKR) corresponding to amino acids 271-285 of rat GluR1), the synaptic localization of AMPA receptor clusters was unchanged across a wide range of antibody concentration (Fig. 6B). When the antibody concentration was high (1:20, 1:50 diluted), AMPA receptor cluster staining increased, probably due to the increased association with commercial antibodies on the surface of neurons in the absence of internalization. Interestingly, neurons treated with anti-GluR2 antibodies (Chemicon; immunogen is recombinant fusion protein with putative N-terminal portion of GluR2 from AA 175-430), especially when a secondary antibody was included to cross-link the primary antibody, a significant decrease of synaptic AMPA receptor clusters density was observed (Fig 6A, 6C). These results suggest that some, but not every, commercially available antibody has similar effects as patient antibodies, possibly due to binding to a specific extracellular epitope on AMPA receptors.
Discussion

In this study, we examined the effects of autoantibodies from anti-AMPAR encephalitis patients on hippocampal neurons. Here we demonstrate that patients’ AMPAR antibodies cause a selective decrease in surface density and synaptic localization of AMPAR and its associated protein Stargazin, via increased internalization rate of AMPAR clusters. Consistent with structural changes, patients’ CSF specifically decreased synaptic AMPAR mediated excitatory postsynaptic currents, while NMDAR mediated currents were not affected. While commercial available antibodies can compete with patients’ antibodies to bind with AMPA receptors, they don’t necessarily have similar effect as patient’s antibodies in decreasing synaptic AMPA receptors. These results together suggest that the in the model of anti-AMPAR encephalitis, the loss of AMPA receptors eliminates AMPAR-mediated synaptic function resulting in the memory and other behavioral deficits observed in patients with anti-AMPAR encephalitis.

We have previously shown that the antibodies from anti-NMDA receptor encephalitis selectively decrease synaptic NMDA receptor clusters by capping and cross-linking induced internalization (Hughes et al., 2010). In this study, our results suggest that the antibodies from anti-AMPA receptor encephalitis patients also have similar effects. In addition our data suggest that the patients’ antibodies against GluR1 or GluR2 epitopes have similar effects on reducing surface/synaptic GluR1 and GluR2 subunits, without obvious selectivity on either one subunit or compensation from the other one. This is probably because hippocampal AMPA receptors are mainly GluR1/GluR2
heteromers (Lu et al., 2009). Once the patients’ antibodies associated with any one of the subunits, they could initiate capping and cross-linking resulting in endocytosis of the whole AMPA receptor.

Under physiological conditions, such as during LTD, it has been suggested that AMPA receptors are first moved to extrasynaptic sites followed by endocytosis through clathrin dependent pathways ((Ehlers, 2000; Shi et al., 2001; Park et al., 2004)). The endocytosis of AMPA receptors have at least one GluR2 dependent pathway that requires GluR2 C-terminals interaction with clathrin (Lüthi et al., 1999; Lee et al., 2002) and GluR2 independent pathways (Jia et al., 1996; Meng et al., 2003), which may involve AMPA receptor activity-dependent ubiquitination of GluR1 and subsequent internalization and their trafficking to the lysosome (Ehlers, 2000; Schwarz et al., 2010). What pathway is used in this pathogenic condition is a future question to address. In addition, whether similar endocytotic pathways are utilized in anti-NMDA receptor encephalitis and anti-AMPA receptor encephalitis or even more antoimmune encephalitis are not known. At least, obvious loss of surface GABAb receptors was not observed in culture model of anti-GABAb receptor encephalitis (Lancaster et al. Neurology 2010 abstract, in press) suggesting that different mechanisms exist.

Preliminary analyses suggest that the location of the epitope of patients’ antibodies is the N-terminal extracellular domain of these AMPA receptor subunits (Gleichman et al., 2009), but details are still under investigation. The commercial antibodies with epitope on amino acids 271-285 of rat GluR1 do not have similar effects
as the patient’s antibodies and conversely, the commercial antibody against N-terminal portion of GluR2 from AA 175-430 has partial effects as the patients’ antibodies, suggesting that the epitope recognized by patients’ antibodies probably overlaps with that recognized by the commercial anti-GluR2 antibody. Future studies on patients’ antibodies epitope will help to address this question. However, the available commercial antibodies and immunized animals may provide useful animal models to study anti-AMPA antibody effects on circuits, learning, memory and behavior.

The consequences of loss of AMPA receptor expression have 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 et al., 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, AMPA receptor 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 AMPA receptors (Mainen et al., 1998). While AMPA receptor subunit knockout mice have not provided a satisfying explanation for the role of AMPA receptors in synaptic plasticity related to learning and memory, the fact that patients with AMPA receptor antibodies have short-term learning and memory deficits argues that further studies at the circuit and behavioral levels are warranted.
Methods

Preparation of patient and control CSF and IgG

Patient or control cerebrospinal fluid (CSF) was collected and filtered using protein A/G sepharose columns. CSF was diluted 1:15-100 to treat neurons in vitro. In some experiments, Patients’ serum was used to treat neurons (Fig. 1D; Fig.2G). Each CSF was tested for antibody reactivity by staining mouse or rat brain sections or HEK cells expressing GluR1/GluR2 heteromers of the AMPAR as previously described (Lai et al., 2009).

Cell culture and patient antibody treatment

Primary rat hippocampal neuron and astrocyte cultures were prepared from embryonic day 18-19 as previously described (Goslin et al., 1988). Briefly, hippocampi were in Ca\(^{2+}\) free HBSS containing 1% papain for 20 min., triturated in Basal Media Eagle (BME; Invitrogen) supplemented with B-27 (Life Technology) and plated at 100,000 or 400,000 (for biotinylation) cells per ml in Neural Basal (NB) supplemented with 10% FBS (Hyclone), B-27, 1% Penicillin and Streptomycin (Life Technologies) and 1% L-Glutamine (Life Technologies) on poly-L-lysine coated (Sigma) coverslips in 24-well plates. Culture media was changed to Neural basal (Life Technologies) supplemented with B27 at 4 \textit{div}. Cells were maintained at 37 °C, 5% CO\(_2\), 95% humidity; medium was changed weekly. Control or patient CSF was used at 1:15 – 1:100 dilutions to treat neurons. Serums were used at 1:200 dilutions.
Immunostaining for pre- and postsynaptic components

To label surface AMPAR clusters and measure the subsequent internalization, neurons were incubated with commercial anti-GluR1 (Calbiochem) antibodies for 2 hours. This commercial antibody can associate with AMPA receptors but not cross link and do not induce significant reduction of surface AMPA receptors. After 2 hours, unbound antibodies were washed off and neurons where treated with control or patient’s CSF containing anti-GluR1 antibodies for 24 hours. After treatment, remaining surface AMPA receptor can be labeled by applying FITC conjugated secondary antibodies against commercial anti-GluR1 (Calbiochem) while the neurons are alive and without permeabilization. After fixation and permeabilization, internalized AMPA receptor clusters can be visualized by applying TRITC conjugated secondary antibodies.

To stain surface AMPAR clusters, control or treated neurons were washed in NB plus B27 and were incubated with commercial extracellular anti-GluR1 or anti-GluR2 antibodies for 30 min., washed and incubated with appropriate fluorescently conjugated secondary antibodies for 30 min., and washed in PBS. Neurons were then fixed in 4% paraformaldehyde, 4% sucrose in PBS, pH = 7.4 for 15 min., permeabilized with cold 0.25% Triton X-100 for 5 min., and blocked in 5% normal goat serum (Invitrogen) for 1 hour at RT. Additional immunostaining was performed with various combinations of primary antibodies: to label glutamate receptors and other postsynaptic proteins, anti-NR1 (1:1000; Chemicon), anti-GluR1 against intracellular epitope (1:10; CalBioChem)
or anti-GluR2/3 with against epitope (1:100; Chemicon), anti-GluR1 against extracellular epitope (1:10; Calbiochem), anti-GluR2 against extracellular epitope (1:500; Millipore), anti-PSD-95 (1:500; Affinity BioReagents), anti-Stargazin (1:500; Chemicon); to label presynaptic terminals, mouse anti-SV2 (1:200; DHSB); guinea pig anti-VGLUT 1 (1:5000; Chemicon), or mouse anti-Bassoon (1:400; Stressgen Bioreagents). Antibodies were visualized after staining with the appropriate fluorescently conjugated secondary antibodies (1:200, Jackson ImmunoResearch).

Confocal imaging, image analysis and statistical analysis

For all experiments, 6-12 randomly selected pyramidal neurons, identified by morphology (Elmariah et al., 2004; Elmariah et al., 2005) in each condition were confocally imaged (Leica TCS 4D system) on each of 2-3 coverslips in 3-5 independent experiments. Images were thresholded automatically using an iterative thresholding technique (Bergsman et al., 2006), and the number and area of individual immunostained pre- or postsynaptic clusters were determined using interactive software (custom-written ImageJ macros). Clusters with pixel overlap of pre- and postsynaptic markers were considered colocalized and thus synaptic. Cluster density was compared among conditions using the Kruskal-Wallis nonparametric ANOVA test followed by Dunn’s pairwise multiple comparison test, unless otherwise indicated. All values are presented as mean ± s.e.m.

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Biotinylation of surface proteins and analysis by Western blot

Neurons were treated with 1:200 diluted patient serum with anti-GluR1 or anti-GluR2 antibodies for 1 day, washed with PBS supplemented with 0.1 mM CaCl₂ and 1 mM MgCl₂ (rinsing buffer) and incubated for 30 min. at 4 °C with 1 mg/ml Sulfo-NHS-Biotin (Thermo Scientific) in rinsing buffer. Neurons were then washed with rinsing buffer + 100 mM glycine (quenching buffer), incubated in quenching buffer for 30 minutes at 4°C to quench excess biotin, then 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 cocktail III, Calbiochem) at 4 °C for 1 hour. Lysates were cleared of debris by centrifugation at 12400 x g for 20 min. An aliquot of the remaining supernatant was taken for the lysate fraction, and a second aliquot was incubated with avidin-linked agarose beads (Immobilized Monomeric Avidin, Thermo Scientific) overnight at 4 °C. After centrifugation, the supernatant was removed and the beads (surface fraction) were washed 1X RIPA buffer, 2X high-salt wash buffer (500 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.1% Triton X-100, pH 7.5), and 1X no-salt wash buffer (50 mM Tris, pH 7.5). The surface fraction was eluted from the beads with 2X sample buffer and proteins separated on an 8% gel using SDS-PAGE. Samples were transferred to nitrocellulose membranes and probed for antibodies against NR1 (1:1000, chemicon), GluR1 (1:200, Calbiochem), GluR 2/3 (1:200, 07-598, Upstate), Stargazin(1:500, Chemicon), GABA₆Rs (1:1000, chemicon) and actin (1:2000, Sigma). Actin and GABA₆Rs were used as loading controls for total and surface
fractions, respectively. Blots were incubated with HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (1:3000, Cell Signaling), and signals were visualized using chemiluminescence (SuperSignal Chemiluminescent Substrate, Thermo Scientific). All quantified films were in the linear range of exposure, were digitally scanned and signals quantified using NIH ImageJ.

*Whole cell electrophysiological recordings of synaptic NMDA and AMPA receptor mediated currents*

Whole cell voltage clamp recordings were performed from 14 – 21 div pyramidal neurons treated for 24-48 hours with patient CSF containing anti-NR1 antibodies, control CSF or left untreated. Briefly, neurons were incubated in extracellular physiology solution (in mM: 119 NaCl, 5 KCl, 2 CaCl$_2$, 2MgCl$_2$, 30 Glucose, 10 HEPES, pH = 7.4) for AMAPR minis. For NMDAR minis, no MgCl$_2$ is included and Glycine (1 µM) is added to reveal all NMDA receptor currents. Voltage-clamp recordings were made at RT (22-25 °C) using glass microelectrodes (resistance 4-6 MΩ) filled with a Cesium substituted intracellular solution (in mM: 100 Cesium gluconate, 0.2 EGTA, 5 MgCl$_2$, 2 ATP, 0.3 GTP, 40 HEPES, pH = 7.2). Pipette voltage offset was neutralized before the formation of a gigaohm seal. 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, using the whole cell application of HEKA software. Criteria for cell inclusion in the data set included a series resistance ≤ 30 MΩ and
stability throughout the recording period. Currents were amplified, low-pass filtered at 2.5 kHz, and sampled at 5 Hz using pCLAMP. Miniature excitatory spontaneous currents (mEPSCs) were recorded at -70mV in the presence of TTX (1 µM), Picrotoxin (10 µM), APV (50 µM) and CNQX (10 µM) were bath applied to block NMDAR and AMPAR mediated currents respectively. mEPSC events were detected and analyzed using MiniAnalysis (Synaptosoft, Leonia, NY), which employs a threshold-based event-detection algorithm. NMDAR and AMPAR components of mEPSCs were separated temporally by their distinct kinetics (Hestrin et al., 1990; Watt et al., 2000; Yang et al., 2003). All values are presented as mean ± s.e.m.
Figure 1: Patients’ anti-GluR1 or anti-GluR2 antibodies selectively decrease synaptic and surface AMPA receptor clusters

**A.** Hippocampal neurons immunostained for GluR1 or GluR2 containing AMPAR clusters and postsynaptic PSD-95. Synaptic AMPARs are defined as the colocalization between GluR1 or GluR2/3 staining and PSD-95 staining. Anti-GluR2
(middle panel) or anti-GluR1 (right panel) patient CSF treatment for 24 hours reduced synaptic GluR2 as well as GluR1 clusters density without affecting PSD-95 density (N = 6-18 neurons from 1-3 independent experiments for each condition). Scale bar = 10 μm.

**B.** Quantification of density of synaptic GluR1 clusters defined as colocalization between GluR1 and PSD-95 clusters per 20 μm dendrite from neurons treated with control, anti-GluR1 or anti-GluR2 patient’s CSF (compared with control, anti-GluR2 patient’s CSF treatment = 0.74 ± 0.08, Mann-Whitney test, p = 0.08; anti-GluR1 patient’s CSF treatment = 0.64 ± 0.05, p = 0.04).

**C.** Quantification of density of synaptic GluR2/3 clusters defined as the colocalization between GluR2/3 and PSD-95 clusters per 20 μm dendrite from neurons treated with control, anti-GluR1 or anti-GluR2 patient’s CSF (GluR2 patient’s CSF treatment = 0.54 ± 0.06, p < 0.0001; GluR1 patient’s CSF treatment = 0.39 ± 0.03, p = 0.0002).

**D.** Western blot analyses of surface biotinylated and total lysate AMPAR protein. Patient serum treatment for 1 day reduces surface AMPAR protein. Actin was used as the loading control.

**E.** Surface NMDAR protein after treatment with serum from anti-GluR1 or anti-GluR2 patients, showing a decrease in AMPAR protein (1 anti-GluR1 patient, anti-GluR2 patient, 1 control, 1 experiment).
Figure 2: Patients’ anti-GluR1 or anti-GluR2 antibodies decrease the clusters density of Stargazin but not other synaptic proteins.
A. Hippocampal neurons immunostained for the presynaptic marker vGlut or the postsynaptic markers PSD-95, NR1 or Stargazin. Synaptic Stargazin is defined as the colocalization between Stargazin staining and PSD-95 staining. Anti-GluR2 patient CSF treatment for 24 hours reduces synaptic Stargazin cluster density without affecting PSD-95 and NR1 density (12-36 neurons from 2-3 independent trials for each condition). Scale bar = 10 µm.

B. Quantification of density of excitatory synapses defined as colocalization between postsynaptic PSD-95 and presynaptic vGlut per 20 µm dendrite from neurons treated with control or patient’s CSF (patient’s CSF treatment = 93 % ± 10 % of control CSF treatment, p = 0.3).

C. Quantification of density of PSD-95 clusters per 20 µm dendrite from neurons treated with control or patient’s CSF (control = 16.7 ± 0.93, patient = 15.2 ± 0.7, Mann-Whitney U test, p = 0.16).

D. Quantification of density of vGlut clusters per 20 µm dendrite from neurons treated with control or patient’s CSF (control = 10.2 ± 0.7, patient = 9.1 ± 0.7, Mann-Whitney U test, p = 0.16).

E. Quantification of density of NR1 clusters per 20 µm dendrite from neurons treated with control or patient’s CSF (control = 17.0 ± 1.1, patient = 16.1 ± 1.2, Mann-Whitney U test, p = 0.57).

F. Quantification of synaptic Stargazin defined as the colocalization between Stargazin staining and PSD-95 staining per 20 µm dendrite from neurons treated with
control or patient’s CSF (control = 14.2 ± 3.1, patient = 6.3 ± 1.5, Mann-Whitney U test, p = 0.02).

G. Western blot analyses of surface biotinylated Stargazin, NMDAR and GABAb receptor protein. Patient serum treatment for 1 day reduced surface Stargazin protein.

H. Quantification of surface Stargazin, NMDAR and GABAb receptor protein after treatment with serum from anti-GluR1 or anti-GluR2 patients, showing a decrease in Stargazin protein (1 anti-GluR1 patient, 1 anti-GluR2 patient, 1 control, 1 experiment; no statistics were performed).
Figure 3: Patients’ antibodies decrease AMPA receptor but not NMDA receptor mediated synaptic transmission
A. mEPSCs recorded in physiological saline with TTX, picrotoxin, APV, an NMDAR antagonist to isolate synaptic AMPAR mediated currents (upper trace). Under the same recording conditions, treatment of hippocampal neurons with patient CSF for 1 day dramatically reduces synaptic NMDAR mediated currents (bottom traces). N = 4 neurons treated with control CSF, 4 neurons treated with patient CSF, 1 patient and 1 control sample.

B. Representative average mEPSCs from neurons treated for 1 day with control CSF (left) or patient CSF (right). Neurons treated for 1 day with patient CSF have less AMPAR-mediated synaptic current than neurons treated with control CSF.

C. Effect of patient antibodies AMPA receptor mediated synaptic currents amplitudes (control = 17.6 ± 3.7, patient’s CSF treated = 10.9 ± 0.7; asterisk indicates significant difference, Student’s t-test, p = 0.04).

D. Effect of patient antibodies AMPA receptor mediated synaptic currents frequency (control = 3.2 ± 0.4, patient’s CSF treated = 2.3 ± 1.4; Student’s t-test, p = 0.48).

E. mEPSCs recorded in physiological saline with TTX, picrotoxin, CNQX, an AMPAR antagonist to isolate synaptic NMDAR mediated currents. Treatment of hippocampal neurons with control (upper trace) and patient CSF (bottom traces) for 1 day have similar synaptic NMDAR mediated currents. N = 4 control CSF treated neurons, 5 patient CSF treated neurons, 1 patient, 1 control sample.
F. Representative average mEPSCs from neurons treated for 1 day with control CSF (left) or patient CSF (right). Treatment of hippocampal neurons with control (left trace) and patient CSF (right traces) for 1 day have similar synaptic NMDAR mediated currents.

G. Effect of patient antibodies on NMDA receptor mediated synaptic currents amplitudes (control = 12.8 ± 1.2, patient’s CSF treated = 12.1 ± 1.7; Student’s t-test, p = 0.56).

H. Effect of patient antibodies AMPA receptor mediated synaptic currents frequency (control = 6.1 ± 0.7, patient’s CSF treated = 6.1 ± 0.3; Student’s t-test, p = 0.94).
Figure 4: Patients’ antibodies increase the internalization of AMPA receptor clusters

**A.** Hippocampal neurons live labeled for surface GluR1 for 2 hours, then treated with control or patient CSF for 24 hours, followed by immunostaining for the remaining surface GluR1 in live neurons, then fixed, permeabilized and immunostained for internal GluR1 and postsynaptic marker PSD-95. Patient CSF treatment for 24 hours increases internal GluR1 cluster density. N = 9 neurons from 1 trial for each condition, Scale bar = 10 µm.
B. Quantification of density of surface GluR1 clusters per 20 µm dendrite from neurons treated with control or patient’s CSF (control treated = 15.9 ± 1.1, patient’s CSF treated = 19.4 ± 1.3, Mann-Whitney U test, p = 0.06).

C. Quantification of density of internal GluR1 clusters per 20 µm dendrite from neurons treated with control or patient’s CSF (control treated = 3.2 ± 0.4, patient’s CSF treated = 6.7 ± 1.3, Mann-Whitney U test, p = 0.01, asterisk indicates significant difference compared to control CSF treated neurons).
Figure 5: Commercial antibodies pre-block patients’ antibodies

A. Neurons were fixed but non-permeabilized, pre-incubated for control, anti-GluR1 or anti-GluR2 patient’s CSF, then immunostained for surface GluR1. Both AMPAR Patient CSF preincubation for one hours decrease surface GluR1 clusters density staining. Scale bar = 10 µm. 6 neurons from 1 trial of treatment for each condition.

B. Quantification of density of surface GluR1 clusters per 20 µm dendrite from neurons pre-incubated with control or patient’s CSF. (Control CSF pre-incubated = 40.4 ± 4.4, GluR1 patient’s CSF pre-incubated = 27.7 ± 3.0, compare to control, Mann-Whitney test, p = 0.03; GluR2 patient’s CSF pre-incubated = 29.4 ± 1.8, p = 0.04)
Figure 6: **Commercial antibodies do not have similar effects as patients’ antibodies**

A. Neurons were treated with PBS, commercial anti-GluR2 or anti-GluR1 antibodies with and without secondary antibodies to crosslink the primary antibodies, or secondary antibodies alone for 24 hours. Neurons were immunostained with C-term anti-GluR1 antibodies and presynaptic marker Synapsin. Commercial anti-GluR2 antibodies + secondary treatment for 24 hours decrease synaptic GluR1 clusters density staining. Scale bar = 10 µm. 5-6 neurons from one trial of experiment for each condition.

B. Quantification of density of synaptic GluR1 clusters per 20 µm dendrite from neurons treated with commercial GluR2 antibody (ANOVA followed by Dunn’s multiple comparison test, p < 0.0001, asterisk indicates significant difference)
C. Quantification of density of synaptic GluR1 clusters per 20 \( \mu \text{m} \) dendrite from neurons treated with commercial GluR1 antibodies (ANOVA followed by Dunn’s multiple comparison test, \( p < 0.0001 \), asterisk indicates significant difference).
Conclusions and future directions

The work discussed in the previous chapters extended our understanding of how functional neural circuits are established and maintained. In particular, I have investigated the spatial distribution of functional properties of presynaptic terminals along axon arbors and suggest that the mechanisms that determine synaptic strength differ spatially. I have also examined the structural and functional consequences of the loss of postsynaptic glutamate receptors that occurs in two forms of human autoimmune encephalitis. My results suggest that patient antibodies selectively cause reduction of surface receptor clusters, their synaptic localization, and the synaptic currents mediated by these receptors. My work extends our understanding of the repertoire of pre- and postsynaptic mechanisms that are required to establish and maintain functional neural circuits during development and in diseases that compromise nervous system function.

The work investigating the spatial distribution of presynaptic function raises important questions for future research into the formation and maintenance of functional neural circuits (Chapter 2). One of them, raised from the observation of a proximal–distal difference in presynaptic strength, leads to the question of what is the physiological implication of this difference. One hypothesis is that the loss of action potentials due to branch point failures along axon arbors results in a compensatory increase in distal presynaptic strengths. Previous work using simulations suggested that action potentials can be lost due to branch point failures and en passant terminals (Lüscher and Shiner,
1990). Though there was the observation that action potentials invaded proximal axon arbors and all their branches efficiently within 100 µm from the cell body (Cox et al., 2000), direct examination of action potential activities over longer distances along the axon arbors >1000 µm is still missing. These together give rise to the hypothesis that the possibility that distal segments of axons see fewer action potentials from the soma underlies the higher presynaptic strengths in distal terminals. Measuring action potentials in small axons of central neurons is difficult to carry out; however, the following experiments can address the previous question without directly measuring action potentials in axon arbors. First, further detailed investigation of the relationship between presynaptic strengths and spatial distribution is needed. My research examined the most proximal and the most distal terminals and their presynaptic strengths. Future works should examine the relationship between the presynaptic strengths and the terminals’ distance to the cell body, the number of branch points between the terminals and the soma, and the number and density of en passant terminals along the axon arbors. A second set of experiments could investigate the changes in presynaptic strengths during activity manipulations. Activity blockade using TTX, to completely eliminate action potentials in cultures, can be used to see whether the proximal-distal difference of presynaptic strengths can be eliminated. A third set of experiments could examine changes in presynaptic strengths in response to spatially distinct stimulation, for example comparing the presynaptic response when the action potential is triggered locally in axon arbors and when it is triggered from the cell body. The differences in presynaptic
strengths measured when the stimulation locations are different would indicate loss of action potentials along axon arbors in between the soma and the terminal of interest. Together, these results could help to address the possible causes of differences in proximal and distal presynaptic strengths that I observed in my research.

Other interesting future research includes the following. It will be of interest to examine how the proximal-distal difference is established during development and maturation of neural circuits. Also, it will be important to examine pre- and postsynaptic mechanisms in a spatially specified manner in vivo to address how synaptic properties at different spatial locations along axons and dendrites are specified and how different mechanisms are orchestrated to achieve this spatial heterogeneity. Techniques to specifically label a small number of neurons and their synapses (Li et al., 2010; Marshel et al., 2010) and to precisely activate individual neurons and/or synapses (Knöpfel et al., 2010) have already set the stage for this type of study.

The identification of cellular and synaptic mechanisms underlying anti-NMDAR encephalitis (Chapter 3 and Chapter 4) and anti-AMPAR encephalitis (Chapter 5) raises many important questions for future research. One interesting question is through what pathways these receptors are internalized, and are they degraded or recycled. NMDA receptors and AMPA receptors are trafficked into and out of postsynaptic sites during physiological processes. Internalization of NMDA receptors has been shown to be mediated by clathrin via endocytotic signal on C-terminal of NR2A or NR2B subunits (Roche et al., 2001; Lavezzari et al., 2004). NMDA receptors then sort into different
intracellular pathways after endocytosis (Lavezzari et al., 2004). Under physiological conditions, such as during LTD, it has been suggested that AMPA receptors are first moved to extrasynaptic sites followed by endocytosis through clathrin dependent pathways (Ehlers, 2000; Shi et al., 2001; Park et al., 2004). The endocytosis of AMPA receptors can follow a GluR2-dependent pathway (Lüthi et al., 1999; Lee et al., 2002) or a GluR2-independent pathway (Jia et al., 1996; Ehlers, 2000; Meng et al., 2003; Schwarz et al., 2010). Whether the internalization of the patients’ antibodies in encephalitis is mediated by a pathway that was already identified in physiological conditions, or by a pathway specific for pathological conditions, is a question to be addressed. One set of experiments could investigate whether known endocytic machinery is involved in internalization of these receptors. Pharmacological blockade or RNAi could be used to interfere with the function or expression of clathrin to allow for investigation into whether the internalization is dependent on clathrin. In addition, experiments could be performed using known NMDAR or AMPAR mutants that can block specific internalization pathways of these receptors (Lavezzari et al., 2004). These experiments will help to address whether the same endocytotic signals on these receptors are needed in pathological situations. A second set of experiments can examine where the internalized receptors are localized, and whether they are degraded or recycled. Receptors on the neuronal surface can be labeled and treated with patients’ antibodies to induce internalization, followed by labeling of internalized receptors and immunocytochemistry for markers of other subcellular structures, such as Rab-5.
(recycling endosome), Rab-9 (late endosomes), Lamp1 (lysosomes), P4D1 (ubiquitin) and proteosome subunits. Together, these results can illuminate the mechanisms and pathways by which NMDAR or AMPAR are internalized, degraded or recycled during autoimmune encephalitis.

Other important directions include the following. One is the generation of *in vivo* animal models to examine how the autoantibodies cause the cellular and physiological defects in neural circuits and how these give rise to the repertoire of behavioral and cognitive deficits in the patients. Generation of the animal models can help to address several important questions. One interesting question is to examine the alterations of synaptic transmission and functional plasticity that are caused by these autoantibodies and that give rise to behavioral deficits. This animal model is more relevant to understanding human behavior, cognition, and disease than knockout animal models. Further interesting questions concern the examination of the cascade of events leading to peripheral autoantibody production, the penetration of autoantibodies through the blood-brain-barrier (BBB), and potential intrathecal synthesis of autoantibodies. A third important direction is to study the process of recovery and relapse, and to examine which neural effects are reversible, and which tend to be refractory to treatment. Since many other severe neurological diseases such as schizophrenia and autism are not reversible, in contrast to autoimmune mediated encephalitis, studying the recovery and relapse of autoimmune mediated encephalitis may be helpful for treatment of other neurological diseases. Ultimately, knowledge obtained by studying the mechanisms underlying these
autoimmune mediated encephalitides will help to further understanding and improve treatment of other neurological diseases such as schizophrenia and autism.
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