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NMDA receptors in the central nervous system

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Abstract

NMDA-type glutamate receptors are ligand-gated ion channels that mediate a major component of excitatory neurotransmission in the central nervous system (CNS). They are widely distributed at all stages of development and are critically involved in normal brain functions, including neuronal development and synaptic plasticity. NMDA receptors are also implicated in the pathophysiology of numerous neurological and psychiatric disorders, such as ischemic stroke, traumatic brain injury, Alzheimer's disease, epilepsy, mood disorders, and schizophrenia. For these reasons, NMDA receptors have been intensively studied in the past several decades to elucidate their physiological roles and to advance them as therapeutic targets. Seven NMDA receptor subunits exist that assemble into a diverse array of tetrameric receptor complexes, which are differently regulated, have distinct regional and developmental expression, and possess a wide range of functional and pharmacological properties. The diversity in subunit composition creates NMDA receptor subtypes with distinct physiological roles across neuronal cell types and brain regions, and enables precise tuning of synaptic transmission. Here, we will review the relationship between NMDA receptor structure and function, the diversity and significance of NMDA receptor subtypes in the CNS, as well as principles and rules by which NMDA receptors operate in the CNS under normal and pathological conditions.

Keywords

Ionotropic glutamate receptor; neurotransmitter; NMDA; ion channel; regulation; structure-function; disease; synaptic transmission

1 Introduction

Glutamatergic neurotransmission in the CNS is mediated by metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors (iGluRs). The iGluRs are ligand-gated ion channels permeable to cations (Na^+, K^+, and Ca^{2+}) that can be divided into three functional classes, namely the α-amino-3-hydroxy-5-methyl-4-isoxasolepropionic acid

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(AMPA) receptors, kainate receptors, and N-methyl-D-aspartate (NMDA) receptors [1,2] (Fig. 1a,b). These functional classes were historically named on the basis of their pharmacological properties (i.e. the activating agonist), but the division was firmly established by subsequent cloning that demonstrated strong correlation between the sequence identity and the pharmacological properties of subunits in these functional classes. The δ (delta) receptors are also considered iGluRs, primarily based on sequence identity, but their function is not fully understood [3–5]. The δ (delta) receptors appear to play important roles in synapse organization and some forms of synaptic plasticity [6–8], but it is uncertain whether they are capable of forming functional ion channels [9–11]. NMDA receptors exhibit voltage-dependent Mg^{2+}-block, high permeability to Ca^{2+}, and require simultaneous binding of the co-agonists glycine and glutamate for activation. These hallmark features distinguish NMDA receptors from AMPA/kainate receptors (i.e. non-NMDA receptors) and have profound impact on their physiological roles in the CNS.

In most central synapses, the release of glutamate activates excitatory postsynaptic currents (EPSCs) with a time course that can be described primarily by two exponential components corresponding to activation of AMPA and NMDA receptors. Activation of AMPA receptors mediates a fast component with rapid rise time and decay, whereas activation of NMDA receptors mediates a slower component with slower rise time and a time course lasting for tens to hundreds of milliseconds [12–15] (Fig. 1c). Activation of postsynaptic kainate receptors typically result in EPSCs with a slower time course than AMPA receptors and a comparable, but generally faster time course than NMDA receptors [16]. At resting membrane potential, the NMDA receptor ion channel is blocked by physiological levels of extracellular Mg^{2+}, but synaptic release of glutamate and the resulting rapid activation of AMPA/kainate receptors can depolarize the membrane potential and thereby relieve voltage-dependent Mg^{2+}-block of NMDA receptors [17,18] (Fig, 1d). Thus, NMDA receptors serve as coincidence detectors that require simultaneous presynaptic release of glutamate and postsynaptic depolarization in order to produce the slow Ca^{2+}-permeable component of the EPSC [19,20].

The NMDA receptors can mediate substantial Ca^{2+}-influx during the EPSC due both to their high Ca2+ permeability and prolonged time course. The resulting increase in intracellular Ca^{2+} can trigger multiple downstream signaling events in the postsynaptic neuron, which are central to the roles of NMDA receptors under both normal and pathophysiological conditions [21,22,2,1,23]. The rise in intracellular Ca^{2+} triggers both short-term and long-term effects, which are accompanied by changes in synaptic efficacy and neuronal morphology (i.e. synaptic plasticity) [24]. Robust NMDA receptor-mediated Ca^{2+}-influx for a short duration can lead to long-term potentiation (LTP) of synaptic efficacy, whereas less pronounced Ca^{2+}-influx for a longer duration can result in long-term depression (LTD) [25,26]. Thus, the frequency and duration of synaptic NMDA receptor activation can result in either potentiation or depression of synaptic efficacy, which is considered a cellular correlate of memory and learning [27,28].

Glutamate is sufficient for activation of AMPA and kainate receptors, whereas the NMDA receptors are unique in that they require simultaneous binding of two distinct agonists, glutamate and glycine/D-serine, for activation [29–35]. In the CNS, NMDA receptors

mainly rely on synaptic release of glutamate for activation, since extracellular glycine (or D-serine) is thought to be continuously present. Whether glycine or D-serine serves as the endogenous co-agonist may depend on brain region and subcellular compartment [36–38]. For example, it has recently been suggested that D-serine is the predominant co-agonist in synapses, whereas glycine is more prevalent at extrasynaptic sites [39]; more work is needed to determine whether this is a principle that transcends anatomical regions. Furthermore, glycine and D-serine are not present at concentrations that fully saturate the NMDA receptor co-agonist binding sites, at least in some brain regions [40,41]. Thus, the co-agonist requirement enables an additional mechanism of NMDA receptor regulation, in which activation is controlled by phasic changes in glutamate concentrations (i.e. synaptic release), but the magnitude of activation can be modulated by changes in the tonic concentration of glycine/D-serine. Given the central roles of NMDA receptors in normal brain function, it is not surprising that their dysregulation has been linked to a number of pathophysiological conditions [2,1,42,23]. In acute conditions, such as ischemia, seizures, and traumatic brain injury, the increase in extracellular glutamate that follows increased release and impaired uptake can produce profound NMDA receptor-mediated Ca^{2+}-flux into the neuron, which may promote neuronal death [43–46]. Impairment of neuronal health by glutamate-mediate signaling is often referred to as “excitotoxicity” [47]. Under chronic conditions of enhanced neuronal susceptibility, as in Parkinson’s and Alzheimer’s diseases, the NMDA receptor-mediated excitotoxicity may contribute to impairment of neuronal health over many years (e.g. see [48]). NMDA receptor antagonists have been proposed to be beneficial under such conditions involving excitotoxicity (e.g. see [49]), but side effects, such as psychosis, memory impairment, anesthesia, and neuronal cell death, can accompany strong and non-selective NMDA receptor blockade, thereby limiting the clinical usefulness of such drugs for chronic conditions [50,51].

Interestingly, the side effects of high-affinity NMDA receptor channel blockers resemble symptoms exhibited by patients suffering from schizophrenia. The observations of these “psychotomimetic” properties of the channel blockers PCP and ketamine have led to the “NMDA receptor hypofunction model of psychosis”, which proposes that multiple symptoms associated with in schizophrenia may be caused by lower than normal NMDA-receptor-mediated glutamatergic activity in key brain circuits [52,51,53]. In theory, enhancing NMDA receptor function, perhaps selectively in key brain circuits, could be beneficial for treating cognitive disorders and schizophrenia. However, NMDA receptor agonists have not been fully studied in this context due to the risk that excessive stimulation may cause excitotoxicity, and indirect methods to enhance NMDA receptor function through block of glycine uptake have been inconclusive. Moreover, only very recently have subunit-selective NMDA receptor positive allosteric modulators been identified that allow this idea to be further investigated (see below). In this regard, subunit-selective modulators of NMDA receptors may be therapeutically beneficial in some CNS disorders, since these modulators would target NMDA receptor subtypes in specific neuronal population or brain regions associated with the disease without affecting NMDA receptors in other regions [54–56].

1.1 NMDA receptor subunit diversity

The arrival of the action potential at the presynaptic terminal triggers the release of glutamate into the synaptic cleft. Termination of glutamatergic neurotransmission is mediated by diffusion and rapid removal of glutamate from synaptic and extrasynaptic sites via reuptake by excitatory amino acid transporters (EAATs; i.e. glutamate transporters) [57]. Synaptically-released glutamate reaches a very high peak concentration (~1 mM) for a brief duration (~1 ms) [58]. In this short period of time, glutamate will bind iGluRs and initiate receptor conformational changes that lead to opening of the ion channel (i.e. ion channel gating). However, the NMDA receptor-mediated component of the EPSC continues for tens to hundreds of millisecond after synaptic glutamate is removed, during which time, NMDA receptors transition between glutamate-bound open and closed conformational states until glutamate eventually unbinds and the EPSC is terminated. Thus, the time course of the EPSC is governed by glutamate binding affinity, the connectivity and lifetime of the receptor in pre-gating conformations that must be traversed before unbinding, and the rates into and out of the desensitized states following agonist binding [59–61]. For NMDA receptors, these functional properties are controlled by the subunit composition [62–64] (Fig. 2). Subunit diversity among NMDA receptors and assembly of different receptor subtypes with distinct functional properties enable precise tuning of the synaptic response and allows variation in the physiological roles of NMDA receptors at synaptic versus extrasynaptic sites, in different neuronal cell types and brain regions, and during neuronal development.

Seven genes that encode NMDA receptor subunits have been identified, which include GluN1, four different GluN2 (GluN2A-D), and two GluN3 subunits (GluN3A-B) [2,1] (Fig. 1a). All NMDA receptors are obligatory heteromeric assemblies of four subunits that form a central ion channel pore, and the majority of NMDA receptors in the CNS are composed of two glycine-binding GluN1 and two glutamate-binding GluN2 subunits (i.e. GluN1/2 receptors) [65–67] (Fig. 1b). However, the glycine-binding GluN3 subunits can also assemble with GluN1 and GluN2 subunits to form GluN1/2/3 receptors or with GluN1 alone to form GluN1/3 receptors [68–72].

1.2 The GluN1 subunit

The glycine/D-serine-binding GluN1 subunit is ubiquitously distributed in the brain and is an obligatory subunit in all NMDA receptor subtypes. GluN1 has eight different isoforms that arise from alternative splicing of three exons within of a single gene product [73–76] (Fig. 3a,b). Exon 5 encodes 21 highly charged amino acids in the GluN1 amino-terminal domain (ATD) named the N1 cassette, exon 21 encodes 37 amino acids in the carboxyl-terminal domain (CTD) named the C1 cassette, and exon 22 encodes 38 amino acids in the CTD named the C2 cassette. Deletion of exon 22 eliminates a stop codon and causes a reading frame shift, which results in the inclusion of 22 alternative amino acids named the C2’ cassette. Different GluN1 splice variants have distinct regional and developmental expression patterns [77–79] and display differences in NMDA receptor function and pharmacology (see below; Fig. 3b,c).

NMDA receptors with GluN1 subunits that contain the N1 cassette (i.e. exon 5) have reduced agonist potency (i.e. increased EC_{50}) and are less sensitive to inhibition by protons

and extracellular zinc [80,81]. Consistent with the effect on agonist potency, the presence of the N1 cassette accelerates deactivation of glutamate-activated NMDA receptor responses, which shortens the time course of EPSCs [82–84] (Fig. 3c). Furthermore, the N1 cassette has a negative impact on modulation of NMDA receptor function by GluN2B-selective antagonists, such as ifenprodil [85,86], and potentiation by extracellular polyamines, such as spermine [82,81,74,87,88] (Fig. 3d,e). Alternative splicing of exons 21 and 22 dramatically alter the length and the amino acid sequence of the intracellular GluN1 CTD, which mediates interactions with several intracellular proteins, including PSD-95, calmodulin, and the neurofilament subunit NF-L [1]. Many of these proteins are involved in surface trafficking and anchoring of receptors at synaptic sites, and alternative splicing of exons 21 and 22 can therefore mediate changes in the subcellular distribution of NMDA receptors [89–91]. Contrasting with the changes observed upon inclusion of exon 5, there has not been convincing demonstration that alternative splicing of exons 21 and 22 has strong effects on the functional and pharmacological properties of NMDA receptors.

1.3 The GluN2 subunits

The four different glutamate-binding GluN2 subunits (GluN2A-D) have pronounced differences in both developmental and regional expression levels and endow NMDA receptors with strikingly different pharmacological and functional properties [92,62,93,63,64,94,95,79] (Fig. 2). Thus, the GluN2 subunits essentially dictate the physiological roles of NMDA receptor subtypes in the CNS. Since GluN1 is an obligatory subunit in all NMDA receptors, the significant sites of structural variation among subtypes are located in the GluN2 subunits of the receptor complex. Efforts to pharmacologically manipulate specific NMDA receptor subtypes in the CNS therefore focus on the development of ligands that can distinguish NMDA receptors on the basis of GluN2 subunits [96,56,54,55,97]. In recent years, the therapeutic rationale for the development of GluN2-selective ligands has been reinforced by increasingly precise localization of GluN2 subunits in neuronal populations relevant to CNS diseases.

The GluN2 subunits impart NMDA receptor subtypes with differences in sensitivity to voltage-dependent Mg^{2+} block [63,98,99,64] and inhibition by endogenous modulators, such as protons and extracellular Zn^{2+} [80,81,100]. The potency of glutamate and glycine/D-serine, as well as other agonists, is influenced by the GluN2 subunits [101–103]. For example, the EC_{50} for glutamate activating NMDA receptors containing two GluN1 and two GluN2D subunits (i.e. diheteromeric GluN1/2D receptors) is 6- to 10-fold lower (i.e. glutamate is more potent) compared to that for GluN1/2A, whereas GluN1/2B and GluN1/2C receptors show intermediate EC_{50} values [2,1,104–106]. GluN1/2A and GluN1/2B have higher single channel conductances compared to GluN1/2C and GluN1/2D receptors [2,1,104–106]. In addition, the probability that the channel will be open when all the agonist binding sites are fully occupied by agonists (i.e. the open probability) is ~0.5 for recombinant GluN1/2A, ~0.1 for GluN1/2B, and <0.05 for GluN1/2C and GluN1/2D [2,1,104–106] (Fig. 2). Importantly, the time constants of deactivation (τ_{decay}) are also highly dependent on the GluN2 subunits; τ_{decay} is ~50 ms for GluN1–1a/2A, ~400 ms for GluN1–1a/2B, ~290 ms for GluN1–1a/2C, and >1 s for GluN1–1a/2D [64,62,94] (Fig. 2). Thus, the GluN2 subunits control functional NMDA receptor properties relevant to synaptic

transmission. Furthermore, the amino acid sequence of the intracellular CTD displays pronounced variation among GluN2 subunits and harbor distinct interaction sites for phosphatases, kinases, and proteins responsible for surface trafficking and anchoring at synaptic sites [1]. The GluN2 subunits therefore also affect subcellular localization, cell-surface expression, and recycling/degradation of NMDA receptor subtypes.

1.4 The GluN3 subunits

GluN3A and GluN3B were cloned based on similarity to GluN1 and GluN2 subunits and were the last NMDA receptor subunits to be discovered (reviewed in [107,71,70,72,69,68]). The GluN3 subunits bind glycine and D-serine [108–110], but the functional properties and physiological roles of GluN3-containing NMDA receptors remain elusive.

Triheteromeric NMDA receptors that are assembled from a combination of GluN1, GluN2, and GluN3 subunits have been consistently reported in both the CNS and heterologous expression systems on the basis of biochemical and functional experiments [111–122]. It is therefore surprising that the subunit stoichiometry of triheteromeric GluN3-containing NMDA receptors has not been resolved; it is unknown whether GluN3 replaces GluN1, GluN2, or both GluN1 and GluN2 in these receptors. Despite this gap in our understanding, numerous studies suggest that the inclusion of GluN3 in the NMDA receptor complex reduces Mg^{2+}-block and Ca^{2+}-permeability as well as response amplitudes (reviewed in [107,71,70,72,69,68]). Thus, the GluN3 subunits appear to have dominant-negative effects on NMDA receptor function.

The GluN3 subunits can also assemble with GluN1 in heterologous expression systems to form functional diheteromeric NMDA receptors that contain two GluN1 and two GluN3 subunits [123,65,124], but their existence in the CNS has not been firmly established. These GluN1/3 receptors have been termed “excitatory glycine receptors”, since they can be activated by glycine alone without the requirement of glutamate binding [123]. In recombinant systems, the GluN1/3 receptors are characterized by low Ca^{2+}-permeability and relative insensitivity to extracellular Mg^{2+} [123,125–127]. Interestingly, agonist binding to the GluN1 subunit of GluN1/3 receptors triggers strong desensitization, whereas agonist binding to the GluN3 subunits is sufficient for activation [127–130]. Thus, in contrast to the more conventional GluN1/2 receptors [30,35], simultaneous binding of agonist to all subunits does not appear to be a requirement for activation of GluN1/3 receptors. While many aspects of the physiological roles of GluN3-containing NMDA receptors remain elusive, it is clear that GluN3 subunits endow NMDA receptors with strikingly unique functional properties.

1.5 Diheteromeric and triheteromeric NMDA receptors

The seven NMDA receptor subunits can assemble to produce receptor subtypes with distinct physiological roles across neuronal cell types and brain regions, thereby mediating changes in synaptic transmission and subcellular localization during neuronal development. At least two different GluN2 subunits are expressed in most, if not all NMDA receptor-expressing cells, and a large proportion of native NMDA receptors in the adult CNS are triheteromers that contain GluN1 and two different GluN2 subunits [131–142,84]. Examples of

triheteromeric NMDA receptors identified in an increasing number of studies are the GluN1/2A/2B, GluN1/2A/2C, GluN1/2B/2D subtypes, but the existence of NMDA receptors with other compositions of GluN2 subunits have not been ruled out [140,135,137,142,143,139,136,134,144,145,138,146,147,131,133,148,132,149]. These subtypes, which are expressed in distinct neuronal populations, have been detected using co-immunoprecipitation and by intriguing functional and pharmacological observations that are not mediated by diheteromeric NMDA receptors.

Despite their prevalence in the CNS, there is a gap in our knowledge of triheteromeric NMDA receptors due to our inability to study a homogenous population of these receptors in heterologous expression systems [148,62,140,150,151]. The nature of the problem is that co-expression of GluN1 with two different GluN2 subunits (e.g. GluN2A and GluN2B) generates three populations of functional NMDA receptors, which are composed of two different diheteromeric receptors (e.g. GluN1/2A and GluN1/2B) as well as triheteromeric receptors (e.g. GluN1/2A/2B) [150,62,151]. The majority of our knowledge regarding function, pharmacology, and regulation of recombinant NMDA receptors is therefore almost exclusively derived from studies on diheteromeric receptors that are assembled from GluN1 and only one type of GluN2 (e.g. GluN1/2A). The properties of diheteromeric NMDA receptors are well-described, but little is known about how co-assembly of two different GluN2 subunits affects properties, such as deactivation time course, Mg^{2+}-block, and the activity of subunit-selective allosteric modulators. Similarly, phosphorylation sites and trafficking properties of the intracellular GluN2 CTDs have been extensively studied, but the regulation of triheteromeric NMDA receptors that possess two distinct GluN2 CTDs is largely unknown (see [152]).

Recently, significant insight into functional and pharmacological properties of triheteromeric NMDA receptors has been gained using a method to tightly control cell surface expression of NMDA receptors with defined subunit composition [151,153]. This method has provided evidence of surprising pharmacological and functional properties of triheteromeric NMDA receptors that are distinct from the properties of the respective diheteromeric receptors [151,153–158] (Fig. 4). Furthermore, the method is enabling exciting, new opportunities to develop therapeutic agents that target disease-relevant triheteromeric NMDA receptors [159,160,156,157,155,158].

2 NMDA receptor structure and function

AMPA, kainate, and NMDA receptor subunits share a common membrane topology; each subunit consists of a large extracellular ATD, a bi-lobed ligand binding domain (LBD), a transmembrane domain (TMD), and an intracellular CTD (Fig. 5a). The TMD is formed by three transmembrane helices (M1, M2, and M4) and a membrane re-entrant loop (M2). The ion channel pore of iGluRs is mainly lined with residues in the membrane re-entrant loop from all four subunits. Among NMDA receptor subtypes, the residues in the pore region, which determines basic ion permeation properties, are highly conserved. One key determinant of ion permeation, which partially defines Ca^{2+} permeability and Mg^{2+}-block, resides at the apex of the membrane re-entrant loop M2 and is sometimes referred to as the Q/R/N site on the basis of amino acid residues found at this position in AMPA, kainate, and

NMDA receptors. The ATD adopts a clamshell-like structure formed by the first ~350 amino acids of the subunit and plays an important role in subunit assembly and as a modulatory NMDA receptor domain. In NMDA receptors, the ATD harbors binding sites for several allosteric modulators, including extracellular zinc and polyamines, as well as GluN2B-selective antagonists (e.g. ifenprodil) (Fig. 5b). The LBD is formed by two segments of the polypeptide chain (S1 and S2), which fold into a kidney-shaped structure composed of an upper lobe (D1) and lower lobe (D2) relative to the cell membrane, and the agonist binding site is located in the cleft between the two lobes (Fig. 5c,d). The relationships between domain structures, their intra- and inter-subunit interactions, and receptor function and pharmacology have been extensively studied for more than two decades. Recently, crystallographic and cryo-EM studies have provided the first glimpses at the domain organization of NMDA receptors and mechanisms by which these domains and allosteric modulators influence receptor function [66,67,161,162].

2.1 Structure and function of GluN1 and GluN2 ligand binding domains

Recombinant proteins that comprise the NMDA receptor LBDs have been generated by combining S1 and S2 segments with a short artificial di-peptide linker [109,163,164] (Fig. 5c,d). These water-soluble LBD proteins retain ligand binding activities comparable to those in full-length NMDA receptors, indicating structural identity between the binding pockets of isolated LBDs and the corresponding intact receptor. LBD crystal structures from GluN1, GluN2, and GluN3 subunits have been solved both in complexes with agonists, antagonists, as well as allosteric modulators [165–167,157,168,108,109,169,170,163,164,155,171,158]. In addition to NMDA receptor subunits, numerous crystal structures for AMPA and kainate receptor subunits have been determined (reviewed in [172–174]). These studies have provided insight to the molecular determinants of full agonists and partial agonists, as well as the mechanism of action for competitive antagonists. Furthermore, the LBD structures have afforded new opportunities to consider the molecular determinants of subunit selectivity. The first structure of the GluN2A LBD was solved in complex with the GluN1 LBD, and provided the first view of the glutamate binding site of the NMDA receptor and the first structural information about a GluN1-GluN2 protein-protein interface within the NMDA receptor complex [164]. Glycine and glutamate bind in the cleft between the two clearly defined lobes (D1 and D2) of the GluN1 and GluN2A LBDs, respectively (Fig. 5c,d). Residues from loops within the upper lobe (D1) form most of the upper half of the binding pocket, and residues from the lower lobe (D2) form most of the lower half of the pocket. Despite being tucked away between the lobes, multiple water molecules are found in close vicinity of the agonists, some of which form hydrogen bonds with the ligand. The glycine binding pockets in GluN1 and GluN3 are smaller and more hydrophobic compared to the GluN2 glutamate binding pocket [164,163,165,168,108]. Residues lining the glutamate binding pocket are fully conserved among the GluN2 subunits, and consequently, agonists or competitive antagonists with strong selectivity among the different GluN1/2 receptor subtypes have not been identified. In order to develop subunit-selective ligands, it is presumably necessary to engage other regions of the NMDA receptor with structural variation, such as inter- or intra -subunit interfaces that are non-conserved among GluN2 subunits.

Interestingly, the heterodimer interface between GluN1 and GluN2 modulates receptor function. Three separate areas of contact between GluN1 and GluN2A are identified in the LBD heterodimer crystal structures (sites I, II, and III) [164]. The two outer regions (sites I and III) consist of hydrophobic residues from GluN1 and GluN2, and nonpolar interactions between these residues mediate heterodimerization of the soluble GluN1 and GluN2A LBDs [164]. Mutations of Y535 in GluN1 demonstrate that modification of site II, which is located at the center of the LBD heterodimer interface, results in increasing or decreasing rates of NMDA receptor deactivation [164,175]. Crystallographic studies recently showed that site II of the GluN1/2A LBD heterodimer harbor binding sites for both positive and negative allosteric modulators with strong selectivity for GluN2A-containing NMDA receptors [157,170,155,158]. These results suggest that the stability and dynamics of the LBD interface can control NMDA receptor function, similar to what has been found for AMPA and kainate receptors.

NMDA receptors are sensitive to the redox potential and reducing conditions can cause marked enhancement of NMDA receptor function [176–179]. This sensitivity is mainly mediated by a pair of cysteine residues within the GluN1 subunit (C744 and C798) that are conserved among all iGluR subunits [180,181]. These two residues form a disulfide bond (i.e. are oxidized) in the GluN1/2A LBD heterodimer structure, and relief (by reduction) of the conformational constraints imparted by this disulfide bond in GluN1, but not GluN2, enhances receptor function [180,182]. Several other disulfide bonds exist in LBD crystal structures of GluN1 and GluN2 subunits, but functional effects of their reduction or oxidation have not yet been described.

Multiple ligands have each been crystallized in complex with GluN1 and GluN2 LBDs, providing a structural basis for the effects of partial agonists, agonists, and competitive antagonists [165,169,167,163,164,166,168]. Binding of glycine and glutamate to their respective binding sites are associated with a rapid LBD rearrangement, involving closure of the angle between the two lobes D1 and D2, akin to a clamshell-like closure (Fig. 5c,d). This agonist-mediated LBD closure mediates formation of interactions between residues from the upper and lower lobes that further stabilizes the agonist-bound LBD structure [183,184]. The energy associated with agonist binding and LBD closure causes the receptor to undergo a series conformational changes that can lead to opening of the ion channel pore (i.e. channel gating). Thus, LBD closure as a result of agonist binding is the initial conformational change that triggers ion channel gating. Binding of competitive antagonists, such as the glycine site antagonist DCKA and the glutamate site antagonist D-AP5, stabilizes a more open cleft conformation of the bi-lobed LBD that is incapable of triggering ion channel gating and presumably resembles the LBD conformation in the absence of bound ligand (i.e. *apo*-state) [166,168,163,171]. The stabilization of the LBD in a closed conformation by agonists and an open conformation by competitive antagonists in NMDA receptors is similar to what has been found for soluble LBDs from AMPA and kainate receptor subunits (reviewed in [172–174]). However, despite this similarity, the domain closures in structures of GluN1 and GluN2 LBDs bound by partial agonists are not similar to those observed for AMPA and kainate receptor subunits. While most structures of AMPA receptor LBDs show partial domain closure that correlates with the efficacy of the partial agonist (reviewed in [172]), no such relationship is observed in GluN1 and GluN2 LBD structures. Multiple structures show

that partial agonists, such as D-cycloserine, ACPC, and ACBC in GluN1 as well as NMDA and Pr-NHP5G in GluN2, bind with virtually identical degrees of domain closure in GluN1 and GluN2 LBDs compared to the complexes with full agonists glycine and glutamate, respectively [169,167,165]. However, while crystal structures capture only one low-energy conformation of the LBDs, recent single-molecule FRET and molecular dynamics studies have provided insight to the dynamic behavior of the NMDA receptor LBDs [168,185–188]. These studies suggest that the LBDs fluctuate between open and closed conformations in the absence of ligand (i.e. in the *apo*-state). The probability that the LBD adopts a fully closed conformation increases by binding of full agonist, whereas binding of partial agonists mainly enables the LBD to adopt conformations with intermediate domain closure. That is, a conformational selection mechanism can presumably account for partial agonism in NMDA receptors, since LBD conformations capable of triggering ion channel gating are selected with greater propensity by full agonists compared to partial agonists.

2.2 Ligand binding to GluN3 subunits

Glycine-activated diheteromeric NMDA receptors assembled from two GluN3 and two GluN1 subunits have been widely studied in heterologous expression systems (reviewed in [71,70,69,72]. However, structural and functional properties of triheteromeric GluN1/2/3 receptors are largely unresolved. For example, it is unknown how agonist or antagonist binding to the GluN3 LBD affects the function of GluN1/2/3 receptors, in terms of their activation, deactivation, and desensitization properties. However, LBD crystal structures have established that structural variation exists between the agonist binding sites of GluN1 and GluN3 subunits, even though they are both glycine-binding subunits [168,108,163,165]. Functional studies on recombinant GluN1/3 receptors suggest that these structural differences can be exploited for the development of GluN3-selective ligands targeting the agonist binding site [130,171].

As mentioned above, agonist binding to the GluN3 subunits is sufficient for activation of GluN1/3 receptors. This feature has enabled pharmacological studies on the GluN3 agonist binding site in isolation by abolishing ligand binding to GluN1 using mutagenesis [130]. This approach identified agonists and antagonists with moderate preferences between the agonist binding sites of GluN1 and GluN3 by comparing ligand activities between wild type GluN1/2 receptors and GluN1/3 receptors with mutations that render GluN1 incapable of ligand binding (hereafter denoted GluN1*/3) [130]. In addition, these studies brought to light interesting discrepancies between ligand binding to the isolated, soluble LBD proteins and full-length subunits in intact receptors. The isolated GluN1 LBD protein binds glycine with lower affinity (26 μM) compared to the isolated GluN3A LBD (0.04 μM) [109]. By contrast, the potency of glycine is higher for full-length GluN1 (e.g. in GluN1/2A the glycine EC_{50} is 1.2 μM) compared to for full-length GluN3A (e.g. GluN1*/3A EC_{50} is 57 μM) [130]. The competitive glycine site antagonist DCKA bind with higher affinity to the isolated GluN1 LBD (0.54 μM) compared to the isolated GluN3A LBD (647 μM), and the binding affinities are estimated to be 0.07 μM for GluN1 in GluN1/2A and 35 μM for GluN3A in GluN1*/3A [109,171]. Thus, in case of GluN1 and GluN3 subunits, there are marked differences in the pharmacology of isolated, soluble LBDs and full-length subunits in intact receptors. The underlying basis of these differences is poorly understood and raises

caveats to evaluation of pharmacology in isolated, soluble LBDs that are excised from the intact NMDA receptor.

2.3 Structures of intact tetrameric NMDA receptors

Crystal structures of GluN1/2A LBD heterodimers and GluN1/2B ATD heterodimers have provided important insight to the overall receptor structure (reviewed in [189]). However, it is only recently that the first structures of intact GluN1/2B receptors firmly established the subunit arrangement and domain organization [67,66]. These structures show that subunits in GluN1/2B receptors are arranged in an alternating pattern (i.e. 1-2-1-2) (Fig. 6). In addition, the NMDA receptor structure shares many of the characteristics of AMPA and kainate receptors. First, the receptor seemingly adopts a layered structure composed of the TMD layer and two extracellular layers formed by LBDs and ATDs. Second, there is a symmetry mismatch between the TMDs and the extracellular portion of the receptor; the TMDs have a quasi-4-fold symmetry, whereas the extracellular portion has a 2-fold symmetry (Fig. 6). The TMDs are arranged symmetrically around the ion channel pore, but the extracellular portion of the receptor adopts a dimer-of-dimer arrangement (i.e. two GluN1/2 heterodimers). Third, there is a subunit crossover between the LBD layer and the ATD layer (Fig. 6). Furthermore, the NMDA receptor structure has several unique features compared to the structures of AMPA and kainate receptors [67,66]. First, there are extensive contacts between the two GluN1/2 LBD heterodimers in the intact NMDA receptor, which are not observed in AMPA receptor structures. Second, the NMDA receptor ATDs are arranged differently and have unique subunit interfaces compared to AMPA and kainate receptors. Third, the ATDs forms extensive contacts with the upper lobe of the LBD, giving the NMDA receptor a more compact appearance compared to AMPA and kainate receptors and creating a protein-protein interface at which modulators can bind [160]. Thus, the crystal structures of the intact NMDA receptor reveal multiple unique intra- and interdomain contacts that can provide explanations to allosteric interactions between subunits and allosteric modulation by small-molecule ligands.

Although the crystal structures of intact NMDA receptors provided major advances in our understanding of the structure-function relationship, they are limited by capturing only one low energy conformational state among many in the NMDA receptor activation cycle. In the crystal structures, agonists were bound to GluN1 and GluN2B subunits and GluN2B-selective negative allosteric modulators (NAMs) were bound at interface between GluN1 and GluN2B ATDs [67,66]. The structures therefore represented the agonist-bound inhibited state of the receptor with the ion channel in the closed conformation. However, recent breakthroughs in the cryo-EM methodology have enabled determination of structures at resolutions sufficient to assign the relative positioning of domains, thereby affording insight to agonist-bound active and inactive states as well as NMDA receptors in states inhibited by competitive antagonists or GluN2B-selective NAMs [162,161]. These cryo-EM structures provide the first dynamic pictures of conformational changes in intact NMDA receptors and provide insight the structural mechanism of ion channel gating (i.e. receptor activation) and allosteric modulation.

2.4 Channel gating in NMDA receptors

The portion of the receptor that controls whether the ion channel pore is open or closed with respect to ion permeation is often referred to as the activation gate. demonstrate that all three transmembrane helices (M1, M3, and M4) and the membrane-reentrant pore forming loop (M2) are involved the process that transitions the NMDA receptor pore to a permeant configuration, a process often referred to as gating [190–197]. Among these regions, the transmembrane helix M3 forms a helical bundle crossing that occludes the pore, and thus the M3 helices must move in order to allow permeation of ions through the channel pore [198–200] (Fig. 7). M3 contains a highly conserved nine amino acids motif (SYTANLAAF), and structural and functional studies have demonstrated that the activation gate is located within this motif [198]. Dilation of the helix bundle formed by M3 from each of the four NMDA receptor subunits is presumably the conformational change that opens the ion channel and allows ion permeation [198,201,202]. However, the structural mechanisms that control opening and closing of the NMDA receptor ion channel are not fully understood. Agonist binding induces closure of the bi-lobed NMDA receptor LBD, and this LBD closure initiates a sequence of conformational changes that result in multiple short-lived, intermediate conformations during the transition from the closed to the open state of the ion channel [203–205,60,206,207], akin to a wave of conformational changes connecting agonist binding to ion channel gating. However, there is as yet poor understanding of which elements and conformations represent rate limiting steps *en route* to gating. Moreover, the lifetimes of some of these intermediate conformations are too brief (i.e. they are unstable) to be observed in crystal structures or functional experiments, which has confounded attempts to link the sequence of protein conformational changes to kinetically distinct functional pre-gating steps. However, the field is poised for major advances that should occur as new, more detailed structural information emerge and efforts to conceptualize functional models take stock of structural principles. Nonetheless, the presence of intermediate states can be detected using Hidden Markov modeling of single-channel recordings, and the lifetimes of these states differ significantly among NMDA receptor subtypes in a GluN2 subunit-dependent manner [60,208,83,209,207].

Agonist binding steps and the sequence of protein conformational changes that lead to gating can be described as reaction schemes representing agonist binding as well as the transition between different conformational states of the receptor. The first widely accepted kinetic model for the NMDA receptor gating cycle was proposed by Lester and Jahr [61]. This model was designed solely to account for the macroscopic current response waveform, and consisted of two independent, but identical glutamate binding sites, one open state, one closed state, and one desensitized state. This simple formulation adequately described key features of the macroscopic time course for NMDA responses, but was not designed to capture the complexity observed in single channel recordings. The utility of the model was further limited given the lack of glycine binding steps, which are required for receptor activation. Benveniste et al. [210] developed models that took into account glutamate and glycine binding steps, as well as allosteric interaction between the glutamate and glycine binding domains. These models captured additional features of NMDA receptor pharmacology and response time course, including an apparent glycine-dependent desensitization (see below).

Newer and more complex models, which incorporate both glutamate and glycine binding steps, have been proposed that provide a better description of single channel data by the incorporation of multiple steps between binding and gating [204,206,205,60,203]. In some studies, single-channel and macroscopic responses to full and partial agonists suggest that agonist binding to either GluN1 or GluN2 controls different steps in the receptor gating scheme [206,205,60,203]. These models can also account for some of the actions of allosteric modulators by explicitly representing the modulator bound and unbound receptor as independent states [211–213]. Additional models that exclusively enable modulators to bind to the open state have also been described for channel blockers and other use-dependent modulators [214–219].

The modular nature of the glutamate receptor structure, coupled with the established ability of AMPA receptors subunits to operate independently [220–222], raises the possibility that subunit-independent conformational changes may progress within the sequence of steps leading to channel opening. Some studies suggest that subunit-specific structural changes are required in all four subunits for channel opening, and that these conformational changes occur in any order to arrive at an intermediate state, which can then transition to the open state of the ion channel [206,205,60,223,203]. However, other models can account for single-channel and macroscopic properties by incorporating just a few sequential gating steps in a linear reaction scheme with an implicit order for fast and slow gating steps [207,204]. Importantly, all kinetic models for NMDA receptor gating, which faithfully represent both single channel data and macroscopic responses, require multiple pre-gating steps as well as multiple open states. Thus, opening of the NMDA receptor ion channel is not directly coupled to agonist-induced closure of the LBD, but rather the receptor proceed through a sequence of protein conformational changes that connects agonist binding to ion channel gating.

2.5 Structural determinants of ion permeation and channel block

In the open conformation, the NMDA receptor ion channel pore can be divided into the extracellular vestibule and the intracellular vestibule, separated by a narrow constriction. The ion permeation pathway is formed by pore-lining residues that determine ion selectivity and channel conductance. The narrow restriction, also referred to as the selectivity filter, resides at the apex of the membrane re-entrant loop M2 (i.e. the Q/R/N site), approximately halfway across the transmembrane electric field, and is a key determinant of single-channel conductance, Ca^{2+}-permeability, and channel block by Mg^{2+} and organic cations (reviewed in [224,1]) (Fig. 7). In both GluN1 and GluN2 subunits, the residue at the position of the Q/R/N site is an asparagine (N), whereas this residue is glycine (G) in GluN3 subunits. Interestingly, the contribution of residues at the apex of M2 to ion permeation is asymmetric between GluN1 and GluN2 subunits [225–228]. The narrow constriction is mainly formed by the Q/R/N site asparagine in GluN1, whereas in GluN2, it is formed by the asparagine residue adjacent to the Q/R/N site (i.e. Q/R/N +1 site). Thus, the narrow constriction is formed by non-homologous residues in GluN1 and GluN2 subunits. For example, mutations at the Q/R/N site in GluN2 dramatically reduce Mg^{2+}-block and have weak effects on Ca^{2+}-permeability, but the same mutations at the Q/R/N site in GluN1 have the opposite effects [227,228]. In addition, substitutions of the asparagine residue at the Q/R/N +1 site in GluN2

strongly reduce Mg^{2+}-block [228]. Thus, functional data suggest a structural asymmetry, in which the apexes of M2 in GluN1 and GluN2 are slightly staggered [225].

In terms of physiologically relevant ions, the NMDA receptor ion channel is permeable to Ca^{2+}, Na^{+}, and K^{+} ions. GluN1/2 receptors have similar permeability to K^{+} and Na^{+} ions (P_K/P_{Na} = 1.14), but are ~2–5 times more permeable to Ca^{2+} relative to monovalent ions (P_{Ca}/P_X = 1.8–4.5), depending on the GluN2 subunit [229–233]. Interestingly, despite being highly permeable to Ca^{2+}, NMDA receptors also exhibit voltage-dependent block by external Ca^{2+}, which is readily observed in single-channel recordings as a reduction in channel conductance [234–236,208]. The concurrent high Ca^{2+}-permeability and Ca^{2+}-block of NMDA receptors are not necessarily incompatible properties, but could be expected if multiple Ca^{2+} binding sites exist within the ion channel pore [234,229]. One Ca^{2+} binding site is presumably located at the Q/R/N site in the pore, and a cluster of charged GluN1 residues, the DRPEER motif, have been suggested to form another, more external Ca^{2+} binding site [237,67]. The external Ca^{2+} binding site is located C-terminal to the transmembrane helix M3 in GluN1 at the external entrance to the ion channel. Removal of the net negative charge in DRPEER using mutagenesis (i.e. ARPAAR) reduces the fractional Ca^{2+} currents in NMDA receptors, consistent with an important role of this motif in mediating high Ca^{2+}-permeability [237].

It has been suggested that diheteromeric GluN1/3 receptors form a unique narrow constriction in the extracellular vestibule of the ion channel pore [238]. This narrow constriction, which is presumably not found in GluN1/2 receptors, is proposed to be a main structural determinant for the dramatically reduced Ca^{2+}-permeability and minimal Mg^{2+}-block of GluN1/3 receptors [238]. Co-expression of GluN3 subunits with GluN1 and GluN2 subunits also produce NMDA receptors with reduced single-channel conductance, decreased Ca^{2+}-permeability, and diminished Mg^{2+}-block (reviewed in [71,70,68,72,69]). However, it is unknown whether the GluN3-specific narrow constriction is formed in the extracellular vestibule or these NMDA receptors, which are presumably triheteromeric GluN1/2/3. Furthermore, the extent and mechanisms by which GluN3 subunits influence permeation properties of triheteromeric GluN1/2/3 receptors have not been quantitatively evaluated and remains poorly understood.

NMDA receptor ion channels are blocked by divalent cations Zn^{2+} and Mg^{2+} in a membrane potential-dependent manner (i.e. voltage-dependent) (Fig. 1d). GluN1 and GluN2A mutations in the re-entrant pore loop M2 that reduce channel block by extracellular Mg^{2+} have similar effects on Zn^{2+}-block, suggesting shared molecular determinants [100,239]. While Mg^{2+}-block of NMDA receptors is centrally implicated in neuronal function, the channel block by Zn^{2+} is low affinity, rapidly reversing, and has far less physiological implications [240,241]. GluN1/2A and GluN1/2B are more strongly blocked by external Mg^{2+} compared to GluN1/2C and GluN1/2D [63,99,242,98,243]. At a holding potential of −100 mV, the IC_{50} values for block by Mg^{2+} are 2.4 μM, 2.1 μM, 14.2 μM, and 10.2 μM for GluN1/2A, GluN1/2B, GluN1/2C, and GluN1/2D, respectively [99]. The GluN2 subunit-specific effects on Mg^{2+}-block are likely influenced by multiple structural elements, but a main determinant is a single residue, which is a serine in GluN2A/B and a leucine in GluN2C/D (i.e. the S/L-site) [243]. The S/L-site does not face the ion channel pore, but is

located on the internal side of the M3 transmembrane helix, and mutagenesis data suggest that this residue interacts with tryptophan residues in the GluN1 membrane re-entrant loop M2 [243]. In addition to channel block by Mg^{2+}, the subunit-subunit interaction between GluN1 and the GluN2 S/L site is also a main determinant of GluN2 subunit-specific variation in Ca^{2+}-permeability and channel conductance [243]. The structural mechanism by which the GluN2 subunits control block by external Mg^{2+} is unknown, but it is possible that the GluN2 S/L site and other structural elements influence the binding sites for permeant ions in the channel pore, since these binding sites are different between GluN2 subunits and have been shown to profoundly modulate Mg^{2+}-block [244–248].

Numerous organic cations with diverse chemical structures are capable of binding and blocking the NMDA receptor ion channel pore in voltage-dependent manner [219,249,250]. Most, if not all, of these compounds are positively charged at physiological pH, and almost exclusively block activated NMDA receptors with open channels. This mechanism of channel block has been termed “use-dependent” or “uncompetitive”. The open channel blockers are further classified into three categories based on their interaction with the channel: (1) “Sequential” or “foot-in-the-door” blockers, such as aminoacridine derivatives, bind to the channel only when it is open and prevent channel closure [251–254]. (2) Partial trapping blockers, such as memantine and amantadine, impede channel closure without completely preventing it [217,255,218,256–258]. (3) Trapping blockers, such as MK-801, phencyclidine (PCP) and ketamine, are trapped inside the pore as the channel returns to the closed state and agonists unbind [259]. Some channel blockers can also interact with the gate to facilitate channel closure [218,255].

Open channel blockers are generally considered non-selective among NMDA receptor subtypes [216]. However, some channel blockers, at least ketamine and memantine, may display some selectivity under physiological conditions, since 5- to 10-fold selectivity for GluN2C/D-containing receptors over GluN2A/B-containing receptors have been reported in the presence of 1 mM extracellular Mg^{2+} [260]. This observation may be clinically significant, since NMDA receptor channel blockers have been shown to have neuroprotective effects in animal models of CNS disorders that involve excessive stimulation of NMDA receptors, such as traumatic brain injury, epilepsy, and stroke. Unfortunately, human clinical trials have been disappointing due to patient heterogeneity, dose-limiting side effects, and a narrow temporal window for intervention, which may have confounded interpretation. High-affinity NMDA receptor channel blockers, such as phencyclidine (PCP) and ketamine, are dissociative anesthetics, but their clinical use is limited by strong psychomimetic side effects (see below). By contrast, low-affinity channel blockers, which shows fast blocking/unblocking kinetics [261], appear to have a greater therapeutic index with respect to psychomimetic effects, which may be due to less channel block under conditions of normal synaptic transmission [262]. One such low-affinity blocker, memantine, has been approved for clinical use in the treatment of moderate to severe Alzheimer’s disease. However, the mechanism by which NMDA receptor channel block by memantine may contribute to a symptomatic benefit for advanced Alzheimer’s disease patients is not well understood.

2.6 Modulation by the amino-terminal domain

Similar to the LBD, the ATD also adopts a bilobed kidney-shaped structure with upper and lower lobes termed R1 and R2, respectively [263,264]. Crystal and cryo-EM structures of intact iGluRs revealed a unique dimer-of-dimer arrangement of the NMDA receptor ATDs compared to those in AMPA and kainate receptors [67,161,265,266,66,162]. This arrangement, which is also present in crystal structures of heterodimers formed by soluble GluN1 and GluN2B ATDs, is characterized by a subunit interface formed by extensive contacts between the upper R2 lobes from GluN1 and GluN2, whereas the lower R1 lobes, which connect to the LBDs, are almost completely separated. The ATDs are resting immediately above the LBDs and strong interactions are formed between the LBD and ATD layers. By contrast, AMPA and kainate receptor ATDs associate through interactions between both upper R1 and lower R2 lobes in a back-to-back fashion and there is very little contact between the LBD and ATD layers. Numerous studies have revealed important roles of the NMDA receptor ATD as a modulatory domain that affects function and harbors modulatory binding sites for ions and small-molecule ligands (reviewed in [267,56,55,189]). Modulatory roles or ligand binding sites have not been identified for AMPA and kainate receptor ATDs, even though molecular dynamics simulations predict they should be capable of similar conformational changes as NMDA receptor ATDs [268,269]. Consistent with these differences, mutant subunits with deletion of the ATD have dramatic impact on the functional properties of NMDA receptors [94], whereas little to no changes are observed in AMPA and kainate receptors [270].

Many of the GluN2-specific differences between NMDA receptor subtypes are in large part due to variation in the weakly conserved GluN2 ATDs [271,94]. Studies with NMDA receptors containing chimeric GluN2 subunits have revealed that swapping of the ATD between GluN2A and GluN2D, which have widely different properties, shifts the open probability, deactivation time course, agonist potency in the direction of the subunit contributing the ATD [94,271]. Little is known about how the ATD controls NMDA receptor function, but the mechanism presumably involves a combination of intra- and inter-subunit allosteric interactions between the ATDs and LBDs that can affect the dynamic behavior and stability of the GluN1/GluN2 LBD heterodimer [272,273]. Functional and structural studies suggest that the ATDs adopt distinct conformations, depending on the GluN2 subunit, which may underlie some GluN2-specific functional and pharmacological NMDA receptor properties [274,151].

Extracellular Zn^{2+} is an endogenous modulator that inhibits NMDA receptors in a voltage-independent manner through a binding site in the GluN2A and GluN2B ATDs [275–278,80,279,280,263]. The affinity of Zn^{2+} to the GluN2A ATD is in the low nanomolar range, whereas the affinity to the GluN2B ATD is in the low micromolar range. Crystal structures and functional data have identified the binding site for Zn^{2+}, which is located at the mouth of the cleft formed by the two lobes R1 and R2 [263]. Several experimental observations support a mechanism of Zn^{2+}-modulation that involves opening and closing motions of the angle between the two lobes R1 and R2 as well as twisting motions around the hinge region of the ATD clamshell [280,263,273]. Binding of Zn^{2+} stabilizes a

conformation of the GluN2 ATD, which presumably is accompanied by structural changes at the GluN1/2 LBD subunit interface [273].

Crystal structures of both isolated ATDs and intact NMDA receptors established that GluN2B-selective NAMs, such as ifenprodil and Ro 25–6981, bind the subunit interface between GluN1 and GluN2B ATDs [67,281,264,66]. Interestingly, only one residue in the ifenprodil binding pocket is different between GluN2A and GluN2B, but sensitivity to ifenprodil is not introduced by converting this or other residues in GluN2A to that in GluN2B [264,282]. This observation further supports that the ATD arrangement in GluN2A- and GluN2B-containing receptors is likely fundamentally different and highlights that the mechanism of subunit-selectivity for ifenprodil and its analogs remains unresolved. Recent cryo-EM structures of intact NMDA receptors, supported by functional studies and computational analyses, suggest that the mechanism of ifenprodil inhibition involves closure of the GluN2B ATD clamshell and changes in the arrangement of the GluN1/2B ATD heterodimers [161,282] (see below).

Polyamines, such as spermine and spermidine, enhance NMDA receptor function in a GluN2B-selective manner through a binding site, suggested to be located in the vicinity of clusters of negatively charged residues in the lower R2 lobes of GluN1 and GluN2B ATDs [283]. Although the precise location of this binding site for positive allosteric modulation remains to be identified, it has been shown using FRET that spermine binding opens the GluN2B ATD clamshell [284]. Furthermore, a model has been proposed where the positively charged spermine shields the negatively charged residues in GluN1 and GluN2B ATDs, thereby potentially eliminating electrostatic repulsion between the two lower R2 lobes [283]. Consistent with this model, other cations can also potentiate GluN2B-containing NMDA receptors in manner similar to spermine; for example, millimolar concentrations of extracellular Mg^{2+} enhance GluN1/2B responses under conditions with no channel block [285].

Functional and structural investigations appear to converge on a structural model for NMDA receptor modulation by Zn^{2+}, ifenprodil, and spermine, in which modulator binding regulates receptor function through GluN2 ATD clamshell opening and closing motions and rearrangement of the ATD layer. It is not fully understood how these conformational changes affect other structural elements of the receptor, but several studies propose that downstream changes occur at the subunit interface of GluN1/2 LBDs. Interestingly, the activity of all three allosteric modulators (Zn^{2+}, ifenprodil, and spermine) is reduced for NMDA receptors containing GluN1 with exon 5 (e.g. the GluN1–1b splice variant) [81,85]. Recent structures of intact NMDA receptors show that the 21 amino acids, which are encoded by exon 5, are located just above the GluN1/2 LBD heterodimer interface between the ATD and LBD layers, well-positioned to influence allosteric coupling between GluN2 ATD clamshell motions and GluN1/2 LBDs [67,66]. In addition, mutational analyses identified GluN2C residues from both the ATD and LBD that influenced the activity of PYD-106, which is a recently developed GluN2C-selective positive allosteric modulator (PAM), and molecular modeling proposed a binding site located in a pocket residing at the intra-subunit ATD/LBD interface of GluN2C [160]. Thus, the ATD is the major structural determinant of GluN2-specific variation in functional and pharmacological properties of NMDA receptors. The

mechanism of allosteric modulation by the NMDA receptor ATD remains an important focus in structure-function studies, and drug discovery efforts are poised to identify novel ATD ligands with therapeutic potential. In particular, it is unknown how structure and ATD arrangement differs among the various NMDA receptor subtypes.

2.7 Control of assembly by the amino-terminal domain

The mechanism and progression of subunit assembly of two GluN1 and two GluN2 subunits in an alternating 1-2-1-2 arrangement around the central ion channel pore is not well-understood. Three main models of the steps required for NMDA receptor assembly have been proposed: 1) It has been suggested that GluN1-GluN1 and GluN2-GluN2 homodimers initially form and then associate to form the tetrameric receptor [286–289]. 2) Alternatively, two initial GluN1-GluN2 heterodimer are formed that subsequently associate to generate the tetrameric arrangement [290]. 3) Lastly, it has been suggested that a GluN1-GluN1 homodimer is initially formed to which GluN2 subunits are sequentially added to form the tetrameric NMDA receptor [291,292]. While there is some supporting experimental data for each model, this data is as yet insufficient to make a clear distinction between these models. Regardless of sequence, it appears that the NMDA receptor ATD is the main determinant of the initial subunit dimer formation [288,286,292]. This feature of the ATD in NMDA receptors appears to be shared in AMPA and kainate receptors, where the role of the ATD in subunit assembly has been extensively studied [293,294]. Interestingly, the NMDA receptor ATD may also influence receptor trafficking, since the GluN2A ATD has been shown to contain a retention signal that prevents exit from the endoplasmic reticulum unless it is masked by assembly with the GluN1 ATD [295].

3 Mechanisms of NMDA receptor regulation

Many functional and membrane trafficking properties of NMDA receptors are regulated by extracellular ions, phosphorylation, and intracellular binding proteins. Here, we will describe regulation of NMDA receptor function by extracellular ions and molecules, and highlight key phosphorylation sites and their implications for protein-protein interactions important for neuronal functions.

3.1 Desensitization of NMDA receptors

The definition of desensitization is a decrease in the receptor response in the continued presence of a stimulus. NMDA receptors exhibit several different types of desensitization with distinct mechanisms, including glycine-dependent desensitization, Zn^{2+}-dependent desensitization, Ca^{2+}-dependent desensitization, and glycine/Ca^{2+}/Zn^{2+}-independent desensitization.

Glycine-dependent NMDA receptor desensitization can be observed in the presence of subsaturating glycine concentrations, and is abolished in a saturating concentration of extracellular glycine [296]. This type of desensitization occurs due to a negative allosteric interaction between the GluN1 and GluN2 subunits such that the binding of glutamate decreases the affinity for glycine [297,210]. Thus, when glutamate binds GluN2, the affinity for the glycine binding site in GluN1 becomes lower, and in the absence of high

concentrations of glycine, the current diminishes and relaxes to a new equilibrium as glycine unbinds from the receptor. The time course for the desensitization therefore reflects glycine unbinding, which is within the range of the synaptic NMDA receptor time course, suggesting glycine-dependent desensitization could impact synaptic signaling. Recent crystal and cryo-EM structures of intact NMDA receptors offer plausible structural models for the negative allosteric coupling between glutamate and glycine binding sites [66,67,161,162], but the structural mechanism of glycine-dependent desensitization is still not fully understood. Extracellular Zn^{2+} mediates a rapid component of desensitization that occurs by a mechanism similar to glycine-dependent desensitization [298]. It has been proposed that a positive allosteric interaction exists between the glutamate binding site in the GluN2 LBD and the Zn^{2+} binding site in the GluN2A ATD, which enables binding of glutamate to enhance Zn^{2+} binding [299,300]. Thus, glutamate binding will, in the presence of subsaturating concentrations of Zn^{2+}, cause a relaxation of the receptor response to a new equilibrium as more Zn^{2+} ions bind and inhibit the receptor in a concentration-dependent fashion. The time course of Zn^{2+}-dependent desensitization therefore reflects the time course for Zn^{2+} binding following a glutamate-dependent shift into a Zn^{2+} binding site with higher affinity.

NMDA receptors also undergo Ca^{2+}-dependent inhibition, which is often referred to as Ca^{2+}-dependent desensitization or inactivation, since this type of desensitization requires intracellular Ca^{2+} and develops slowly over seconds [301–304]. The magnitude of Ca^{2+}-dependent desensitization varies among GluN2 subunits, and is more prominent for GluN2A-containing than for GluN2D-containing receptors and appears to be absent for GluN2B- and GluN2C-containing NMDA receptors [305,306]. It has been hypothesized that a local increase in the intracellular Ca^{2+} concentration occurs in the immediate vicinity of the NMDA receptor, which results in inhibition by stimulating uncoupling of the receptor from filamentous actin in a manner sensitive to second messenger systems [307]. Furthermore, calmodulin binding to the GluN1 CTD have been suggested to play an important role in this form of desensitization. Thus, Ca^{2+}-dependent desensitization is abolished in NMDA receptors containing GluN1 splice variants in which calmodulin binding sites are absent [308,309], and mutations within calmodulin binding sites in the GluN1 CTD similarly disrupt Ca^{2+}-dependent desensitization [310,311].

Most ligand-gated channels undergo a form of desensitization that reflects a conformational change to a relatively stable and sometimes long-lived agonist-bound receptor state with a closed ion channel. NMDA receptors can also desensitize in the continued presence of agonist by a mechanism that is independent of glycine, Zn^{2+}, and Ca^{2+} (i.e. the types of desensitization discussed above). This desensitization develops with time, is sensitive to intracellular dialysis, and is thus more prominent in excised outside-out membrane patches compared to whole-cell patches [312,313]. However, desensitization can also be influenced by mutations in the conserved SYTANLAAF motif, the preM1 region, and other positions deep within the ion channel pore, the LBD, and the TMD-LBD interface [195,314,315], suggesting that this desensitization reflects a conformational change in the agonist-bound receptor.

3.2 Regulation of NMDA receptor function by protons

Extracellular protons potently (IC_{50} = ~50 nM) and completely inhibit NMDA receptor function [316–319]. Thus, neuronal NMDA receptors are tonically inhibited by protons at physiological pH 7.4, which corresponds to approximately the proton IC_{50}. NMDA receptors can therefore respond to small changes in extracellular pH under physiological conditions. Moreover, extracellular pH is dynamic and changes with neuronal activity, given that synaptic vesicles are acidic and various transporters can generate proton gradients [320]. Furthermore, pathological conditions, such as seizure or ischemia, reduce extracellular pH (i.e. increase proton concentration) to levels that are sufficient to strongly inhibit NMDA receptor function [320].

As with many other NMDA receptor properties, the inhibition by extracellular protons depends on the GluN2 subunit [81]. GluN2A-, GluN2B-, and GluN2D-containing NMDA receptors are inhibited with proton IC_{50} values near physiological pH (7.2 –7.4), whereas GluN2C- containing receptors are much less sensitive to protons (IC_{50} value at pH 6.2) [81,321]. In addition, proton inhibition is reduced for NMDA receptors with the GluN1–1b isoform, which has an additional 21 amino acids inserted in the ATD [81]. Proton inhibition is voltage-independent and is also independent of actions at the agonist binding site. The location of the structural determinant for proton inhibition (i.e. the proton sensor) is unknown and it is possible that multiple sites within the NMDA receptor work in concert to mediate the actions of protons. However, residues within the ion channel gate, near the linkers that couple the TMD to the LBD, and in the GluN1-GluN2 LBD dimer interface have been shown by mutagenesis to influence pH sensitivity [321,273], suggesting that NMDA receptor gating elements are tightly coupled to the proton sensor. This idea is supported by evidence that channel blockers are sensitive to the protonation state of the receptor while entering the pore [216].

Several studies suggest that actions of ATD modulators may reflect a subtle change in the pKa of the proton sensor that either enhances or reduces tonic proton inhibition at physiological pH (see below). In this regard, both extracellular Zn^{2+} and ifenprodil appear to enhance proton sensitivity, which will increase tonic inhibition at physiological pH, whereas binding of extracellular polyamines, such as spermine, reduce proton sensitivity, which results in potentiation. For example, spermine potentiation of GluN1/2B strongly correlates with the degree of proton inhibition and is most robust at pH values that produce strong tonic inhibition (i.e. pH < 8). This is consistent with a mechanism in which polyamines enhance receptor function by relieving proton inhibition [81,322,323]. Similar functional evidence support a mechanism for inhibition by extracellular Zn^{2+} and ifenprodil in which receptor function is reduced by enhancing proton inhibition [212,85,86,324,80,275].

3.3 Regulation of NMDA receptor function by extracellular Zn^{2+}

GluN2A-containing NMDA receptors are highly sensitive to extracellular Zn^{2+}, and numerous studies have reported variable IC_{50} values in the low nanomolar range (e.g. [298,278,80,100]). A key provision in these studies was the need for a buffer system to accurately control Zn^{2+} concentration and unambiguously determine the IC_{50} value for Zn^{2+} inhibition, since hundreds of nanomolar Zn^{2+} contaminates physiological saline solutions

under most experimental conditions [80,100]. Thus, in order to remove effects of extracellular Zn^{2+} in functional experiments, many studies include Zn^{2+}-chelators, such as tricine or EDTA, in the extracellular recording solution. The high affinity of these chelators for Zn^{2+} means that 10's of micromolar of chelator will bind virtually all of the nanomolar contaminating Zn^{2+} ions, but minimally alter millimolar concentrations of Ca^{2+} or Mg^{2+} (e.g. see [325]). The concentration-inhibition relationship for Zn^{2+} at GluN1/2A receptors is biphasic, since Zn^{2+} binding to the high affinity site in the ATD causes incomplete inhibition, whereas higher micromolar concentrations of Zn^{2+} result in voltage-dependent channel block [278]. The incomplete inhibition by high affinity Zn^{2+} binding is related to enhancement of proton sensitivity, since Zn^{2+} binding causes a leftward shift of the proton inhibition curve such that inhibition is more complete at acidic pH compared to at alkaline pH [212,80,275,277]. For example, maximal inhibition of GluN1/2A by extracllular Zn^{2+} is ~62% at physiological pH 7.3 compared to ~76% at pH 6.8 [277]. Interestingly, high affinity Zn^{2+} inhibition is maintained in triheteromeric GluN1/2A/2B receptors, albeit with less maximal inhibition and a somewhat different relationship to the extracellular pH [151,150,153].

3.4 NMDA receptor phosphorylation and membrane trafficking

The intracellular CTDs of NMDA receptor subunits contain numerous sites for posttranslational modifications (e.g. phosphorylation, nitrosylation, and palmitoylation) and for protein-protein interactions, which have important implications for receptor localization, trafficking, and signaling (reviewed in [1,326,327]). The intracellular CTDs display very little conservation among subunits, and subcellular localization and trafficking of each subunit therefore appear to be uniquely regulated. Furthermore, the intracellular CTD of GluN1 is modified by alternative RNA splicing, which removes or inserts regulatory sites with important effects on receptor trafficking.

Experimental evidence suggest that the precise subcellular localization of NMDA receptor subtypes is determined by protein-protein interactions between the extreme C-terminus of GluN2 subunits and PDZ domain-containing proteins, including the MAGUK proteins PSD-93, PSD-95, SAP97, and SAP102. Members in the MAGUK protein family have widely different subcellular localization and exhibit GluN2-specific variation in their preferential association with NMDA receptor subtypes. For example, the scaffolding protein PSD-95 is primarily expressed at the postsynaptic density (PSD), whereas SAP102 is more evenly distributed between synaptic and extrasynaptic sites. Furthermore, PSD-95 and SAP102 have been proposed to preferentially bind GluN2A and GluN2B subunits, respectively [328]. The differential interaction of GluN2A and GluN2B subunits with MAGUKs is not firmly established and has been questioned in several studies (e.g. [131]), but has been suggested to underlie differences in the subcellular localization of these subunits. For example, GluN2B-containing receptors appear to move more freely in and out of synaptic sites compared to GluN2A-containing receptors [329]. It has therefore been suggested that GluN2B can be found at both extrasynaptic and synaptic sites, whereas GluN2A is enriched at synaptic sites [329–331]. In addition to MAGUK proteins, numerous other binding partners have been implicated in the subcellular localization and membrane trafficking properties of NMDA receptors (reviewed in [1]).

The cytoplasmic CTDs of NMDA receptor subunits are differentially regulated by posttranslational modifications including phosphorylation, palmitoylation, and nitrosylation (reviewed in [1]). These modifications can affect the ability to bind intracellular proteins involved in membrane trafficking, and can therefore mediate changes in subcellular localization and surface expression. An example of the type of regulation, which has important implications on synaptic plasticity, is CaMKII phosphorylation of GluN2B subunits on Ser1303, which is located in the CaMKII binding site [332,333]. Transient NMDA receptor-mediated influx of Ca^{2+} induces autophosphorylation of CaMKII, which enhance its enzymatic activity and results in persistent activation of CaMKII long after cytoplasmic Ca^{2+} levels return to baseline [334,333]. Once activated, CaMKII rapidly and reversibly undergoes a translocation to the spine, where it binds the CTD of the GluN2B subunit [335–338]. Multiple lines of evidence show that disruption of autophosphorylation and activation of CaMKII, as well as its binding to the GluN2B subunit significantly impairs NMDA receptor-dependent LTP and affects memory in mice, consistent with a key role of CaMKII as a key mediator of some types of synaptic plasticity (reviewed in [339]). The mechanism by which the interaction between the NMDA receptor and CaMKII contributes to synaptic plasticity is largely unresolved and therefore continues to be a primary focus in studies that aim at advancing our understanding of NMDA receptor-dependent synaptic plasticity. Numerous other kinases (e.g. PKA, PKB, and PKC), many protein tyrosine kinases (e.g. Fyn and Src), and phosphatases (e.g. STEP) have been implicated in the regulation of NMDA receptors, and the consequences of modification by these proteins on neuronal function continue to be extensively studied (reviewed in [340]). In addition to CaMKII, other calcium-sensing proteins can interact with NMDA receptor subtypes to mediate downstream signaling and regulate synaptic plasticity. RAS-GRF1 and RAS-GRF2 are two such calcium sensors that selectively bind the GluN2B CTD and thereby initiate ERK- and CREB-mediated signaling pathways in response to NMDA receptor-mediated Ca^{2+}-influx [341–343]. Thus, the implications of NMDA receptor phosphorylation and membrane trafficking on neuronal function are incredibly complex and highly dependent on NMDA receptor subunit composition.

There is a growing body of evidence for metabotropic signaling through the NMDA receptor (i.e. not mediated by ion flux), resulting from direct changes in the interaction of the receptor with other signaling complexes [344,345]. Conformational changes induced by agonist binding are required to gate the NMDA receptor ion channel, but emerging evidence suggest that the conformational changes induced by these various ligands may also have effects that are independent of ion channel flux. For example, glycine binding, but not glutamate binding, have been shown to prime NMDA receptors for internalization [346]. In this case, glycine binding to GluN1 promotes association of the NMDA receptor with clathrin-mediated endocytic machinery that is independent of glutamate binding and receptor activation. Receptor endocytosis is then triggered upon binding of both glutamate and glycine and receptor activation. More recently, evidence has emerged that metabotropic NMDA receptor signaling may play a role in synaptic depression [346]. In this case, the metabotropic effects appear dependent on glutamate binding to GluN2 [347], which results in rearrangement of the associations of protein phosphatase 1 (PP1) and CaMKII with the C-terminal tails of the NMDA receptor to modulate kinase activity [348]. It has been proposed

that the Aβ peptide, a putative pathogen in Alzheimer's disease, may cause synaptic depression and dysfunction via this mechanism [344]. At the physiological level, it may be speculated that these subunit specific metabotropic signaling mechanisms interact with ionotropic signaling mechanisms. Thus, metabotropic signaling provides another layer of signal integration by these important NMDA receptor complexes.

4 Pharmacological manipulation of NMDA receptor subtypes

Small-molecule modulators with selectivity for the different NMDA receptor subtypes (i.e. the GluN2 subunits) are powerful pharmacological tools to dissect the roles for NMDA receptors in neurophysiology, behavior, development, and diseases. In this regard, decades of studies aimed at developing glutamate-site agonists, competitive antagonists, and channel blockers have not identified such pharmacological tools with sufficient GluN2 subunit-selectivity; in part due to the fact that these sites are fully conserved among GluN2 subunits. However, extensive pharmacology has been developed around ifenprodil that was shown to be a GluN2B-selective NAM in 1993 [349], and until recently, ifenprodil and analogs were the only available and widely used pharmacological tool compounds with strong GluN2 subunit-selectivity. However, since approximately 2010, there has been an acceleration in the discovery of novel NMDA receptor allosteric modulators with GluN2 subunit-selectivity and multiple new binding sites on the receptor for positive and negative allosteric modulators have been identified [55,54,350,56] (see below).

4.1 GluN2A-selective allosteric modulators

NVP-AAM077 (also known as PEAQX) is a competitive antagonist that interacts with the glutamate binding site. Although it was initially reported as having a high degree of selectivity for GluN2A over GluN2B [351], subsequent evaluation of the binding affinity of NVP-AAM077 at GluN1/2A and GluN1/2B receptors found more modest selectivity (K_B values were 15 nM for GluN1/2A and 78 nM for GluN1/2B) [352]. This, and other studies, suggested that the level of selectivity (5-fold) of NVP-AAM077 is insufficient for dissection of synaptic responses mediated by GluN2A- and GluN2B-containing receptors [352,353]. Many studies evaluating native receptors and excitatory synaptic transmission have been performed using NVP-AAM077, however, the results of this body of work should be carefully interpreted with regards to the experimental design, level of selectivity assumed, and conclusions drawn.

TCN-201 and TCN-213 were the first non-competitive GluN2A-selective inhibitors (or GluN2A-selective NAMs) that were identified [354]. TCN-201 has a binding affinity of 27–70 nM at GluN2A-containing receptors, with >1000-fold selectivity over other GluN2 subunits [355,356,158]. Inhibition by TCN-201 is surmounted by glycine binding, which is paradoxical since the subunit selectivity depends on the glutamate binding GluN2 subunit [356,354,355]. However, the TCN-201 binding site is located in the LBD heterodimer interface between GluN1 and GluN2A subunits, with key interacting residues around 16 Å from the glycine binding site in GluN1 [356,157,158]. Quantitative analyses show that the functionally-observed interaction between TCN-201 and glycine was best described by an allosteric model of antagonism rather than a direct competition model [356,355]. Recent

analyses of crystal structures of receptor states that are activated and inhibited by the GluN2A-selective NAMs demonstrated a mechanism in which NAM binding to the modulatory site stabilizes the open conformation of the GluN1 LBD, thereby facilitating glycine unbinding and receptor inactivation [158]. Furthermore, these structures revealed that two residues in the interface between GluN1 and GluN2A LBDs play principal roles in the allosteric mechanism of GluN2A-selective NAMs by forming a molecular switch that controls the difference between low- and high-affinity NAM binding; this difference is the primary driving force for allosteric inhibition. MPX-004 and MPX-007 are newer GluN2A-selective NAMs closely related to TCN-201 that have improved potency (79 and 27 nM, respectively, determined in 3 μM glycine) compared to TCN-201 (340 nM in 3 μM glycine) [357]. The MPX compounds provide nearly complete block of GluN2A-containing NMDA receptors and have improved solubility compared to TCN-201 [357,158]. In recent years, the GluN2A-selective NAMs have been used to probe the GluN2B to GluN2A developmental switch, the expression of GluN2A in subcortical and subthalamic nuclei, as well as the role of GluN2A in nicotine reinstatement, cortical spreading depression, and hippocampal plasticity [357,355,358,359,84,360,361].

A high throughput screen performed by Genentech to identify GluN2A-selective positive allosteric modulators (PAMs) identified several structurally-related compounds, here referred to as GNE compounds. These compounds are GluN2A-selective PAMs with at least 10-fold selectivity over other GluN2 subunits that bind the LBD heterodimer interface between a GluN1 and GluN2A subunits, similar to the GluN2A-selective NAMs [157,170]. Interestingly, the GNE compounds interact with the same residue (GluN2A V783) in the GluN2A subunit that mediates the selectivity and inhibition by TCN-201, MPX-004, and MPX-007 [157,158]. This valine is non-conserved across the GluN2 subunits, and introduction of this residue into GluN2B via site-directed mutagenesis is sufficient to confer both inhibition and potentiation to GluN2B-containing NMDA receptors [157,356]. GluN1/2A LBD heterodimer crystal structures in complex with GNE compounds and the GluN2A-selective NAMs show that the binding modes of both positive and negative allosteric modulators are distinct within this pocket, a finding reinforced by the results of mutagenesis studies [158,157,170]. Interestingly, some GluN2A-selective PAMs (i.e. GNE compound analogs) also affect the function of AMPA receptors with similar potencies as for NMDA receptors [157]. The different GNE compounds display variation in the efficacy of GluN1/2A receptor potentiation (up to 6-fold potentiation of receptors activated by EC_{30} of glutamate) and potency (EC_{50} values between 0.02–60 μM) [170]. Furthermore, the series of modulators show reduced efficacy when receptors are activated by saturating concentration of agonist. For example, GNE-0723 shows ~5 fold potentiation of an EC_{30} response compared to ~2 fold potentiation of an EC_{100} response, which is presumably due to an increase in agonist potency mediated by the modulator [170,157]. A complex relationship appears to exist between GNE compound structure, efficacy, and the degree of prolongation of glutamate deactivation rate, which could reflect increased glutamate affinity and potency [170,157]. Two GNE compounds (GNE-6901 and GNE-8324) were evaluated on NMDA receptor-mediated responses in hippocampal neurons [157]. These GNE analogs differed in their ability to prolong the deactivation rate of NMDA receptors, and also showed differences in their ability to alter short- and long-term synaptic plasticity. More studies

could help fully elucidate the mechanism of action of this interesting series of GluN2A-selective PAMs and demonstrate their usefulness in studies on the physiological roles of GluN2A-containing NMDA receptors. The pharmacology of GluN2A-selective modulators is summarized in Table 1.

4.2 GluN2B-selective allosteric modulators

Ifenprodil and its mechanistically-similar analogs have been tremendously useful tool compounds since the discovery in 1993 that they are non-competitive GluN2B-selective inhibitors (i.e. GluN2B-selective NAMs) [349]. The IC_{50} for ifenprodil is in the nM range with 200–400 fold selectivity for the GluN1/2B receptor over GluN1/2A [349]. The observed inhibition of GluN1/2A at high concentrations is caused by low-affinity non-selective channel block [349]. The high-affinity ifenprodil binding site is located in the interface between the GluN1 and GluN2B ATD heterodimer [362,264]. Ifenprodil inhibition is dependent on agonist concentrations; at saturating glutamate and glycine concentrations, maximally effective concentrations of ifenprodil produce incomplete inhibition, with 10–20% residual response [349,363,85,362], whereas the glycine concentration is inversely correlated with the extent of observed inhibitory effect [349]. The actions of ifenprodil at different glutamate concentrations are also complex; ifenprodil causes an increase in glutamate-site agonist affinity, which produces less inhibition with lower agonist concentrations [363]. This positive allosteric interaction between ifenprodil and glutamate binding is similar to that observed for Zn^{2+} acting at the GluN2A ATD and can lead to apparent potentiation at low agonist concentrations [100,300,363]. Many newer GluN2B-selective NAMs acting at the ifenprodil site have been synthesized with improved potency and selectivity (e.g. Ro 25–6981 and CP-101,606) [364,365], and additional mechanistic features such as pH-sensitivity (e.g. see [366]). Recently, crystal structures suggest that GluN2B-selective NAMs can be divided into two classes with distinct binding modes at the GluN1-GluN2B ATD heterodimer interface; one class containing ifenprodil, CP-101,606 and Ro 25–6981 and a second class typified by EVT-101 [281]. EVT-101 is and orally active compound with potent inhibition at low nanomolar concentrations [281], however, a thorough study of EVT-101 properties and mechanism of action has not been published. GluN2B-selective NAMs have been intensely studied by academic research groups and pharmaceutical companies in an effort to identify new series with therapeutic benefits as well as to expand our understanding of the role of GluN2B in normal physiology and disease, a topic thoroughly summarized in a number of excellent reviews [367–369]. GluN2B-selective inhibitors have also been evaluated in clinical trials, with mixed and complex results [370,371,369,372]. The pharmacology of GluN2B-selective modulators is summarized in Table 2.

4.3 GluN2C/D-selective allosteric modulators

Spurred by the description of NVP-AAM077 and other glutamate-site competitive antagonists, studies of related compounds were performed in order to find similar antagonists with variation in the selectivity at NMDA receptor subtypes [373,374]. This effort lead to the discovery of PPDA, which is similar to the earlier identified competitive antagonist PBPD [375], that had differential selectivity and showed high potency [374]. Several structural modifications were pursued in subsequent studies, yielding compounds

that consistently displayed a preference for GluN2C- and GluN2D-containing NMDA receptors over GluN2A- and GluN2B-containing receptors [376]. UBP-141 was observed to have 5–7 fold selectivity for GluN1/2D over GluN1/2A and GluN1/2B, but was less potent than PPDA. Several studies have used UBP-141 to probe the role of GluN2D in certain physiological processes [143,377–380]. These studies provided important insight to the physiological roles of GluN2D in central neurons, but they also should be interpreted with the caveat of modest subunit-selectivity, which is apparently inherent to glutamate-site competitive antagonist. Further expansion and exploration of the chemical space around the compounds related to UBP-141 led to an investigation of related scaffolds, and the subsequent discovery of several mixed-action modulators, including UBP-710 and UBP-551 [381]. UBP-710 shows divergent action at concentrations of 100 μM and higher, resulting in potentiation of GluN1/2A and GluN1/2B, but inhibition of GluN1/2C and GluN1/2D [381]. UBP-551 appears to be uniquely selective for GluN2D-containing NMDA receptors and potentiates current responses with a biphasic concentration-effect relationship, with maximal potentiation of GluN1/2D observed at 30 μM, a concentration at which other NMDAR diheteromeric receptors are inhibited [381]. The mixed-action UBP compounds possess remarkably unique actions, but their utility is hampered by poor physicochemical properties of the parent scaffold and a lack of high affinity actions or high subunit-selectivity [382,381]. It will be interesting to learn more about mechanism and site of action of this class as molecules with higher potency, selectivity, and improved physicochemical properties are developed.

A series of quinazolin-4-ones (QNZ) are negative allosteric modulators of NMDA receptors that show ~50-fold selectivity for GluN2C- or GluN2D-containing NMDA receptors [383,384]. The prototypical compound QNZ-46 has an IC_{50} of 7.1 and 3.9 μM at GluN1/2C and GluN1/2D, respectively, and has minimal effects on AMPA and kainate receptors. QNZ-46 does not compete with glutamate or glycine binding and inhibition is voltage-independent. Interestingly, the inhibition by QNZ-46 is dependent on glutamate binding, but not glycine binding, and the potency of QNZ-46 is increased when glutamate is bound [384]. Glutamate deactivation is prolonged in the presence of QNZ-46, consistent with a mechanism in which QNZ-46 must unbind before glutamate can unbind [384]. Structural determinants of action appear to reside in the lower lobe of the GluN2D LBD, however, the precise binding site for this series remains to be determined [384].

A series of dihydroquinolone-pyrazoline (DQP) analogues are, like QNZ-46, GluN2C- and GluN2D-selective NAMs [385]. The prototypical analogue in this series, DQP-1105, is ~50-fold selective for GluN2C/D-containing receptors with IC_{50} values of 7.0 and 2.7 μM at recombinant GluN1/2C and GluN1/2D, respectively [385]. Inhibition by DQP-1105 is voltage-independent and is not surmounted by increased concentrations of glutamate or glycine, consistent with a non-competitive mechanism of action [385]. Inhibition by DQP-1105 is dependent on glutamate binding [385], a property it shares with the QNZ class of inhibitors. Similarly, the structural determinants of DQP-1105 action resided in the lower lobe of the GluN2D LBD and largely overlapped with those of the QNZ class of inhibitors [385]. The finding that the QNZ and DQP series share similar structural determinants on GluN2C/D-containing receptors raises the possibility their binding sites may overlap and that the binding pocket could be exploited by a wide array of ligands with distinct binding

modes. Further exploration of the DQP structure-activity relationship led to the synthesis of chiral compounds with nanomolar activity at GluN2C/D-containing receptors, making the DQP series more potent and selective than the QNZ series [386]. DQP-1105 has been used in several recent studies illustrating roles for GluN2C and GluN2D in normal physiology as well as pathophysiology in various nuclei of the brain [379,84,387–389].

A series of tetrahydroisoquinoline PAMs are highly selective for GluN2C/D-containing NMDA receptors [390]. Further exploration of the structure-activity relationship for this class of PAMs resulted in a large family of stereo-selective analogues with strong selectivity for GluN2C/D-containing receptors, some of which have nanomolar EC_{50} values [391,392]. Separation of CIQ, an early prototype in this class of GluN2C/D-selective PAMs, into its two stereoisomers showed that (+)-CIQ contains all the activity observed for the racemic mixture [391,392] and has reduced off-target actions [393]. CIQ has similar potency and efficacy at GluN2C- and GluN2D-containing receptors, as do virtually all related analogues studied to date [391,392]. Importantly, it has been demonstrated that CIQ also potentiaties responses from triheteromeric GluN1/2A/2C and GluN1/2B/2D receptors, albeit with some reduction in efficacy [390]. CIQ has no effect on the deactivation time course for GluN1/2D, but prolong glutamate deactivation for GluN1/2C [390]. Chimeric and mutational studies suggest that the potentiation by CIQ is dependent on residues in the M1 transmembrane helix and a short preM1 helix in the GluN2 subunit [390,191]. However, whether these structural determinants correspond to the CIQ binding site remains to be determined. Racemic CIQ and (+)-CIQ have been used in several studies probing the expression and role of GluN2D in synaptic transmission in various nuclei across the brain and spinal cord [394,395,84,393,396,387,397]. One series of GluN2C-selective PAMs has been described (i.e. PYD compounds) [160,398]. The structure-activity relationship for this series revealed stereo-selective actions and additional analogues with enhanced potency [398]. To date, the PYD series is the only highly-selective positive modulator series that discriminates between GluN2C- and GluN2D-containing NMDA receptors. The prototypical analogue, PYD-106, has an EC_{50} of 16 μM at GluN1/2C and maximally potentiates receptor responses to 200% of control [160]. PYD-111, a closely-related analogue, is slightly more potent with an EC_{50} of 4 μM [398]. Interestingly, PYD-106 is highly selective for the diheteromeric GluN1/2C receptors, but has no effect on triheteromeric GluN1/2A/2C receptors [160]. PYD-106 has a weak allosteric effect on glutamate potency and modestly prolongs the glutamate deactivation time-course (in the sustained presence of glycine) [160]. Chimeric and mutational studies identified structural determinants of PYD-106 actions at a unique site residing at the interface of the GluN2C ATD and the upper lobe of the GluN2C LBD [160]. Modelling of the GluN1/2C structure on the basis of the GluN1/2B crystal structure [67] revealed that residues that affect PYD-106 actions line a large pocket, suggesting a novel modulatory site on the NMDA receptor [160]. The pharmacology of GluN2C/D-selective modulators is summarized in Table 3.

5 NMDA receptor subtypes in the CNS

As described above, the different GluN2 subunits endow the NMDA receptor subtypes with distinct functional properties, unique pharmacology, and markedly different mechanisms of regulation. This feature is a major determinant of the variation observed between distinct

neuronal cell types with respect to the time course of the synaptic NMDA receptor response as well as their changes in response to neuronal activity or other stimuli. Thus, the neuronal cell types in the different brain regions or nuclei can precisely tune their functional properties by expressing different complements of GluN2 subunits. Furthermore, the expression profiles of the different NMDA receptor subtypes undergo marked changes during development to enable modifications of neuronal functions during critical neurodevelopmental periods and maturation of the CNS.

5.1 Distinct expression profiles of NMDA receptor subunits

The different GluN2 subunits have profoundly different regional and developmental expression profiles (Fig. 2a). The GluN2B subunit is widely expressed in the embryonic brain, but becomes restricted to the forebrain in the adult rodent brain [93,399,63,92,400,95]. By contrast, the expression of GluN2A subunit is ubiquitous in the CNS, initially at very low levels at birth, after which the expression increases dramatically during the second postnatal week (P7–P14). Thus, in some regions, such as the cortex and hippocampus, there is a developmental switch in the expression of GluN2B to GluN2A, and synaptic NMDA receptors change from mainly containing GluN2B early in life to also containing GluN2A (see below) [135]. In the adult brain, the GluN2A is present in virtually all regions of the CNS with particular high abundance in the cortex, hippocampus, and cerebellum [93,399,63,92,400,95]. Expression of GluN2C is undetectable at birth, but in the second postnatal week this subunits becomes highly enriched in the cerebellum and the olfactory bulb [93,63,92,400–402]. Similar to GluN2B, the GluN2D subunit is widely expressed early in development, but then expression fades in the second postnatal week. The GluN2D subunit remains expressed into adulthood with the highest abundance in the diencephalon, mesencephalon, and spinal cord [93,63,92,403].

In addition to the aforementioned overall expression profiles, the different GluN2 subunits can be found in distinct neuronal populations in some brain regions. Thus, although the overall expression of a GluN2 subunit may appear low in a specific region, the expression can still be high in a small subpopulation of neurons in that region. For example, the overall expression levels of GluN2C and GluN2D appear to be low in the cortex and hippocampus, but more precise anatomical localization of these subunits suggest that they are specifically expressed in some populations of glial cells and interneurons in these regions [63,404,401,405,393]. Similarly, GluN2B and GluN2D are highly expressed in cerebellar Golgi cells, although they are considered to have less overall abundance in the cerebellum [137,406]. In recent years, increasingly precise identification of GluN2 subunit expression and subcellular localization in distinct neuronal populations have been reported as more refined methods of detection and pharmacological tool compounds become available.

Weak expression of GluN3A can be detected in several regions of the embryonic brain, and expression increases throughout the brain during the early postnatal development [407–410,116,411]. GluN3A expression peaks around postnatal day 8 (P8) in rodents, but then diminishes with time. By adulthood, GluN3A is weakly, but widely, expressed in the CNS. By contrast, expression of GluN3B slowly increases during the late stages of postnatal development and becomes widely expressed in the adult CNS [119,412–414]. Thus, there is

an apparent developmental switch from expression of GluN3A to GluN3B in the rodent brain during the first two postnatal weeks. In addition, GluN3B is also highly expressed in motoneurons in the rodent spinal cord, but here expression starts at embryonic day 16 (E16) [413]. Recent studies suggest that the GluN3 subunits also have distinct subcellular distributions with GluN3B found primarily in the postsynaptic membrane and GluN3A found mostly at extrasynaptic and presynaptic sites [415]. The contrasting expression profiles of the GluN3 subunits suggest they serve distinct physiological roles in the CNS. It should be noted, however, that the expression profiles of GluN3 subunits appears to vary markedly between brain regions [407–410,116,411,119,412–414], and also appears to be different in rodents compared to primates and humans [416,111,417,418].

Functional properties and trafficking of NMDA receptor subtypes are influenced by alternative splicing of the GluN1 mRNA. Differences between the regional and developmental distributions of GluN1 isoforms in the CNS have been described (Fig. 3a) [93,92,63,77,78]. However, the functional significance of these differences remains unclear and not as well characterized as those of GluN2 subunits. Consistent with its inclusion in all NMDA receptor subtypes, the GluN1 subunit is ubiquitously expressed in the CNS throughout development [93,92,63,77,78]. The GluN1–2 isoforms are widely distributed in the rodent brain, whereas low expression of GluN1–3 isoforms appears to be restricted to the sensorimotor cortex, the neocortex, hippocampus, and selected thalamic nuclei at later developmental stages. There is an apparent complementary distribution of GluN1–1 and GluN1–4 isoforms with GluN1–1 primarily expressed in more rostral regions, such as the cortex and hippocampus, and GluN1–4 in more caudal regions, such as the basal ganglia and cerebellum. The expression of GluN1-a and GluN1-b isoforms largely overlap, but marked variation in the relative abundance is observed between regions and even between neuronal cell types in the same region. For example, GluN1-a and GluN1-b isoforms have strikingly distinct developmental expression profiles in the hippocampus, and in the adult rodent brain, GluN1-b appears to be the major isoform in the CA3, while GluN1-a is the major isoform in the CA1 and dentate gyrus [78]. These differences in expression profiles are likely to have functional significance, since the deactivation time course of NMDA receptors containing GluN1-b (e.g. GluN1–1b) is accelerated compared to receptors containing GluN1-a (e.g. GluN1–1a) (Fig. 3c) [82,83], and GluN1–1b-containing receptor are less sensitive to endogenous negative allosteric regulators [80,81].

5.2 The GluN2B to GluN2A developmental switch

The increase in GluN2A expression during the second postnatal week in the rodent cortex and hippocampus results in a switch in the subunit composition of synaptic NMDA receptors from primarily being GluN2B-containing to also being GluN2A-containing. This switch is accompanied by the appearance of triheteromeric GluN1/2A/2B receptors, which contain two GluN1, one GluN2A, and one GluN2B subunit [132,135]. At early developmental stages, the time course of the EPSC (i.e. deactivation time constant) and the sensitivity to GluN2B-selective NAMs, such as ifenprodil, suggest that diheteromeric GluN1/2B is the primary NMDA receptor subtype in central synapses of the cortex and hippocampus [419,132,420,149,421]. However, the marked acceleration of the EPSC time course and reduced ifenprodil sensitivity observed during the second postnatal week are consistent with

a switch in the synaptic content from GluN2B-containing to GluN2A-containing NMDA receptors. That is because the deactivation time constants of both diheteromeric GluN1/2A and triheteromeric GluN1/2A/2B are markedly faster than diheteromeric GluN1/2B (Fig. 4) [133,151]. Furthermore, maximal inhibition by GluN2B-selective NAMs is retained, but significantly reduced for triheteromeric GluN1/2A/2B compared to diheteromeric GluN1/2B [151,150].

The “GluN2B to GluN2A developmental switch” is evolutionarily conserved and occurs in many brain areas of frogs, birds and mammals, including cortex, hippocampus, amygdala and cerebellum. Numerous studies have reported that the timing of the switch, which varies between brain regions, is coincident with changes in specific learning abilities. The prevalent hypothesis is therefore that the GluN2B to GluN2A developmental switch is a major factor in the synaptic maturation, which is important for the refinement and fine tuning of neuronal circuits. The developmental switch in NMDA receptor subunit composition closes a critical period for the refinement of connections in the key brain regions, resulting changes in synaptic plasticity [422,423]. However, in some brain circuits, the changes in synaptic plasticity during critical developmental periods are not corresponding to the switch from GluN2B to GluN2A expression (e.g. [419]), suggesting that other NMDA receptor subunits (e.g. GluN2C/D or GluN3 subunits) may have important roles in the refinement and fine tuning of these neuronal circuits.

The switch in the GluN2 subunit composition of synaptic NMDA receptors is experience-dependent and can occur acutely following synaptic activity or sensory input. For example, the change from synaptic GluN2B- to GluN2A- containing NMDA receptors is not observed in the visual cortex of dark-reared rats until they are exposed to light [424]. Thus, the EPSCs in the visual cortex of dark-reared rats have slower time course and higher sensitivity to ifenprodil compared to light-reared rats. Remarkably, returning the animals to the dark can restore the synaptic content of GluN2B to levels observed in animals that have not been exposed to light [425]. Thus, the experience-dependent GluN2 subunit switch appears to be bi-directional, at least in some brain regions [420].

The mechanisms that mediate the exchange of synaptic GluN2B-containing NMDA receptors with GluN2A-containing receptors are not fully understood and this remains an area of intense investigation. Similarly, detailed insights to the consequences of changes in GluN2 subunit composition on the refinement of synaptic plasticity and neuronal circuits are still lacking. However, many excellent reviews discuss our accumulated understanding of these processes and highlights important studies in these areas of NMDA receptor research [426,326,23].

6 NMDA receptors in disease

NMDA receptors have been considered in the context of numerous neurological conditions, either as a potential causative feature, exacerbating component, or therapeutic target [23,1,2,427,42]. However, the interest in NMDA receptor modulators as therapeutics has grown significantly in recent years. Contributing to this interest has been the growing clinical evidence that the NMDA receptor channel blocker ketamine could act as a radically

new treatment for depression. Indeed, discovery of the antidepressant activity of ketamine has been characterized as “the most important psychiatric discovery in half a century” [428,429]. Here, we will highlight two emerging mechanistic themes in this area of drug discovery. These are the growing awareness of the significance of metaplasticity in the therapeutic response to NMDA receptor modulation and the progress in linking NMDA receptor subtypes to CNS disorders.

6.1 Depression

Short intravenous infusions of the pan-NMDA receptor channel blocker ketamine (0.5 mg/kg over 40 min) has now been repeatedly demonstrated to yield a robust antidepressant response that 1) develops within hours, 2) may last for days to weeks, and 3) is effective in up to 70% of patients [430–432]. This ketamine regimen also is reported to reduce suicidal ideation [433,434] and have benefit in patients suffering bipolar depression [435], obsessive-compulsive disorder [436], and post-traumatic stress disorder [437]. The antidepressant response appears to be sustainable with repeated doses [438–440] and clinical studies are beginning to define the optimal dose and treatment chronicity [441]. Side effects include those expected for an NMDA receptor channel blocker, including cognitive disruption and neuropsychiatric symptoms; however, these appear to be mild and manageable at effective exposures [442]. Indeed, intravenous infusion may be the most significant limitation to ketamine use and clinical studies are exploring other routes of administration [443,444]. Furthermore, another NMDA receptor channel blocker, lanicemine [445,446], and GluN2B-selective negative allosteric modulators (GluN2B NAMs) [447,448] also are reported to have clinical antidepressant activity. Several detailed reviews of the rapid progress in this area have been recently published [429,449].

A remarkable aspect of the antidepressant activity of ketamine is that the clinical response develops and is sustained after the drug has been cleared from the body. In contrast, the psychotomimetic effects track closely with drug residence time and typically resolve shortly after cessation of drug infusion [430]. The antidepressant effects of other NMDA antagonists also persist beyond drug clearance from the body. In fact, it has been shown that the brief ketamine exposure is sufficient to induce a long-lasting change in human brain physiology [431]. These results may indicate that the antidepressant effects of these drugs arise from a metaplastic change in synaptic activity. Metaplasticity is ‘the plasticity of synaptic plasticity’ [450]; that is, the effect that an acute change in synaptic function has on the ability of subsequent stimuli to effect further change [451,452]. The antidepressant effects of NMDA receptor inhibitors may be interpreted as a variation on this theme. The antidepressant response is not the direct result of acute NMDA receptor inhibition, but rather a long-lasting change in synaptic function triggered by the brief inhibition. There is considerable interest in determining the nature of these long-lasting synaptic changes at the molecular level, as these findings might reveal insight into the neurobiology of depression and be applied prospectively to develop new antidepressants. There is speculation that the antidepressant effect of ketamine may be due to an effect of a metabolite that does not inhibit NMDA receptors [453]; however, this has not yet been reconciled with the clinical antidepressant effect of the other chemically and mechanistically diverse NMDA receptor modulators.

Indeed, a fruitful avenue of research is through comparative analysis of these different agents to pinpoint common mechanisms that may account for the antidepressant effects.

Both ketamine and GluN2B NAMs induce persistent increases in synaptic strength after drug washout. This is evidenced by mTOR-driven increases in synaptic protein levels in rodents [454–456], an increase in sensitivity to the induction of LTP in rodents [457], and an increase in sensory stimulus-evoked potentials in rodents [458] and in humans [431]. A working hypothesis is that such an up-regulation of synaptic strength underlies the antidepressant activity. One hypothesis for the mechanism by which ketamine induces synaptic up-regulation derives from its use-dependence for channel block, which confers selectivity for highly active NMDA receptors on PV-positive, fast spiking GABAergic interneurons [459]. It is hypothesized that inhibiting fast-spiking interneurons disinhibit cortical microcircuits, inducing gamma-band cortical activity that drives an LTP-like up-regulation of synaptic strength. While attractive, this hypothesis accounts poorly for the putative antidepressant activity of lanicemine [445] and particularly the GluN2B NAMs. These latter compounds do not induce gamma-band activity in rodents [460,458,461] or primates [462] even at high levels of receptor occupancy. This functional difference between ketamine and the GluN2B NAMs may be accounted for by differences in brain micro-circuitry modulated by these agents. GluN2B is weakly expressed in interneurons arising from medial ganglionic eminence [463] that include the ketamine-sensitive fast spiking PV- and SST-family interneurons that synapse directly with pyramidal neurons [464]. Lack of predominant GluN2B expression on these interneurons may account for the fact that GluN2B NAMs do not induce gamma-band activity. Instead, GluN2B is expressed by CCK-family interneurons that arise from the caudal ganglionic eminence [463]. These interneurons synapse with the fast-spiking interneuron classes to regulate their activity in response to long-range pyramidal neuron inputs [465]. Thus, GluN2B NAMs may be speculated to increase activity of fast-spiking interneurons by decreasing excitatory drive on CCK-family interneurons, the opposite of the putative effect of ketamine. The effects of ketamine and the GluN2B NAMs are also likely to be divergent on pyramidal neurons. Deployment of GluN2B varies across different pyramidal neuron populations [466] and in different synaptic compartments (reviewed in [467]). The deployment of GluN2B subunits is also activity-dependent and is increased at relatively inactive synapses (reviewed in [426,468]). Thus, the pan-NMDA receptor antagonist ketamine and the GluN2B NAMs likely inhibit different receptor pools on pyramidal neurons based on the subunit-selectivity of the NAMs, as well as the activity dependence of ketamine. At present there is no obvious point of convergence between these two compound classes that may account for their striking similarity in terms of functional endpoints in preclinical models and clinical antidepressant efficacy (and side effect profile, see below). However, the fact that points of convergence are apparently so few increases the power of comparative analyses to pinpoint the molecular mechanisms of their antidepressant response. This seems a promising area for continued research.

6.2 Neurodevelopmental disorders

NMDA receptor signaling plays a central role in circuit development of the central nervous system. As noted above, during development, high expression of GluN2B and GluN2D

NMDA receptor subunits is superseded by expression of GluN2A [92,93,63,135,469,470]. This choreography mediates the transition from high levels of synaptic plasticity as circuits are formed and refined to the circuit stability of the adult brain. Consistent with a fundamental role in this developmental progression, variation in genetic loci encompassing *GRIN2A* and *GRIN2B* (i.e. genes encoding GluN2A and GluN2B, respectively) are identified in genome-wide association studies (GWAS) as contributing to the risk of developing the two major neurodevelopmental disorders, autism and schizophrenia [471–474]. The symptoms of autism manifest early in life, whereas those of schizophrenia do not fully manifest until late adolescence or early adulthood. Thus, these two disorders arise from derangements at different epochs of the brain's developmental program. The association of *GRIN2A* and *GRIN2B* genetic variation in the risks for both disorders highlights a role for NMDA receptor signaling in unfolding the entire developmental program. However, there are different scenarios by which variation in NMDA receptor signaling may contribute to these disorders that are important in considering NMDA receptor modulation as a therapeutic strategy. Defective NMDA receptor signaling could impact a specific segment of the developmental program, in which case therapeutic intervention would need to occur during that developmental epoch. Alternatively, defective NMDA receptor signaling could impact a developmental trajectory and so therapeutic intervention would need to occur at some time before the symptoms begin to manifest. Finally, aberrant NMDA receptor signaling may be a factor in the expression of symptoms, in which case NMDA receptor modulation may be effective as a 'symptomatic' therapeutic at any time after symptoms manifest. Of these three scenarios, the most extensively studied therapeutic use of NMDA receptor modulators is as a 'symptomatic' approach to schizophrenia.

6.3 Schizophrenia

The association of NMDA receptor dysfunction with schizophrenia initially arose from the clinical observation that NMDA receptor inhibition in healthy individuals induces a spectrum of symptoms that are strikingly similar to those exhibited by patients suffering schizophrenia [475–477]. These "schizophrenomimetic" symptoms (e.g. see [478]) correspond closely with NMDA receptor occupancy [477,479]. This infers that symptom expression in schizophrenia patients may result from hypofunction of NMDA receptor signaling [480–482]. The NMDA receptor hypofunction hypothesis for schizophrenia has driven a great deal of research to develop drugs to potentiate NMDA receptor signaling to overcome the symptoms of this disorder.

The largest body of work aiming to overcome NMDA receptor hypofunction encompasses strategies to increase agonist occupancy of the GluN1 glycine co-agonist binding site. This has included clinical testing of the natural ligands glycine and D-serine, glycine analogs such as D-cycloserine (DCS), and inhibition of the GlyT1 transporter to increase peri-synaptic glycine levels [483,484]. Unfortunately, the effectiveness of the glycinergic approach has so far proved modest, with the most consistent effect being a reduction in negative symptoms, but with little effect on cognitive or positive symptoms [484,485]. Nonetheless, this clinical research has yielded significant insight that may be critical to further advancement of NMDA receptor potentiator strategies. It has been suggested that treatment with "glycinergics" may trigger metaplastic changes in glutamate signaling that

significantly affect the drug response [486,487]. These effects on drug response include limited efficacy of continuous drug exposure and can cause complex dose responses, such as observed with the GlyT1 inhibitor bitopertin [488]. To exploit the plasticity induced by modulating NMDA receptors, Goff and colleagues have begun to explore intermittent dosing with the glycinergic DCS. In preliminary clinical studies, intermittent DCS treatment also improved negative symptoms. More significantly, intermittent DCS improved memory performance and reduced delusional severity when combined with cognitive behavioral therapy [487,489,490]. Thus, an intermittent DCS dosing regimen may be at least as efficacious as continuous treatment with regard to negative symptoms and may deliver efficacy against positive and cognitive symptoms not observed with continuous exposure regimens. This line of clinical research clearly calls for further study and begs investigation into the underlying molecular mechanisms.

Several mechanisms that may contribute to enhanced efficacy with intermittent DCS treatment. Increasing glycine-site occupancy to acutely increase NMDA receptor activity also increases NMDA receptor internalization rate, which may offset positive effects [491]. An intermittent dosing regimen may reduce drive on internalization and thereby tip the balance towards potentiation. More intriguing is the possibility that intermittent dosing enhances plasticity beyond a simple ‘drug-on’ potentiation [487]. The pharmacology of DCS is complex; the compound is a partial glycine site agonist and a single administration may therefore potentiate or inhibit NMDA receptors, and possibly both, over the exposure time course of a single dose. Furthermore, DCS is a super-agonist at GluN2C-containing receptors and will activate a larger current compared to glycine/D-serine [492,493], suggesting that at these receptors, substitution of DCS for glycine could selectively enhance synaptic NMDA receptor responses. An appropriate single dose may trigger a longer lasting metaplastic change in synaptic activity that results in sustained efficacy. Indeed, it is interesting to draw analogy between intermittent dosing with DCS in schizophrenia and intermittent dosing of NMDA antagonists in depression. In both cases, it is the metaplastic effect of the brief drug exposure, i.e., the ‘drug-off’ effects, that delivers the efficacy.

It is also of interest to understand the underlying mechanism(s) by which NMDA receptor hyopfunction may result in the expression of schizophrenia symptoms. The clinical pharmacology may be informative. First, DCS produce a maximal response that is twice as large as glycine at GluN2C-containing NMDA receptors, resulting in increased NMDA receptor signaling whenever concentrations of DCS allow it to displace glycine from its site these receptors [492,493]. GluN2C is highly expressed in cerebellum and in the thalamic reticular nucleus [63,494,495,401]. It has been speculated that the efficacy of DCS may be derived from agonist activity GluN2C-containing NMDA receptors in these brain regions, prompting an effort to develop other GluN2C-selective PAMs [390,398,160,394]. Another interesting clue to underlying mechanisms is the clinical observation that the GluN2B-selective NAM, CP-101,606, causes cognitive disruption and dissociative effects similar to those caused by ketamine [496,497,448]. Consistent with the clinical data, GluN2B NAMs and NMDA receptor channel blockers share discriminative stimulus properties in animal studies [498,499]. These findings are interesting with respect to the fact that there is little apparent overlap in the neuronal microcircuitry impacted by these two drug classes, as reviewed above. Thus, comparative analyses of the schizophrenomimetic effects of these

drugs may also help pinpoint the microcircuit defects in NMDA receptor signaling relevant to the expression of schizophrenia.

6.4 Epilepsy/aphasia syndromes

GRIN1, *GRIN2A*, and *GRIN2B* have been associated with epilepsy (EpiPM consortium, "Roadmap for precision medicine in the epilepsies", Lancet Neurology 2015 [500]). For example, a deterministic link has recently been made between genetic variation in *GRIN2A* and childhood epilepsy/aphasia syndromes [501–504]. The spectrum of these syndromes includes relatively benign Rolandic epilepsy, the more severe continuous spike-and-waves during slow-wave sleep syndrome (CSWSS), and Landau-Kleffner syndrome (LKS), and very severe epilepsies with significant developmental delay, intellectual disability, and dysmorphic features. Manifestation arises between ages 3–11 during the developmental epoch that is associated with language development [505]. This is also the epoch over which there is significant pruning of cortical excitatory synapses [506], in which NMDA receptor signaling is fundamentally involved. To date, more than 60 mutations in *GRIN2A* have been identified that appear to be causal to these developmental disorders [507]. Significantly, whereas many of these mutations result in receptor truncation or other losses of function, numerous point mutations result in a gain of function. This includes reduced Mg^{2+}-block, enhanced agonist potency, and increased open probability and open time, at least when the receptors are expressed in heterologous expression systems [507,508]. Critical questions remain around how specific variations in *GRIN2A*, including both gain and loss of function, relate to the spectrum of severities in a common group of epilepsies and language disorders.

The discovery of the association of *GRIN2A* mutations with epilepsy/aphasia syndromes immediately suggested NMDA receptor modulators as potential therapeutics. In a first case, Pierson, Yuan, and colleagues [159,509] identified a child through the NIH Undiagnosed Diseases Program suffering early-onset epileptic encephalopathy, manifest as profound cognitive and motor development and intractable seizures resistant to standard anticonvulsant therapies, who had a point mutation in *GRIN2A*. Analyses in heterologous expression systems revealed that the mutation resulted in significantly increased activity of GluN2A-containing receptors, suggesting that inhibition of NMDA receptors may have a therapeutic benefit where other conventional therapeutics had failed. The treatment of this patient with the NMDA receptor antagonist memantine (approved for the treatment of Alzheimer's disease) produced a rapid onset and persistent reduction in the number of seizures suffered by the child [509]. This suggests that the altered function of the GluN2A subunit may have contributed to seizure etiology. Unfortunately, memantine did not have an effect on the child's cognitive or motor disability, suggesting that the *GRIN2A* mutation also had effects on the developmental trajectory, which were insensitive to memantine at the time treatment was initiated. It should be noted that this remains only a single case, and considerable work is needed to determine whether viable treatment options can be developed for these patients with specific mutations in NMDA receptor subunits.

6.5 Rett Syndrome

Rett Syndrome (RTT) is another severe neurodevelopmental disorder in which NMDA receptor dysfunction is implicated and NMDA receptor modulators are of therapeutic

interest. RTT is a severe X-linked neurodevelopmental disorder caused by defects in transcriptional regulation by MeCP2 [510]. Although girls with RTT initially develop on a normal trajectory, developmental stasis and regression begins at 6–18 months that includes a severe reduction in the size and complexity of forebrain pyramidal neuron dendritic arbors, but without apparent reduction in neuron number [511]. Significantly, Bird and colleagues demonstrated in a mouse model that restoration of MeCP2 function in symptomatic animals reverted much of the neurological phenotype [512]. This implies that the fundamental architecture of the brain develops normally prior to the effects of MeCP2 lesion and that restoration of network function is an attainable goal. There are several emerging lines of research that suggest NMDA receptor dysfunction contributes to this network dysfunction and that modulation of these receptors may be an effective therapeutic approach [513]. Blue et al. [514] reported alteration of NMDA receptor expression in MeCP2 mutant mice. Subsequently, the Fagiolini lab reported an imbalance in GluN2A/GluN2B subunit deployment in both cortical pyramidal neurons and interneurons [515,516]. Significantly, manipulating the GluN2A/GluN2B balance through hemizygous *GRIN2A* knock out prevented the development of cortical dysfunction and ameliorated some of the RTT-like phenotype [515]. In another line of research, Katz and colleagues demonstrated that treatment of Mecp2 mutant mice with a low, sub-anesthetic dose of ketamine acutely reversed RTT-like phenotypes, including abnormal patterns of neuronal activation in cortical and subcortical structures as well as sensorimotor dysfunction [517]. Subsequently, Patrizi et al. [518] reported that once daily administration of this same low dose ketamine produced a sustained reduction in RTT-like symptoms and ameliorated structural circuit defects that underlie or contribute to neurological dysfunction. Significantly, in the study by Patrizi et al., neurological testing of mice occurred ~21 h after drug administrations; i.e., after ketamine had been completely eliminated. Thus, it appears that ketamine has beneficial effects in mouse RTT models during both “drug-on” [517] and “drug-off” [518] periods. The latter drug-off effects suggest a potential mechanistic parallel to the effects of ketamine in depression, particularly with respect to the possibility of durability of action beyond the acute period of NMDAR antagonism. Trials have now been initiated to test the safety and efficacy of NMDAR antagonists in RTT patients, including dextromethorphan, a weak NMDAR antagonist, and low-dose ketamine [513].

6.6 NMDA receptors as therapeutic targets

Clearly, the most significant recent advance in the area of therapeutics targeting NMDA receptors has been the emergence of ketamine as a rapidly acting antidepressant. Ketamine is now being used in clinics to treat patients for which standard of care monoaminergic reuptake inhibitors provides little relief. Ketamine is also groundbreaking from a mechanistic perspective. The therapeutic effect of ketamine is not due to an effect of the drug ‘on’ the brain per se, but to the response of the brain to the drug that manifests after the drug is gone. The concept of synaptic metaplasticity has been evolving for decades [519,451,452], and ketamine is the first example where such a metaplastic effect has been harnessed for therapeutic benefit. Indeed, this therapeutic effect may be broad, as clinical data is emerging to suggest that brief ketamine exposure may be beneficial across a range of neuropsychiatric conditions and perhaps in neurodevelopmental disorders. While the discovery of the antidepressant effect of ketamine was serendipitous [430], as were the

implications of metaplasticity as its therapeutic mechanism, Goff and colleagues have been developing a parallel theme of inducing plasticity in exploring the utility of intermittent dosing of DCS in schizophrenia and anxiety disorders [487]. These research paths may be the herald of a new era in the development of CNS therapeutics in which we try and work with the brain instead of trying to overpower it.

The above notwithstanding, there is a tremendous amount of work ahead to build on the theme of harnessing the brain's plasticity to therapeutic benefit. From a very practical perspective, the optimal duration of NMDA receptor inhibition and exposure interval remains to be determined in order to realize therapeutic benefits in depression and other conditions. For example, a single exposure to the GluN2B NAM CP-101,606, a short half-life compound, had a robust and long lasting antidepressant effect [448], whereas a long half-life GluN2B NAM, CERC 301, dosed for 28 days and likely resulting in continuous NMDA receptor occupancy, was without efficacy. Although it is not possible to draw firm conclusions from single studies, it is tempting to speculate that the difference in efficacy in these studies may be due in part to the difference in exposure duration, with the short duration exposure allowing the metaplastic mechanism to emerge.

It is also important to note the significance of back-translational research in advancing this area. Clinical data on NMDA receptor modulators provides a rich frame for preclinical studies into molecular mechanisms of disease as well as for new therapeutic approaches. As an example mentioned above, the similarity in clinical efficacy and side effect profile between ketamine and the GluN2B NAM CP-101,606, in light of the apparently scant overlap in site of action at the level of neurocircuitry, may be leveraged to gain significant insight into the role of NMDA receptor signaling in both depression and schizophrenia [520]. It also appears that the repertoire of NMDA receptor modulators is expanding rapidly [521,55,54,96]. This includes compounds with unique NMDA receptor subtype selectivity and modes of action that include both augmenting and inhibiting receptor activity. Thus, these compounds will provide new tools to interrogate the physiology of NMDA receptor signaling, which in turn may reveal new therapeutic opportunities. As insightfully pointed out by Köhr [522,523], it will be important to consider not only the locus of action of such compounds based on NMDA receptor subtype expression pattern, but also the mechanism of pharmacological action, as each may have a significant impact on the functional effects of these new compounds. Based on the new insights gained from the effects of ketamine and DCS, it will also be important to consider the metaplastic effects of these compounds in addition to their more direct effects on signaling.

7 Conclusions

Emerging information from genetic analyses linking NMDA receptors to specific disease conditions and the discovery of antidepressant effects for NMDA receptor antagonists have fortified and reinvigorated the long-standing focus on NMDA receptors as therapeutic targets. Recent year's remarkable acceleration in the discovery of novel allosteric NMDA receptor modulators as pharmacological tools greatly facilitates studies to achieve new levels of understanding of NMDA receptor subtypes in physiology and disease. Many new modulatory binding sites in NMDA receptors have been identified along the way and

combined with rapidly improving structural crystallographic and cryo-EM data, we are improving our understanding of how agonist binding is linked to channel gating and how the different subunits contribute to conformational changes during gating and allosteric modulation. These developments in the NMDA receptor field offer new perspectives and exciting opportunities to study unique roles for NMDA receptor subtypes, diheteromeric as well as triheteromeric, in distinct neuronal populations and subcellular locations. Furthermore, the converging advances in NMDA receptor pharmacology and clinical and mechanistic understanding of CNS diseases involving NMDA receptor dysfunction are poised to result in the development of new therapeutic agents.

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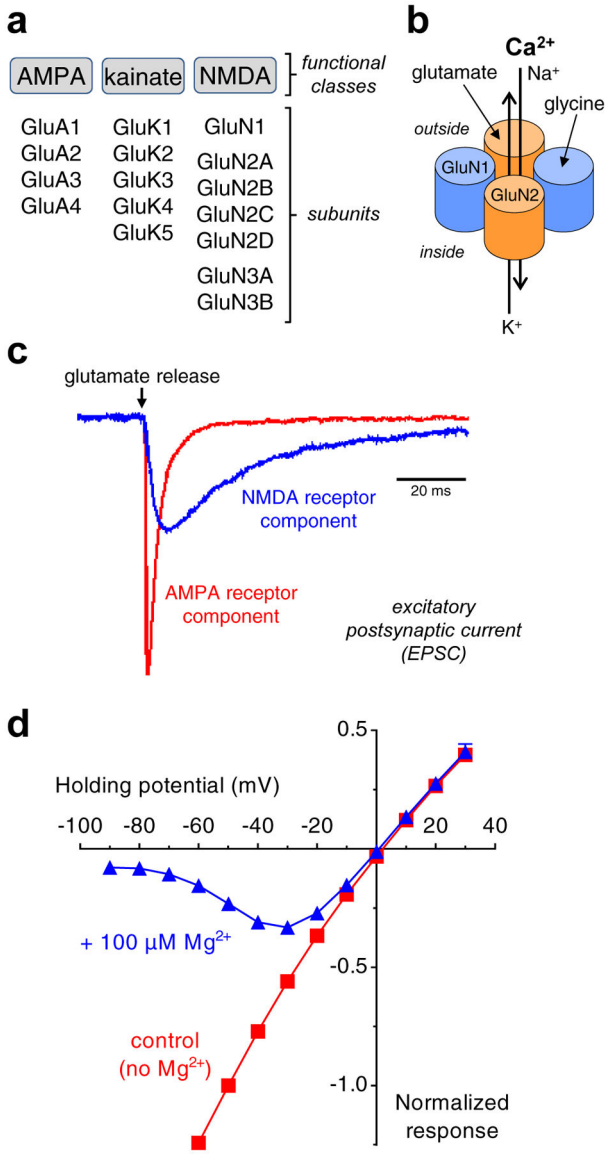


Figure 1. Functional classes of ionotropic glutamate receptors.

a) Ionotropic glutamate receptors are divided into three functional classes, namely AMPA, kainate, and NMDA receptors. Multiple subunits have been cloned in each of these classes. **b**) The majority of NMDA receptors in the CNS are composed of two glycine-binding GluN1 and two glutamate-binding GluN2 subunits, which form a central cation-permeable channel pore. **c**) AMPA and NMDA receptor-mediated components of the EPSC at a central synapse. The slow NMDA receptor-mediated component is isolated in the absence of Mg^{2+} using the AMPA receptor antagonist CNQX, whereas the fast AMPA receptor-mediated component is isolated using the NMDA receptor antagonist AP5. The figure shows unpublished data from Lonnie P. Wollmuth and is adapted with permission from Traynelis et al. [1]. **d**) Relationship between NMDA receptor current response and membrane potential (i.e. I/V-relationship) in the presence and absence of 100 µM extracellular Mg^{2+}. Voltage-dependent Mg^{2+}-block is relieved with depolarization of the membrane potential (i.e. as the

membrane potential approaches 0 mV). Unpublished data from Feng Yi and Kasper B. Hansen.

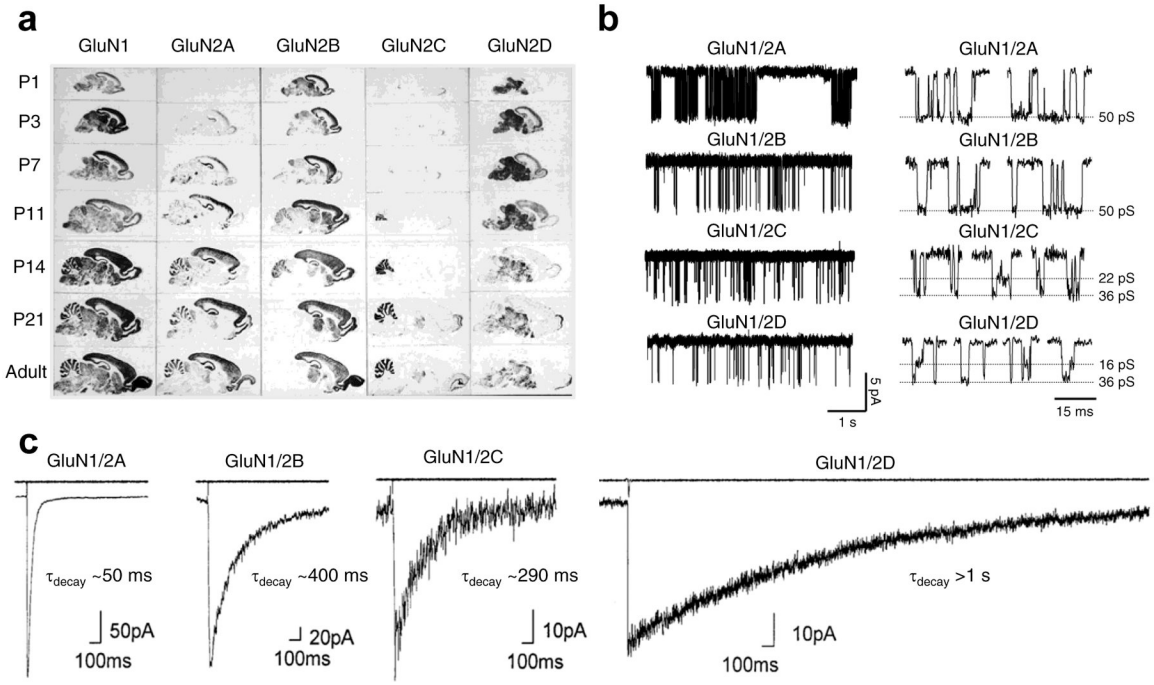


Figure 2. GluN2 subunit-specific expression and functional properties of recombinant NMDA receptor subtypes.

a) Regional and developmental expression of GluN2 subunits in rat brain revealed in autoradiograms using *in situ* hybridizations of oligonucleotide probes for the relevant mRNAs to parasagittal sections. Modified with permission from Akazawa et al. [92]. **b**) Single-channel recordings of currents from diheteromeric NMDA receptor subtypes expressed in HEK293 cells (outside-out membrane patches). Open probability is ~0.5 for GluN1/2A, ~0.1 for GluN1/2B, and <0.05 for GluN1/2C and GluN1/2D. Highlights of individual openings are shown on the left. GluN1/2A and GluN1/2B have higher channel conductance (~50 pS) compared to GluN1/2C (~22 and ~36 pS) and GluN1/2D (~16 and ~36 pS). Adapted with permission from Yuan et al. [524]. **c**) Whole-cell patch-clamp recordings of responses from brief application of glutamate (1 ms of 1 mM glutamate) to recombinant diheteromeric NMDA receptor subtypes expressed in HEK293 cells. The open tip current indicating the duration of the drug application is shown in the upper trace. Adapted with permission from Vicini et al. [62].

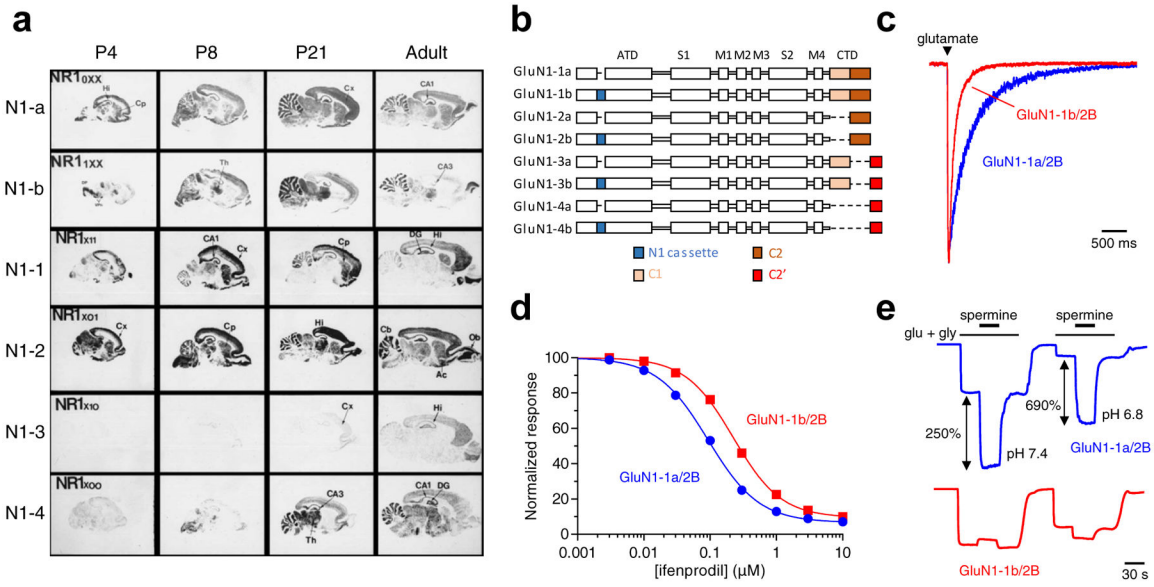


Figure 3. Expression and functional properties of GluN1 splice variants.

a) Regional and developmental expression of GluN1 splice variants in rat brain revealed in autoradiograms using *in situ* hybridizations of oligonucleotide probes for the relevant mRNAs to parasagittal sections. Ac, nucleus accumbens; Cb, cerebellum; Cp, caudate-putamen; Cx, cortex; DG, dentate gyrus; DP, dorsal pons; Hi, hippocampus; Ob, olfactory bulb; Th, thalamus; VPn, ventro-posterial thalamic nuclei. Modified with permission from Paupard et al. [78]. **b**) Linear representation of the GluN1 polypeptide chain for eight alternative splice variants. GluN1 subunits are composed of the amino-terminal domain (ATD), S1 and S2 segments that form the ligand binding domain (LBD), three transmembrane helices (M1, M3, and M4) and a membrane reentrant loop (M2), and the intracellular carboxyl-terminal domain (CTD). The N1 cassette (blue) is 21 amino acids in the ATD encoded by exon 5. The C1 cassette (yellow) is 37 amino acids in the CTD encoded by exon 21, while the C2 cassette (orange) is 38 amino acids in the CTD encoded by exon 22. Deletion of exon 22 creates a shift in the open reading frame, resulting in the alternate exon 22' that encodes the C2' cassette (red; 22 amino acids). **c**) Whole-cell patch-clamp recordings of responses from brief application of glutamate (1 ms of 1 mM glutamate) to recombinant GluN1–1a/2B and GluN1–1b/2B receptors expressed in HEK293 cells. NMDA receptors containing exon 5 (e.g. as in GluN1–1b) display faster deactivation time course compared to receptors lacking exon 5 (e.g. as in GluN1–1a). **d**) Ifenprodil concentration-inhibition relationships for recombinant GluN1–1a/2B and GluN1–1b/2B receptors expressed in *Xenopus* oocytes. Ifenprodil potency is lower for receptors containing exon 5. **e**) Representative recordings for spermine potentiation of responses from recombinant GluN1–1a/2B and GluN1–1b/2B receptors expressed in *Xenopus* oocytes. Spermine sensitivity is dramatically reduced for receptors containing exon 5. Data in **c-e**) are unpublished from Feng Yi and Kasper B. Hansen.

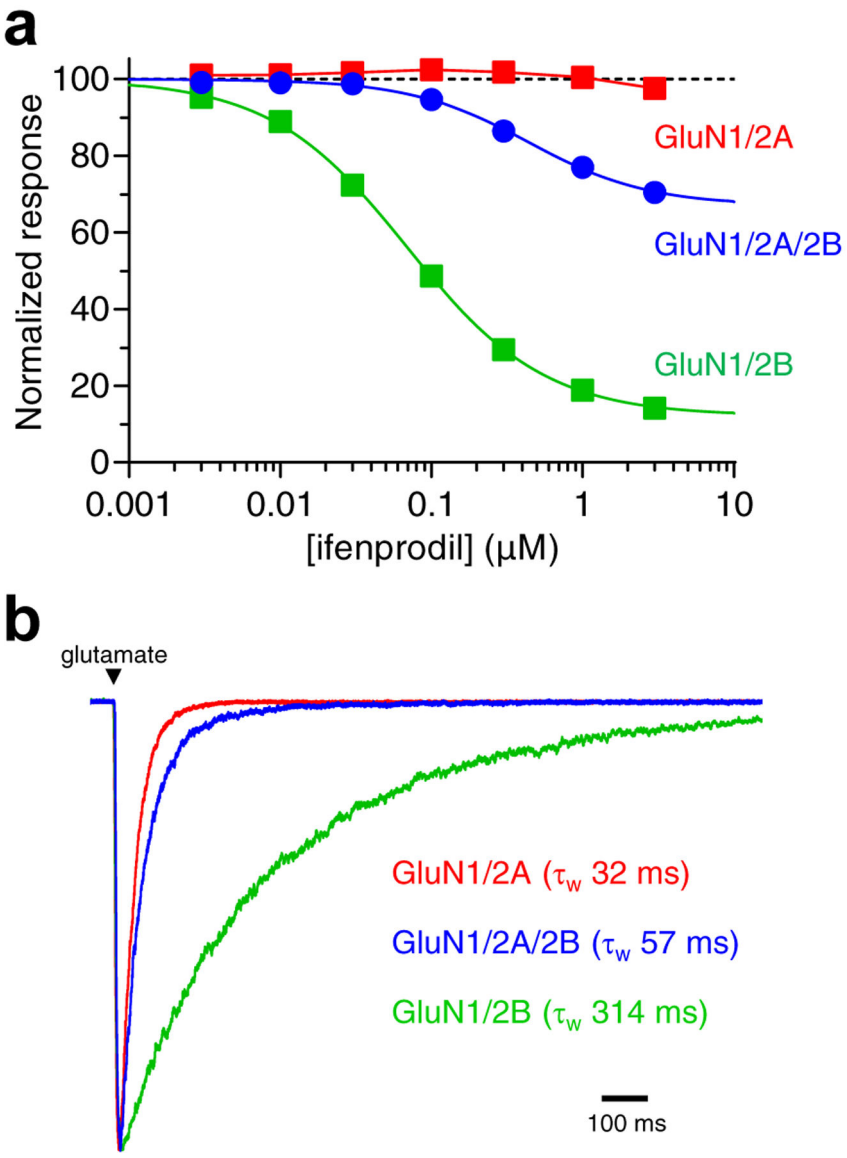


Figure 4. Functional properties of triheteromeric GluN1/2A/2B receptors.

a) Ifenprodil concentration-inhibition relationships for recombinant diheteromeric GluN1/2A and GluN1/2B receptors and triheteromeric GluN1/2A/2B receptors expressed in *Xenopus* oocytes using a method to control subunit composition of NMDA receptors [151]. Ifenprodil efficacy and potency are reduced for triheteromeric GluN1/2A/2B receptors that only contain one binding site for ifenprodil. **b**) Whole-cell patch-clamp recordings of responses from brief application of glutamate (1 ms of 1 mM glutamate) to recombinant diheteromeric GluN1/2A and GluN1/2B receptors and triheteromeric GluN1/2A/2B receptors expressed in HEK293 cells. The deactivation time course of triheteromeric GluN1/2A/2B receptors is similar to diheteromeric GluN1/2A and strikingly different from diheteromeric GluN1/2B. Data are adapted with permission from Hansen et al. [151].

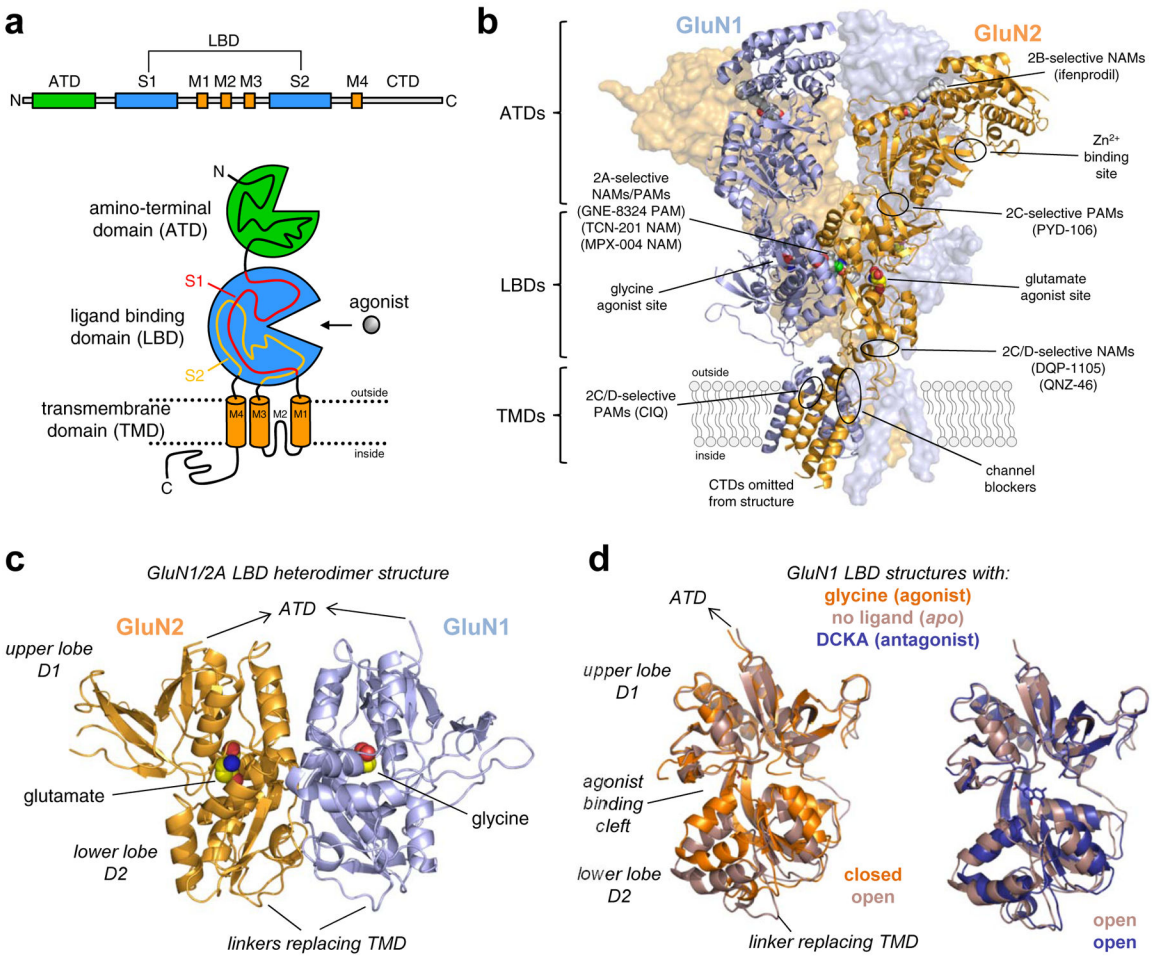


Figure 5. NMDA receptor structure and ligand binding sites.

a) Linear representation and cartoon illustration of the polypeptide chain in iGluR subunits. Each subunit consists of a large extracellular amino-terminal domain (ATD), a bi-lobed ligand binding domain (LBD), a transmembrane domain (TMD), and an intracellular CTD. The TMD is formed by three transmembrane helices (M1, M2, and M4) and a membrane re-entrant loop (M2). The LBD is formed by two segments of the polypeptide chain (S1 and S2), which fold into a kidney-shaped structure composed of an upper lobe (D1) and lower lobe (D2) relative to the cell membrane, and the agonist binding site is located in the cleft between the two lobes. **b**) Crystal structure of the GluN1/2B NMDA receptor (PDB ID 4PE5; [67]), illustrating the subunit arrangement and the layered domain organization composed of the TMD layer and two extracellular layers formed by LBDs and ATDs. Agonist binding sites as well as known and predicted binding sites for positive and negative allosteric modulators (PAMs and NAMs) are highlighted. **c**) Crystal structure of the soluble GluN1/2A LBD heterodimer (PDB ID 5I57; [158]), showing the subunit interface and back-to-back dimer arrangement of the LBDs. Soluble LBD proteins composed of the S1 and S2 segments of the polypeptide chain are produced by deleting the ATD and replacing the TMD with a di-peptide linker. **d**) Overlay of crystal structures of the soluble GluN1 LBD in the *apo*-form (PDB 4KCC; [168]) or in complex with the agonist glycine (PDB ID 5I57; [158])

or competitive antagonist DCKA (PDB ID 4NF4; [166]). The upper D1 lobes are aligned to illustrate the similar conformations of antagonist-bound and *apo*-form structures. Agonist binding induces considerable closure of the LBD compared to the antagonist-bound and *apo*-form structures, and agonist-induced closure of the LBD is required for activation of NMDA receptors. Competitive antagonists bind the LBD without inducing domain closure, thereby preventing agonist binding and receptor activation.

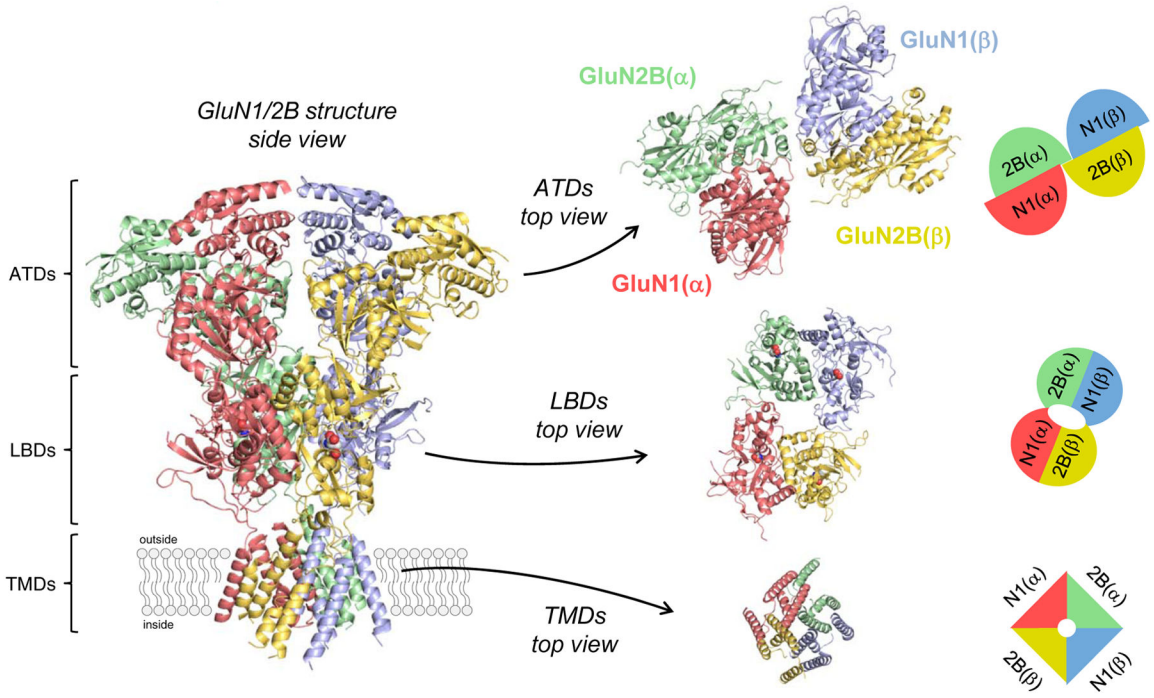


Figure 6. Subunit crossover and symmetry mismatch in the NMDA receptor structure.

Side view of the GluN1/2B NMDA receptor structure (PDB ID 4PE5; [67]) and top views of the ATD, LBD, and TMD layers. The subunits in GluN1/2 receptors are arranged in an alternating pattern (i.e. 1-2-1-2) and there is a symmetry mismatch between the TMDs and the extracellular LBDs and ATDs of the receptor. The TMDs are arranged symmetrically around the ion channel pore with a quasi-4-fold symmetry, whereas the extracellular portion adopts a dimer-of-dimer arrangement (i.e. two GluN1/2 heterodimers) with a 2-fold symmetry. There is a subunit crossover between the LBD layer and the ATD layer in that the GluN1(α) ATD forms a local dimer with the GluN2B(α) ATD, whereas the GluN1(α) LBD forms a local dimer with the GluN2B(β) LBD.

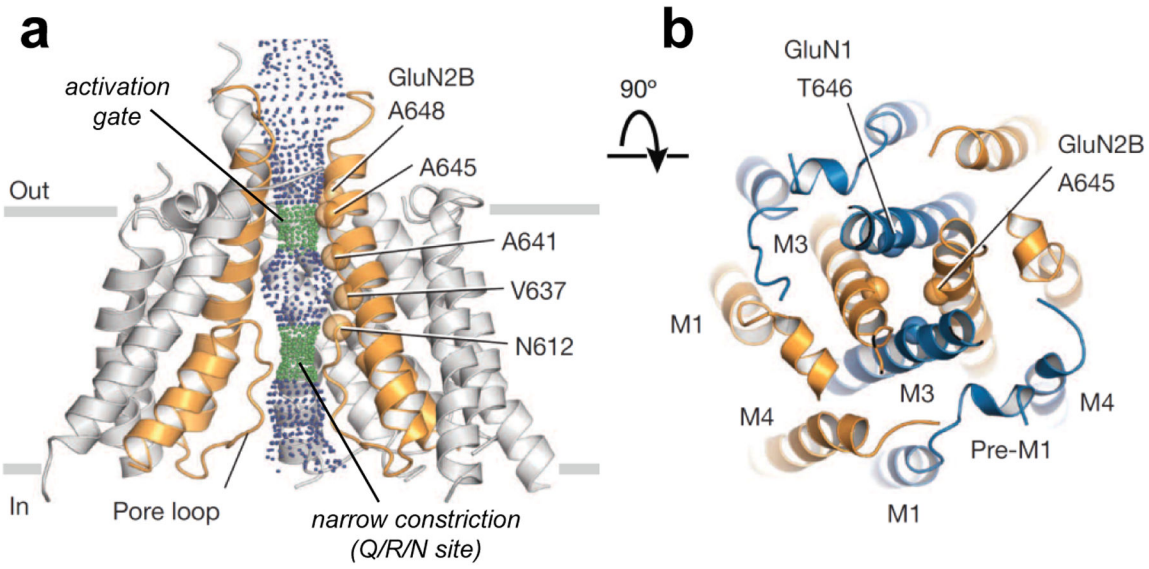
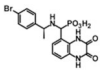
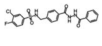
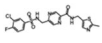
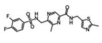
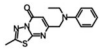
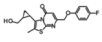
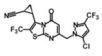
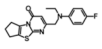


Figure 7. Structural determinants in the NMDA receptor ion channel pore.

a) View parallel to the membrane of the TMDs in the GluN1/2B NMDA receptor structure (PDB ID 4TLM; [66]). The solvent accessible surface is carved along the pore axis using the computer program HOLE and shows the M3 bundle crossing near the extracellular side of the membrane, which presumably forms the activation gate, and the narrow constriction in the pore (Q/R/N site). Green dots indicate a pore radius of 1.15–2.3 Å and blue dots indicate a pore radius greater than 2.3 Å. **b**) View of the TMDs from the extracellular side of the membrane along the pore axis. GluN1 and GluN2B subunits are blue and orange, respectively. The α-carbon of residues T646 and A645, which appear to define the activation gate, are highlighted as spheres. Adapted with permission from Lee et al. [66].

Table 1.

Summary of GluN2A-selective modulators.

Compound			Activity at GluN1/2X (in μM) 2A	2B	2C	2D	
NVP-AAM077		K_B [a]	0.015	0.078	-	-	[352]
TCN-201		K_B [a,b]	0.045 0.070 0.027	NE	NE	NE	[356] [355] [158]
MPX-004		IC_{50} [c,d]	0.079 [c] 0.198 [†]	NE [c] NE [†]	- [c] NE [†]	NE [c] NE [†]	[357]
MPX-007		IC_{50} [c,d]	0.027 [c] 0.143 [†]	NE [c] ND [†]	- [c] NE [†]	NE [c] NE [†]	[357]
GNE-3419		EC_{50} [c]	2.03	NR	NR	NR	[157]
GNE-6901		EC_{50} [c]	0.33	NR	NR	NR	[157]
GNE-0723		EC_{50} [c]	0.021	ND	7.4	6.2	[170]
GNE-8324		EC_{50} [c]	2.43	NR	NR	NR	[157]

- denotes not determined, NE denotes no effect at the highest concentrations evaluated, and ND indicates that the compound displayed some activity, but the affinity or potency could not be determined. NR denotes some activity, but that the numerical affinity value was not reported. Unless otherwise stated (also denoted by †), the values were determined using two-electrode voltage-clamp experiments with *Xenopus* oocytes.

[a] denotes when Schild analysis was used for affinity determination.

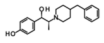
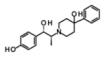
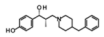
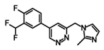
[b] three independent studies are published reporting the K_B of TCN-201 at GluN1/2A.

[c] denotes that potency (i.e. half maximally effective concentration) was determined using a Ca^{2+} imaging assay.

[d] Experiments using MPX compounds were performed in 3 μM glycine.

Table 2.

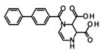
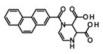
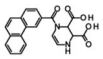
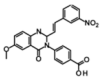
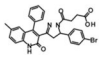
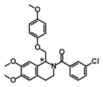
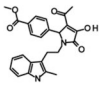
Summary of GluN2B-selective modulators.

			Activity at GluN1/2X (in μM)				
Compound			**2A**	**2B**	**2C**	**2D**	
Ifenprodil		IC_{50}	39.5	0.114	29.1	75.9	[525]
CP-101,606		IC_{50}	NE	0.039	NE	NE	[85]
Ro 25–6981		IC_{50}	52	0.009	-	-	[365]
EVT-101		IC_{50}	-	0.012	-	-	[281]

-denotes not determined and NE denotes no effect at the highest concentrations evaluated. Unless otherwise stated (also denoted by †), the potency (i.e. half maximally inhibiting concentration) was determined using two-electrode voltage-clamp experiments with *Xenopus* oocytes.

Table 3.

Summary of GluN2C/D-selective modulators.

			Activity at GluN1/2X (in μM)				
Compound			**2A**	**2B**	**2C**	**2D**	
PBPD		K_i [e]	15.8	5.0	9.0	4.3	[375]
PPDA		K_i [e]	0.55	0.31	0.096	0.125	[374]
UBP141		K_i [e]	14.2	19.3	4.2	2.8	[376]
QNZ-46		IC_{50}	229 182	ND 193	6 7.1	3 3.9	[383] [384]
DQP-1105		IC_{50}	ND	113	7.0	2.7	[385]
CIQ, (+)-CIQ [*]		EC_{50}	NE	NE	2.7, 9.0 [≠]	2.8, 8.0 [≠]	[390] [391,392]
PYD-106		EC_{50}	NE	NE	16	NE	[160]

NE denotes no effect at the highest concentrations evaluated, and ND indicates that the compound displayed some activity, but the affinity or potency could not be determined. Unless otherwise stated (also denoted by †), the values were determined using two-electrode voltage-clamp experiments with *Xenopus* oocytes.

[e] K_i values were estimated using Cheng-Prusoff correction of the measured IC_{50} values.

[*] The chiral carbon of (+)-CIQ, the active enantiomer, is denoted by the asterisk in the chemical structure.

[≠] The apparent lower potency for (+)-CIQ compared to the racemic mixture is likely due to better estimation of maximum potentiation, since the active enantiomer has increased abundance in solution at concentrations close to the solubility limit (i.e the pure enantiomers can be evaluated at higher concentrations compared racemic CIQ).