Structural Properties of the NMDA Receptor and the Design of Neuroprotective Therapies

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Abstract: NMDA receptors are linked to neuronal loss in stroke and neurodegeneration because their activation can trigger excitotoxic Ca^{2+} dysregulation. Accordingly, NMDA receptor antagonists are neuroprotective, providing a rationale for their clinical application. However, side effects often outweigh benefits. Herein we highlight structural properties in receptors that are used in drug development.

Key Words: Glutamate, excitotoxicity, NMDA receptor, therapeutics, neurodegeneration, drug design.

INTRODUCTION

The treatment of neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases, age-related dementias, and neuronal loss following stroke and trauma remain formidable but crucial challenges. In the United States of America alone, millions of individuals are severely afflicted by these pathologies yet we lack effective therapies. The *N*-methyl-D-aspartate (NMDA) receptor-channel continues to be a common target in on-going efforts to develop neuroprotective agents for use in humans. Widely distributed throughout the brain and spinal cord, NMDA receptors (NRs) are essential in a myriad of processes including neuronal survival, differentiation, adaptation and normal functional responsiveness [1, 2]. For example, mice rendered null for expression of the NMDA receptor subunit 1 (NR1) die within hours of birth due to respiratory failure [3]; those with a targeted NR1 deletion in hippocampus survive but perform poorly in nonspatial memory tasks [4]. Further, pharmacological blockade of NRs for only a few hours during neonatal development triggers widespread neuronal death [5] resulting in long-term neurofunctional deficits [6, 7]. In certain contexts however, activation of NRs initiates intracellular events that lead to excitotoxic cell death. Excitotoxicity can result from conditions causing sustained or excessive activation of receptors, or from normal activation of receptors in otherwise compromised neurons [8]. Based on extensive evidence in animal and cell culture models in which NR antagonists prevent or attenuate neuronal loss after exposure to toxic insults, practitioners anticipated that such treatments would likewise be protective in patients. However, these efforts failed placing patients in comas or eliciting intolerable side effects; the antagonists were too effective, interfering with essential receptor functions [9, 10]. Despite these setbacks, investigators continue to believe that differences in NR subunit composition and expression patterns can be exploited to design improved agents that interfere with pathology while preserving physiological functions. Current drug discovery initiatives are based on expanding our knowledge of drug interactions within discrete structural domains in NMDA receptor-channels, the focus of this review.

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NMDA RECEPTORS: STRUCTURE AND EXCITOTOXICITY

Glutamate is the principal excitatory neurotransmitter in the mammalian CNS. It transduces its effects by activating distinct classes of receptors that can be distinguished pharmacologically. Ionotropic receptor-channels are rapidly activated and consist of α-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA containing GluR1-4 subunits), kainate (KA) and NMDA types, composed of different subunit combinations with different pharmacologic specificities. Most subtypes of AMPA and KA receptors are permeable to Na^+ and K^+ and gate a rapidly-inactivating, depolarizing current. In contrast, NRs are permeable to the ubiquitous second messenger Ca^{2+}, and are both ligand and voltage-gated. Accordingly, extracellular Mg^{2+} binds to the channel pore at or near resting membrane potentials, preventing ion permeation even when glutamate is bound. If the postsynaptic membrane is sufficiently depolarized, typically a function of AMPA/KA receptor activation in mature synapses, the voltage-dependent 'Mg^{2+}-block' is relieved and Ca^{2+} enters the cell. The majority of NRs are concentrated postsynaptically where their regulation is more dynamic than previously believed; some are also presynaptic and extrasynaptic [11-13]. The metabotropic class of glutamate receptors (mGluR 1-8) are slower than ionotropic receptors as they do not contain an integral channel; rather they are coupled to G-proteins and transduce their signals through adenylyl cyclases and phospholipases [14, 15]. For example, activation of mGluR1 and mGluR5 stimulates phospholipase C-mediated hydrolysis of phosphoinositol bisphosphate, generating diacylglycerol and inositol trisphosphate (IP_3) which activate protein kinase C and IP_3 receptors, respectively. Thus, in neurons expressing NRs as well as mGluRs linked to IP_3 generation, Ca^{2+} signaling is influenced by both receptor classes. Experimental data indicate that the combinations of

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receptor classes, subunit composition, locations and dynamic regulation ultimately determine the responses to glutamate, and also to therapeutic agents that act on glutamate receptors.

NR1 (designated ζ in mouse) contains the binding site for glutamate's coagonist glycine and is necessary to form a functional receptor [16, 17]. As depicted in Fig. (**1**), eight possible splice variants can be created through rearrangement of 3 exons (5, 21, 22) encoded on a single NR1 gene. Notably, some of these splice variants show differential inhibition by protons in the physiological pH range [18], exist in discrete synaptic locations, and show activity-dependent synaptic targeting [19, 20]. However, we know little about the selectivity of pharmacological agents for NRs containing different alternatively-spliced variants. There are also NR3 subunits whose physiological significance and pharmacological utility remain to be determined [21]. NR2 subunits include four types (designated as NR2A-D in rat and ε1-4 in mouse) that share ~50% homology with one another [19, 22, 23]. Each contains a glutamate-binding site and has a role in modulating receptor activity [24, 25]. Though the obligatory NR1 subunit is found throughout the brain, expression of NR2 subunits shows regional and temporal specificity. For example, receptors containing NR2B and NR2D are widely expressed in embryonic brain, but NR2A and NR2C emerge as prominent receptors in adult forebrain and cerebellum, respectively [26]. Importantly, the incorporation of NR2 in the receptor complex determines channel conductance, kinetics and pharmacological sensitivities [19, 27]. For example, recombinant receptors containing NR1 and NR2B display higher affinities for glutamate and glycine, higher peak currents and longer offset decay time constants compared to those containing NR2A; those containing NR2D display a reduced sensitivity to Mg^{2+} block, lower elementary conductance, and substantially longer offset decay time constants than those containing NR2A or NR2B. Each NR subunit contains three transmembrane helices plus a re-entrant helical loop, a bi-lobed glycine or glutamate-binding domain and a leucine/isoleucine/valine-binding protein (LIVBP) bi-lobed amino-terminal domain, schematized in Fig. (**2**). In the multimeric complex NR1 subunits appear to associate freely with one another, whereas the LIVBP-like domain is needed for NR2 dimerization and association with NR1 [28]. Alterations in the conformation of NR1 upon binding of NR2 probably account for the observed changes in channel properties in the heteromeric receptor complex [29]. The notion that the three regions depicted in Fig 2 are functional modules is supported by domain-swapping experiments and studies expressing isolated modular constructs [30].

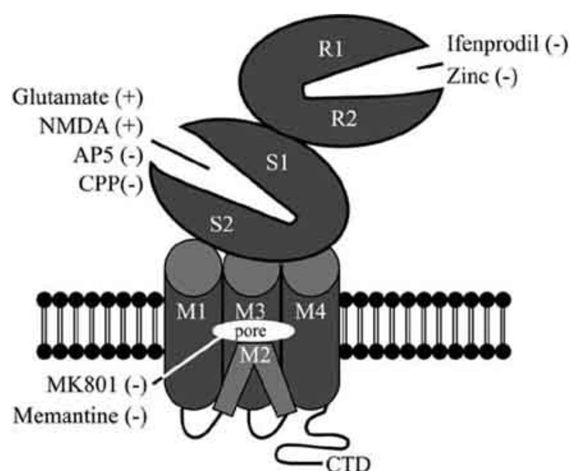

Fig. (2). The domain structure of the NMDA receptor-channel. Each subunit contains a channel pore region composed of 3 helices that span the plasma membrane (M1, M3 and M4) and a re-entrant helical M2 loop. A variety of ligands bind in a noncompetitive or uncompetitive manner to the channel pore including MK801 and memantine, respectively. In NR2 subunits, glutamate, NMDA and the antagonists AP5 and CPP bind competitively in the cleft of a bi-lobed globular module containing S1 and S2 segments. Agonist-induced closure of the cleft is transduced into opening of the channel pore. Antagonists stabilize the open state of the cleft, thus preventing opening of the channel pore. In NR1 and NR3 subunits, glycine binds to this domain. A structurally similar bi-lobed module comprises the amino terminal domain, which contains R1 and R2 segments separated by a hinge. In this module, allosteric modulators such as Zn^{2+} and ifenprodil bind in a noncompetitive manner to influence NR function.

Under physiological conditions, NR stimulation by glutamate leads to an influx of Ca^{2+} and activation of downstream messengers important in survival, maturation and plasticity. However, excessive or sustained glutamate release can increase the intracellular Ca^{2+} concentration to excitotoxic levels that trigger death by necrosis and/or apoptosis [22, 31, 32]. Though all classes of glutamate receptors can contribute to excitotoxic neuronal death, NRs are believed to have a central role. Accordingly, NR antagonists are neuroprotective in a host of excitotoxicity paradigms [33-37]. Although NRs are permeable to both Ca^{2+} and Na^{+}, the influx of Ca^{2+} triggers excitotoxicity [38, 39]. Further, excitotoxic glutamate exposure is known to induce both rapid and delayed accumulation of Ca^{2+} in cultured neurons [35, 40, 41], and the degree of neuronal death is proportional

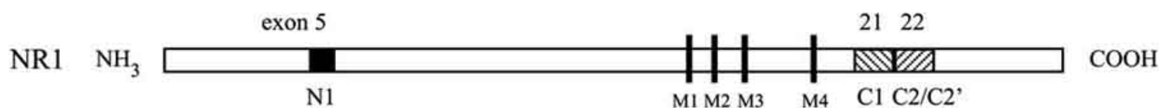

Fig. (1). Schematic diagram of the NR1 subunit showing the locations of alternatively-spliced exon 5 in the amino-terminal and exons 21 and 22 in the carboxy-terminal. Shown also are the relative positions of transmembrane segments M1, M3, and M4, and M2 which forms a re-entrant loop in all NR subunits. Consequently, the carboxy-terminal is located intracellularly and the amino-terminal is located extracellularly.

to intracellular Ca^{2+} activity [42]. Several downstream factors can transduce the death signal and, like NRs, are putative targets for the development of therapeutic drugs. The effects of these factors include free radical formation, increased protease activities, membrane failure, mitochondrial dysfunction, and energy depletion [43-47]. How these events are linked causally and temporally are inherently complex and critically dependent on context. Similarly, successful treatment with NR antagonists is complex and dependent on context. For example, ischemia is an acute excitotoxic insult necessitating immediate intervention. Although treatment with NR antagonists will elicit undesirable side effects, their benefit may supercede these side effects in an acute administration paradigm. In contrast, neurodegeneration progresses over the course of several years necessitating long-term treatment. In these cases, a goal is to develop improved drugs that are well tolerated when used chronically.

Clearly, the ability to selectively inhibit NRs mediating pathology while preserving their physiological actions is of great clinical importance. The remainder of this review will discuss the structure and pharmacological properties of the NMDA receptor-channel with a focus on three regions that show promise in the search to develop clinically effective antagonists: the binding pocket, the channel pore, and the amino terminal domain.

THE GLUTAMATE BINDING POCKET

The glutamate-binding site offers potential for improved drug development, but general antagonists used in clinical trials such as midafotel and selfotel have been ineffective due to intolerable psychotomimetic effects [48]. In searching for selective antagonists, NR2 subunits are a natural target as they contain the glutamate-binding site and confer distinct functional responses by virtue of regional and temporal differences in expression. General antagonists typically show a rank affinity preference for NR2A>NR2B>NR2C>NR2D, with only slight differences in affinities between subtypes [49, 50]. Substituting a squaric acid moiety in place of a polar amino acid group to produce perzinfotel (EAA-090) marginally improves selectivity for NRs containing NR2A over NR2B, to ~10-fold [51, 52]. Investigators continue to make progress towards understanding the chemical determinants of ligand affinity and efficacy at the glutamate-binding site, and possible pharmacological differences between NR2 subunits.

Structural studies were originally based on homologies between glutamate-binding sites in the bi-lobed bacterial leucine/arginine/ornithine-binding protein (LAOBP), the bacterial periplasmic glutamine-binding protein (QBP) and the GluR2 subunit [53, 54]. Homology models of NR2B were derived from these studies and used to identify critical amino acid residues for glutamate binding in NRs. This evolutionary relationship predicted that the glutamate binding pocket lies within the S1 and S2 domains. In NR2 subunits S1 is situated between the amino terminal and first transmembrane spanning region (M1), and S2 is between the M3 and M4 transmembrane domains [54, 55]. In NR1 subunits, the S1-S2 complex forms the modulatory glycine-binding pocket, another target for therapeutic drug design that has been reviewed elsewhere [56]. Recent resolution of the crystal structure of GluR2 in AMPA receptors and NR1 in NRs confirms the central role of S1 and S2 in glutamate binding. These lobes close like a clamshell upon glutamate binding. This conformational change is transduced to the channel pore domain causing it to open; H-bonding and ionic interactions are proposed to be critical to this process [57, 58]. Competitive antagonists stabilize the clamshell structure, preventing both closure of the binding pocket and opening of the ion channel [59, 60]. Although competitive antagonists are not generally considered subtype selective, emerging structural information suggests that design of such agents is possible.

Using a GluR2 based model of NR2B containing all the essential elements for ligand binding (S1 & S2), Laube *et al*., [61] performed a detailed mutational analysis of the glutamate-binding site. They identified several residues that significantly reduce receptor affinity for glutamate and the antagonist 2-amino-5-phosphonopentanoic acid (AP5), indicating that the antagonist is indeed competitive with glutamate. Many mutations significantly increase the EC_{50} value of the ligand being tested, indicating that binding itself is affected, and not the gating properties of the channel. In S1, residues K459 and K462 create an area of positive charge that appears to be involved in AP5 binding by favoring the interaction of a phosphonium group with the putative pocket. In a model proposed by this study, the interaction of K459 and K462 with oppositely charged residues in S2 (N662, E666) has a role in inducing cleft closure upon agonist binding. However, in the presence of antagonists this charge appears to be neutralized thereby interfering with closure of the pocket and precluding channel opening. Although most mutations affect the EC_{50} for glutamate and NMDA alike, a few substitutions discriminated between these, and suggest that hydrophobic interactions with V709 and aromatic residues Y705 and Y736 are important in this regard. Amino acid substitutions located at the interface between S1 and S2 alter agonist efficacy rather than affinity, indicating that this region is important for transducing the ligand-binding signal to the channel pore. Using a similar approach in modeling NR2A, the effect of selected mutations on the potencies of four agonists was examined [62]. Specific mutations in S1 (H466, S492, T494) and S2 (S670, T671) decrease agonist affinity for the site. Of interest, affinities for particular agonists are dependent on the amino acid replacing the wild type-residue. For instance, a S670G mutation in S2 results in a large increase in glutamate potency while a change to alanine in this position does not cause major changes. Mutations in this region also appear to affect the affinity for agonists differently, indicating that binding is not a uniform process for all ligands [62]. Fig. (**3**) summarizes the mutational work of several laboratories, highlighting common regions of interest in NR subunits.

The elucidation of critical residues for ligand binding show they are widely conserved between NR2 subunits, and this adds to the challenge of designing highly selective antagonists [24]. For example, among the residues in S1 and

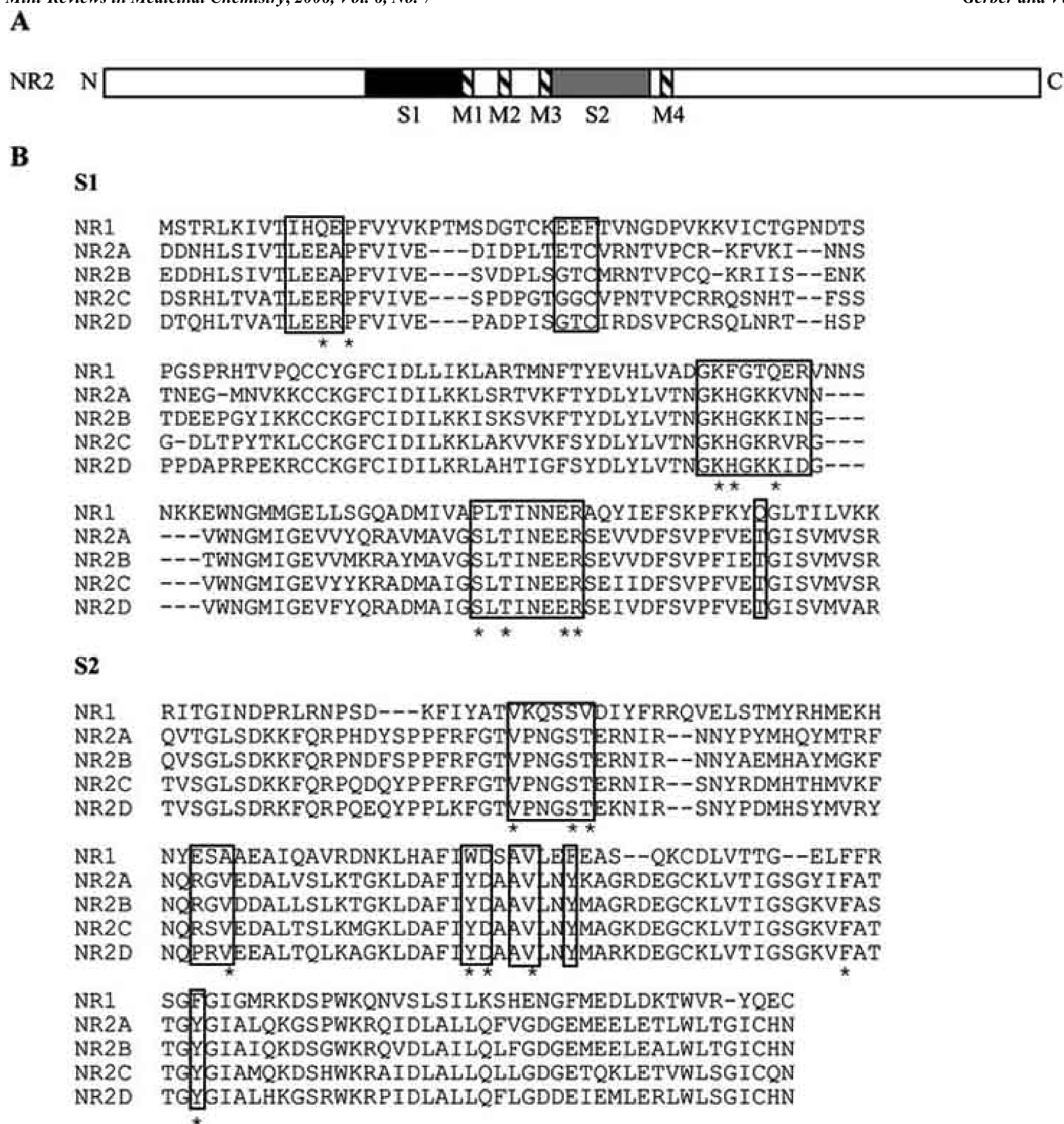

Fig. (3). A. Schematic diagram of a NR2 subunit depicting the relative positions of S1, S2 and M1-4. B. Sequence alignment of the S1 and S2 domains in NR1 and NR2A-D. Boxes depict amino acids lining the ligand-binding cleft based on homology models with GluR2 [63]. Asterisks depict residues implicated in ligand recognition based on mutational analysis [24, 59, 61, 62].

S2, there is only a 36% difference in amino acid sequences, and models of NR2 subunits based on NR1 or GluR2 place only 6-8 of the differing residues within the putative binding cleft [63]. Of these, A414, R712 and G713 in NR2B and their analogues in NR2C and NR2D appear distal to bound glutamate but near enough to the S1/S2 cleft to modify antagonist selectivity when mutated. In this model, antagonists with large side groups extending beyond the cleft towards the protein surface are predicted to interact with one of the variant residues and therefore demonstrate subtype selectivity. In contrast, antagonists that are small, interacting only within the glutamate-binding region, are unlikely to exhibit selectivity [63].

Studies in animals also indicate that certain agents, though generally classified as competitive antagonists, have different physiological profiles in particular brain areas, hinting at different binding properties. Specifically, antagonists with seven versus five bond lengths between their acidic groups are ineffective as inhibitors in certain subpopulations of neurons [64, 65]. These observations led to the idea that structures may influence binding affinities over a wide range (Fig. **4**). For instance, large hydrophobic antagonists such as 1-(phenanthren-2-carbonyl) piperazine-2,3-dicarboxylic acid (PPDA) display affinity for NR2C/D over NR2A/B [66]. The level of selectivity appears to be dependent on the number and linearity of the compound's

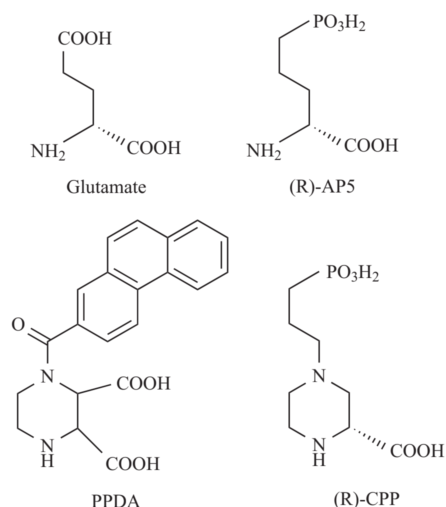

Fig. (4). Chemical structures of agents that bind to the glutamate-binding pocket in NR2 subunits. Shown are glutamate, and the competitive antagonists (R)-AP5, PPDA and (R)-CPP.

benzene rings; each ring may allow more sites of interaction with the receptor. Possibly, the increase in rigidity of the molecule limits conformational states and confers subtype selectivity by reducing the number of receptor-ligand interactions. Altering chain length between acidic groups appears to affect NR2 subunit selectivity as well. Piperazine compounds such as (6)-3-(2-carboxypiperazin-4-y) propyl-1-phosphonic acid (CPP) show an increase in affinity for all NR2 subunits however; the increase is greatest in NR2A/B [67]. Performing these same alterations on straight chain and piperidine-based compounds shows no effect on NR2A/B affinities but greatly decreases affinity for NR2C/D. It has been suggested that the added nitrogen in piperazine and increased chain length alter binding interactions with the phosphonate group, favoring NR2A/B [67]. In 5-phosphonomethyl-1,4-dihydroquinoxaline-2,3-dione (PEAQX or NVP-AAM07), a bromo substitution at the 4 position on the distal benzene ring greatly increases the affinity for NR2A over NR2B, to ~100-fold in humans [66, 68]. Perhaps the alterations in the compounds discussed allows access to the heterogeneous regions in the distant reaches of the binding cleft, a theory proposed in each of the studies. Altogether, these data support the proposition that NR2 subtype selectivity may someday be achieved through a detailed knowledge of ligand binding and careful design of antagonists. Their effective use in patients, however, will also depend upon a greater knowledge of the physiological and pathological roles of receptor subtypes in distinct neuronal types and brain regions.

THE CHANNEL PORE

The channel pore is arguably the domain with the greatest potential for discovery of neuroprotective drugs. Several structurally distinct compounds act as antagonists by binding NMDA receptor-channels that are in the open state; the same sites that bind Mg^{2+} in a voltage-sensitive manner. Thus block by these organic agents is referred to as being both use-dependent and voltage-dependent. Associated with a large number of side effects and low therapeutic indices, most agents are clinically intolerable. However, the low-moderate affinity blockers, amantadine and memantine, are clinically approved for the treatment of Parkinson's and Alzheimer's diseases, respectively. By studying the fundamental principles that govern NR gating and sites of drug interaction within the channel pore, investigators are beginning to provide testable models and theories for the design of improved agents.

Since the crystal structure of the pore region is not available, amino acid substitution and homology modeling experiments have provided the bulk of evidence upon which structure-function models are derived [69, 70]. Glutamate receptors were initially grouped with classical ligand-gated channels such as the nicotinic ACh and $GABA_A$ receptors. All appeared to be pentamers consisting of four membrane spanning hydrophobic segments (M1, M2, M3, M4) that place the amino and carboxyl termini extracellularly, and a Q/R/N site in M2 that is a critical determinant for ion permeation. However, it has since been recognized that ionotropic glutamate receptors share greater structural similarities with potassium (K^+) channels [22, 69]. Notably, NRs are thought to be tetramers, and M2 does not span the membrane but rather forms a re-entrant pore loop such that the ligand-binding amino-terminal is located extracellularly and the carboxyl-terminal intracellularly. The selectivity filter containing critical asparagine residues is positioned at the tip of the M2 loop, and M3 is a major pore-lining domain that is critical for channel gating after glutamate binds. Though not well characterized, M1 and M4 are also directly coupled to the ligand-binding domain and influence channel gating and drug interactions. Using K^+ channels as a model, investigators have revealed important structural differences that account for the unique properties of NRs. For example, unlike K^+ channels, functional activity in NRs appears to require the M4 segment, located at the carboxyl-terminal end of the channel pore domain [71]. Importantly, the unequal contribution of asparagine residues in M2 comprising the pore selectivity filter in NRs appears to be essential in maintaining impermeability to Mg^{2+} ions while gating Ca^{2+} ions. There is also an asymmetry between subunits comprising the pore-lining regions, not observed in other channels. Current evidence suggests that Mg^{2+} binding in this region physically blocks channel conductance and pro-motes channel closure [72]. Two elegant reports by Sakmann and colleagues identified the structural determinants of intracellular as well as extracellular Mg^{2+} binding [73, 74]; both are important for understanding the properties of memantine binding. Using receptors composed of NR1 and NR2A subunits in an oocyte expression system, these studies showed that the binding sites for blockade by intracellular and extracellular Mg^{2+} lie in close proximity in the narrow constriction, but are nonetheless distinct; the primary determinant of intracellular Mg^{2+} binding is an asparagine residue at position 616 (the N-site) in the M2 loop of NR1. Accordingly, when substituting amino acids such as glycine, glutamine or serine, block by intracellular Mg^{2+} is attenuated over a wide range of physiological membrane potentials, whereas similar substitutions in the NR2A subunit do not impact greatly on intracellular Mg^{2+} binding. Alternatively, substitutions in the negatively

charged asparagines at the N-site and especially the N+1 site in M2 in NR2A substantially block inhibition by extracellular Mg^{2+} but exert relatively minor effects on intracellular Mg^{2+} binding. In summary, the N-site asparagines in NR1 and the N+1 site asparagine in NR2 comprise the dominant Mg^{2+} binding sites located at the narrow constriction in the pore, shown schematically in Fig. (**5**). As will be discussed, many organic agents bind to this so-called deep site in a voltage-dependent manner; sometimes they also become trapped. In the tetramers that line the pore, there is evidence for staggering in the vertical axis of homologous regions in NR1 and NR2. This staggering is suggested to be a key structural feature underlying the distinct functional properties of NRs when compared to other glutamate receptors or K^+ channels [75]. The M3 segment forms the core of the extracellular vestibule where a highly conserved SYTANLAAF motif in all NR subunits is a critical determinant of the linkage between agonist binding and channel gating [76, 77]. Experimentally, covalent modification of A652C in this motif in NR1 or analogous mutations in NR2 subunits (using methan-ethiosulfonate ethlyammonium) requires the presence of glutamate and glycine, and such mutations maintain the open state of the channel even after removal from the external solution of glutamate and glycine. NRs also contain an unique DRPEER motif, positioned carboxy-terminal to M3 in NR1, and mutations in this sequence influence channel closing and trapping of organic agents.

Similar to Mg^{2+}, modification of asparagine residues in the selectivity pore influences block by organic channel antagonists. For example, using NR1 and NR2B subunits expressed in oocytes, binding of the high affinity antagonists, dizocilpine (MK801), *N[1]*,*N[4]*,*N[8]*-tribenzyl-spermindine (TB34) and the low-moderate affinity antagonist memantine are all disrupted by site-directed mutagenesis in M2 of N616 in NR1, and N615, N616 and W607 in NR2B. Additionally, mutation of sites in pre-M1, M1, M3, post-M3 and post-M4 reveal differences between memantine and the others, reducing block by MK801 and TB34 but not memantine [78]. These studies and others indicate that there are overlapping as well as discrete binding sites for organic agents, accounting for distinct neurofunctional effects. Consistent with this, a recent comparison of an agent that is poorly tolerated, MK801, versus amantadine in a rodent model of Parkinson's disease demonstrated distinct effects on subthalamic neuronal activity, suggesting differences in mechanisms of blockade [79]. Indeed, the action of amantadine was recently shown to be distinct from other channel blocking agents by causing the channel gate to close more quickly. Its main inhibitory action results from stabilization of the closed state, not inhibition of current *per se* [80]. A comparison of binding affinities and inhibitory effects on learning and memory tasks of MK801 and memantine, used to improve memory in Alzheimer's patients, provides additional perspective on differences between agents. Comparing inhibitory constants (IC_{50}) for inhibition of NMDA-induced currents and NMDA-mediated long-term potentiation (LTP), a model for learning and memory, MK801 was shown to inhibit both with an IC_{50} of 0.13μM. In contrast, memantine inhibits NMDA-induced currents with an IC_{50} of 3μM, whereas a higher concentration is needed to inhibit LTP (IC_{50} of 11.6μM) [81]. Notably, memantine exhibits fast blocking and unblocking kinetics and has a stronger voltage-dependence, compared to MK801 [82]. Thus, the rapid relief of blockade by memantine, but

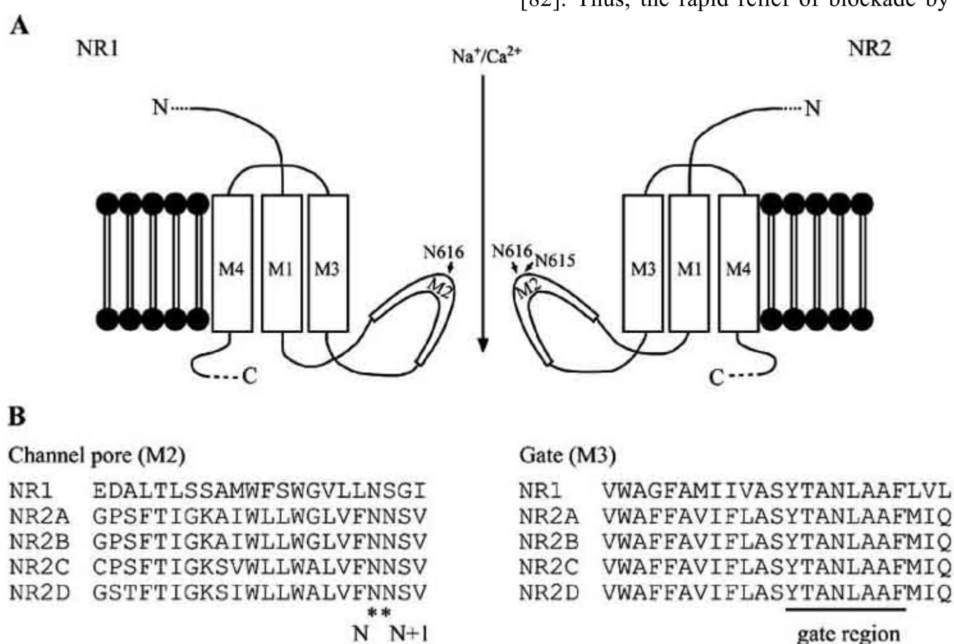

Fig. (5). A. Schematic diagram of the channel pore formed by NR1 and NR2 subunits. Only two of the four NR subunits are shown. The M2 pore region, formed by a re-entrant loop is shown. B. Sequence alignment of the channel pore (M2) and the channel gate (M3) in NR1 and NR2A-D. Asterisks in M2 depict residues implicated in Mg^{2+} binding based on mutational analysis [73, 74]. The bar in M3 depicts motif implicated in linkage between agonist binding and channel gating [76, 77].

not MK801, upon depolarization may contribute to its ability to antagonize chronic low level activation of NRs, thought to be characteristic of certain pathologies, while sparing synaptic activation associated with learning and memory. This property of 'uncompetitive antagonism' may also explain why memantine is not therapeutically effective in models of epilepsy at doses that preserve synaptic NR function [82]. But how can any blocker of NRs, known to be important in models of learning and memory, actually enhance memory in Alzheimer's patients? Animal studies support the notion that NR antagonists can enhance learning performance in certain contexts [83, 84]. Consistent with this, in a study modeling tonic NMDA receptor activation, which interferes with induction of LTP in hippocampal slices and is proposed to occur in chronic neurodegenerative diseases, pre-incubation with memantine, but not MK801, restored LTP [85]. In summary, several studies support the notion that memantine can block moderate pathological activation of NRs at doses that preserve synaptic plasticity whereas MK801 poorly differentiates between physiological and pathological processes [9, 86].

Structural data also point to differences between clinically intolerable agents like MK801 when compared to amantadine and memantine. In general, pore blocking agents interact with the channel in two distinct ways; "trapping blockers" like MK801 and ketamine enter the open channel and become trapped within the pore upon closure, while "foot-in-the-door" blockers such as tetrapentylammonium bind in the open state but do not become entrapped [87, 88]. Antagonists such as memantine or amantadine, shown in Fig. (**6**), are "partial trappers", with intermediate properties. Most agents in this class are V-shaped, composed of two hydrophobic "wing" regions that interact with hydrophobic

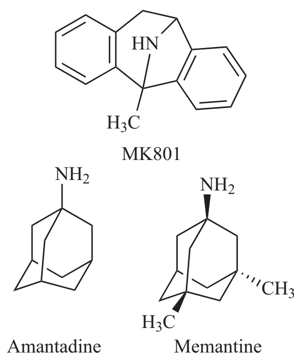

Fig. (6). Chemical structures of antagonists that bind to the channel pore in NR2 subunits. Shown are the structures of the high-affinity antagonist MK801, and the low-moderate-affinity antagonists amantadine and memantine.

binding sites in the channel and a protonated nitrogen at the vertex that is involved in H-bonding [89]. The physical size of the pore blocker appears to have little or no effect on trapping. Two key properties determined by modeling studies are the size of the terminal nitrogen group, as larger groups cannot penetrate the narrow selectivity filter, and its location relative to the most distant atom on the pore axis (chain length) [89]. Experimentally, a small monocationic terminal amine group appears optimal, allowing for channel penetration while alterations in the effective chain length produced less predictable results [89, 90]. These dimensions permit interaction of antagonists with the deep hydrophobic site and intrapore nucleophilic site, believed to contain the N site for H-bonding in M2 [90]. There is also support for the presence of a shallow binding site, the proposed location for interaction of foot-in-the door blockers with the channel [90].

Current electrophysiological data using recombinant receptor subunits supports the theory that the shallow binding site may be utilized by foot-in-the door blockers and, the deep, by trapping blockers [89-91]. Binding to the deep site shows greater voltage-dependence with greater binding/trapping at hyperpolarized potentials. For example, comparison of the binding of dextrorphan with that of MK801 determined that it binds at a substantially faster rate and can bind a closed channel, possibly interacting with a shallower site. Site-directed mutagenesis confirmed that the binding sites are indeed different. This study is consistent with data supporting a deep and a shallower binding site in the channel, accounting for the two different classes of pore blockers [89, 90]. Similar approaches using memantine suggest that it binds to a deeper site than previously believed, but unbinds more easily than agents such as MK801. Like intracellular Mg^{2+}, the NR1 N-site appears to be the most important residue for binding while the NR2 asparagines support less critical electrostatic interactions that influence voltage-dependence but not affinity. Through a protection study using memantine, cysteine mutations in this region are protected from reactions with cysteine-modifying agents, confirming its role as a binding site. The alignment of the carbonyl oxygen in these residues is proposed to stabilize the binding of memantine to the N-site. No differences are observed in memantine binding in receptors composed of different NR2 subunits or alternatively spliced variants of NR1 [91]. In M3, segments of NR1 are also protected indicating a second binding site in which the affinity of memantine is ~300-fold lower. When compared with amantadine the difference in binding affinities between the two sites is much smaller, providing a possible explanation for why memantine is clinically tolerated; it interacts with the receptor at low micromolar concentrations in a highly specific manner. This offers significant advantage when compared to other agents such as amantadine where the difference in affinities between the two sites is smaller, thus requiring a larger concentration of drug and associated non-specific side effects ([91] also see [92]). As such, investigators are actively synthesizing compounds that resemble memantine to further improve clinical efficacy and tolerability in patients. For example, Lipton and colleagues are developing 2nd generation memantine derivatives, the NitroMemantines [9]. With an attached S-nitrosyl group, these unique compounds are thought to have a dual mechanism of action, channel block at the N-site in M2 and the ability to S-nitrosylate C399 greatly increasing their inhibitory potential. Accordingly, NitroMemantines have proven to be more potent than memantine *in vitro* and in animal neuroprotection studies.

THE AMINO-TERMINAL DOMAIN

Complex regulation of NRs occurs through a number of allosteric sites contained within the amino-terminal domain (ATD). There are several known modulators, including phenylethanolamines, zinc ion, and polyamines (Fig. **7**). The ATD has not been crystallized, but initial structural work based on mGluRs revealed homology with a bacterial LIVBP domain capable of binding a wide variety of ligands [93, 94]. A subsequent study by Paoletti and colleagues produced a homology model of the ATD in NR2A indicating the presence of a LIVBP-like domain ~380 amino acid residues from the glutamate-binding module. Similar to the glutamate-binding site, the ATD is a globular cleft-like structure presumably allowing for a venus-fly trap closure upon ligand binding [95], shown in Fig. (**2**). Notably, phenylethanolamines and Zn^{2+} bind with high affinity to the ATD in some NR subunits but not others, resulting in functional inhibition of NRs.

From a clinical perspective, the phenylethanolamines have generated keen interest because they have been useful in stroke patients [10, 96]. Moreover, animals studies indicate that ifenprodil, the best known member of this family, is antinociceptive at lower doses than those that elicit motor incoordination or hallucinations; it has also proven useful in animal models of stroke and edema due to head trauma [97, 98]. Ifenprodil greatly prefers NMDA receptors containing NR2B compared to those containing NR2A by ~400-fold [99]. This high-affinity, voltage-independent binding site is located in the ATD, and a low affinity, voltage-dependent site is likely to reside in the channel pore [99]. High-affinity block of NR conductance by ifenprodil is incomplete, non-competitive and influenced by pH. A key study by Perin-Dureau and associates [100] showed that chimeric NR2A subunits containing the LIVBP-like domain from NR2B demonstrate ifenprodil sensitivity. Also, binding of ifenprodil to LIVBP protects it against proteolysis by trypsin. Together, these results indicate that the binding site is indeed located in the ATD. Within this domain, residues D101, F176, F182 and I150 are crucial, while other mutations affect the degree of inhibition. It was proposed that these residues lie in three theoretical "pockets" optimally spaced to interact with the functional groups of ifenprodil [100-103]. Recent modeling of NR2B based on the homologous structure of mGluR places the critical binding residues in the R1 and R2 lobes and asserts that ifenprodil binds the open state, and stabilizes the desensitized state [104].

Further characterization of the ifenprodil binding site has led to the development of more effective derivatives. In particular, the congener R0 25-6981 demonstrates a >5000-fold affinity for NR2B over NR2A. It attenuates cell death in an *in vitro* seizure model and in animal models of Parkinson's disease [105]. Its interaction with LIVBP in NR2B has been characterized using site-directed mutagenesis, radioligand binding, and NMDA-mediated conductance assays [106]. All mutations are in the central cleft: D101 and D104 in lobe I; F176, F182, T233, K234 in lobe II. Similar to ifenprodil, mutations to alanines of D101 and F176 completely prevent both RO 25-6981 binding and inhibition of NMDA-evoked currents. These data led to the proposition that the two critical mutations interact with the molecule in a fashion similar to the proposed three-pocket model for ifenprodil binding; the COOH of D101 interacts with the basic nitrogen and benzyl group electrostatically and F176 is involved in an aromatic stacking interaction [106]. Mutagenesis to alanines of D104, K234 and F182 residues that are critical for ifenprodil interaction, reduce affinity for RO 25-6981 but do not abolish binding. T233 is also important and it is the only distinct residue when comparing this region in NR2B and NR2A subunits; NR2A has a serine residue at this position. Because of this similarity, the lack of high-affinity phenyethanolamine binding in NR2A has been attributed to an insertion containing two histidine residues that sterically hinder binding of the compound.

Zn^{2+} and ifenprodil are believed to share a common mechanism of modulation at the structural level: the central cleft of the LIVBP-like domain in NR2A and NR2B, respectively [95]. In the ATD, inhibition by Zn^{2+} is high-affinity, voltage-independent, and linked to fast desensitization [30]. There is also a low-affinity, voltage-dependent site in the channel pore region of all receptor subunits [107,

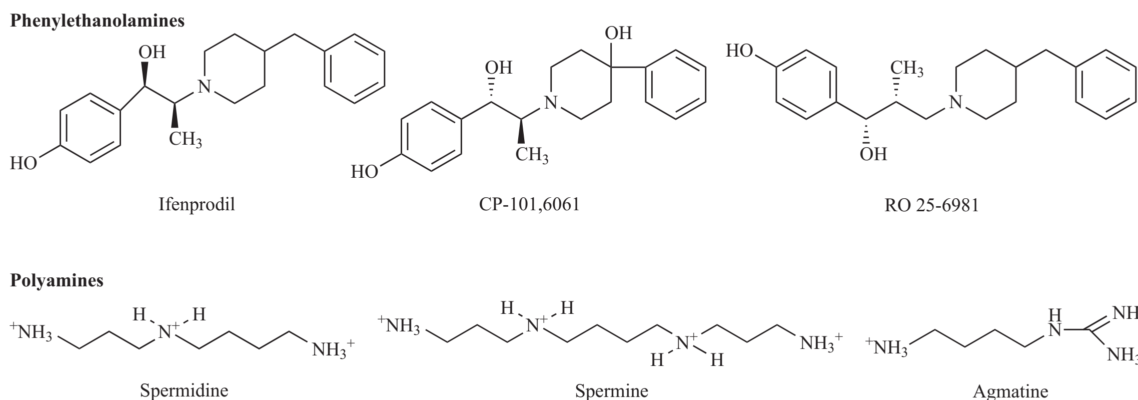

Fig. (7). Chemical structures of allosteric modulators that bind to the amino-terminal domain in NR subunits. Shown are the structures of phenolethanolamines: ifenprodil, CP-101,601, RO 25-6981, and polyamines: spermidine, spermine and agmatine.

108]. High-affinity block of NR conductance by Zn^{2+}, like ifenprodil in NR2B, is incomplete (~40-80% of NMDA-induced current) and influenced by pH. The electrostatic binding of Zn^{2+} in the ATD is subunit specific; NR2A binds with nanomolar affinity, NR2B binds with low micromolar affinity, and NR2C and D do not bind at all [109]. Recent evidence suggests that Zn^{2+} and ifenprodil share several key binding residues that lead to stabilization of the open state of their respective ATDs. Specifically, Paoletti and colleagues [95] showed that six residues control high-affinity inhibition of NR2A by Zn^{2+}, forming two clusters in the hinge/cleft region that face one another. R1 contains H44, D102, D105 and H128. R2 contains K233 and E266. When extracellular Zn^{2+} binds, they close tightly around it. Together, these lobes coordinate Zn^{2+}, and large shifts in Zn^{2+} sensitivity and the degree of fast desensitization are observed when they are mutated.

Polyamines such as spermine and spermidine are ubiquitous agents that modulate cell growth and division, as well as protein synthesis [110]. In part, these effects are due to modulation of NRs. Through complex effects in the ATD that likely involve more than one binding site, spermine and related compounds potentiate NR conductances, increase the probability of channel opening, and increase glycine affinity under specific conditions. They also exhibit a voltage-dependent inhibitory effect similar to that of Mg^{2+} by binding to a site in the channel pore [110-112]. Homology models suggest that the binding site of spermine that potentiates NRs in a glycine-independent manner lies somewhere between the R2 lobes at a series of acidic residues, not in the binding crevice; spermine stabilizes the negatively charged residues maintaining the receptor in either an open or closed state [104]. Although scant, previous work in site-directed mutagenesis of these residues seems to confirm this theory [113]. Notably, alternatively-spliced variants of NR1 that lack exon 5 encoding a 21-amino acid insert in the R2 lobe show reciprocal potentiation by spermine and inhibition by protons [18]. In this model, spermine is thought to 'relieve' proton inhibition which at physiological pH inhibits NR conductance by ~50%. The exon 5 insert is structurally similar to spermine in that it contains several positively charged residues. It is thought to mimic the effect of spermine in NR1 [18, 93]. Importantly, most neuronal types express NR1 lacking exon 5 [50]. Thus, most NRs are sensitive to potentiation by polyamines and inhibition by protons. Binding of polyamines to sites in the ATD is variable, and members of the spermine family display a degree of subunit selectively, preferring NR2B, thus indicating that NR1 alone does not determine binding. In contrast, agmatine, a polyamine containing a guanidine moiety, shows no subunit preference and exhibits behavior more akin to a channel pore blocker but without the property of use-dependence [114]. Huggins and Grant [104] recently developed a new model using dimers suggesting that polyamines bind at inter-subunit boundaries between NR1 (lacking exon 5) and NR2.

Although continued study of the ATD and its interactions with polyamines and ifenprodil may lead to the development selective antagonists, it is a challenging task. As indicated, polyamines are found throughout the CNS and endogenous interactions with polyamine-derived drugs is an important consideration [115]. Moreover, spermine reduces by ~44-fold inhibition of NR1/NR2B-containing NRs by the ifenprodil analogue, CP101,606 [116]. Along these lines, because Zn^{2+} is accumulated and released at glutamatergic synapses [117], ifenprodil derivatives will be forced to compete for the site, possibly lowering their clinical effectiveness. Further complicating matters, there is compelling evidence for triheteromeric NRs with ligand sensitivities that are a blend of their diheteromeric cousins [118]. Thus, depending on the NR2 subunits present in these complexes, the clinical efficacy of ATD-directed drugs could be greatly decreased, or perhaps enhanced to dangerous levels. To date, one ifenprodil derivative, CP-101,606, has been proposed to be selective against dimeric over triheteromeric receptors and should be useful as a template to develop antagonists that discriminate between NRs comprised of NR1/NR2B versus those comprised of NR1/NR2A/NR2B [119]. In preliminary studies, CP-101,606 has shown some promise in animal models of Parkinson's disease by reducing leva-dopa induced dyskinesias [120, 121]. Recent success in reconstituting the ATD of NR2B [122] should aid in the effort to generate new agents by providing more precise structural data about ligand-receptor interactions that determine NR specificity and potency.

CLINICAL CONSIDERATIONS

Neuronal death as observed in stroke, trauma and chronic degenerative diseases has been directly linked to the activation of NRs and Ca^{2+}-mediated excitotoxicity. The prevalence of these pathologies is highest in elderly populations, and as our population continues to age the burden on the health care system and economy will increase. For example, it is predicted that by 2050, there will be 13.2 million cases of Alzheimer's related dementia in the United States alone [123]. Therefore, the development of new pharmacotherapies that markedly slow or halt disease progression is essential. NR antagonists have been studied and used extensively with some successes, notably memantine in Alzheimer's disease. Cocktail therapy using memantine and denozepil is safe and relatively free of major side effects and has shown great promise in the improvement of dementia symptoms [124, 125]. This has been the subject of recent reviews [123, 126-128]. Statistical analysis and compilation of several studies involving patients receiving denozepil therapy that was supplemented with memantine indicated significant improvement in symptoms across all the examined trials [129]. Currently, a cocktail therapy of memantine and aricept are in phase III clinical trials and data indicate that the combination of drugs appears to have a synergistic effect on the improvement of patients' cognitive abilities [126, 127].

Memantine use is not limited to Alzheimer's disease and dementia. Current data suggest it may prove useful in Parkinson's and Huntington's diseases, multiple sclerosis, amyotrophic lateral sclerosis, and as an anticonvulsant therapy [130-133]. It has proven less effective in the treatment of neuropathic pain [134-136], but this situation is complex. In the setting of pain modulation in which the NR is only mildly activated, the use-dependent action of

memantine would not be realized. Alternatively, more severe cases of pain may be amenable to low-dose treatment with memantine [9]. Along these lines, it is important to consider that the effectiveness of different NR antagonists may vary, depending upon the target neurons and brain regions. For example, under equivalent experimental conditions, MK-801, ketamine and memantine are less potent, whereas amantadine is more potent in striatal versus hippocampal neurons, likely accounting for amantadine's better clinical profile in Parkinson's disease [137]. Another important consideration is the diversity in patient populations. As our understanding of NR structure, functional interactions with ligands, and involvement in distinct pathologies continues to evolve, clinical successes will undoubtedly follow.

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