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Review

Mechanism-based targeting of NMDA receptor functions

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Abstract. NMDA receptors (NRs) are key signaling proteins in the central nervous system and represent important targets for drug development in several neurologic disorders. They are critically involved with fundamental brain processes, and thus indiscriminate pharmacological suppression of NR currents has seen only modest therapeutic success so far. Targeting harmful NR receptor activities while sparing the receptor's vital functions requires a better understanding of the complexity of NR activation reaction; of the range of mechanisms that modulate discrete receptor activities; and of the consequences of this modulation on specific receptor functions. A quantitative account of the NR activation pathway was recently proposed and validated. It describes the gating reaction as a sequential, multi-step process rather than a binary, on-off switch. Alongside isoform-specific modulators, state-specific modulators may represent sophisticated interventions with high potential for narrow, functional specificity. Here I review physiologic mechanisms that control NR responses; the salient features of the NR activation reaction; and discuss the model's validity and its implications for drug development and characterization.

Key words. N-methyl D-aspartate receptor; activation kinetics; multi-step gating; single-channel currents; kinetic modeling; synaptic transmission; drug effects; molecular pharmacology.

Introduction

NMDA receptors (NRs) are glutamate-activated ion channels with complex roles in the physiology of the central nervous system (CNS). Synaptic NRs mediate the slow component of excitatory post-synaptic currents (epsc) in the CNS and have critical roles in fast synaptic transmission and the development and remodeling of central excitatory pathways [1–3]. NRs are required throughout life for synapse formation, maintenance and plasticity, and are involved with higher brain functions such as learning, memory and certain behaviors.

Inappropriate NR activation contributes to severe neurologic disorders and pathologies. Insufficient NR-mediated transmission during development has been implicated in the etiology of schizophrenia, whereas increased NR activity promotes seizures and may contribute to epilepsy [4, 5]. Certain forms of chronic pain and addiction that rely on aberrant synaptic plasticity may be initiated by inappropriate NR stimulation [4, 6, 7]. Excessive activation of NRs contributes to the neuronal loss that accompanies hypoxia, brain and spinal cord injury, and chronic neurodegenerative diseases [8]. Strategies for both pharmacologic enhancement and reduction of NR currents hold great promise in the rational design of therapeutic agents [5, 9].

Nonselective NR antagonists have a number of adverse, unacceptable side effects, including hallucinations, increased blood pressure, catatonia and anesthesia. Given the delicate balance between a continuous requirement for physiologic NR activity and the severe consequences of these receptors' inappropriate activation, it is in hindsight understandable that attempting to reduce NR activity with strong, indiscriminate blockers has seen

only modest clinical success and that the therapeutic potential of NR targeted interventions remains largely untapped [9]. In order to control harmful NR activities while maintaining vital NR functions, it is necessary to develop a deeper understanding of the molecular events leading to NR activation, of the mechanisms responsible for drug-induced changes in receptor activities, and of the consequences of NR activity modulation on specific cellular functions.

NMDA receptors generate electrical and biochemical signals

The many important roles demonstrated for NRs in the CNS stem from two main signals generated simultaneously by these receptors: an electrical signal consisting of a depolarizing current and a biochemical signal consisting of calcium influx. Upon binding glutamate and in the presence of glycine, NRs open an intrinsic cation permeable pore, thus causing a transient depolarization of the membrane in which they reside. The kinetic characteristics of this excitatory signal are unique among other ligand-activated ion-channels [1]. First, the current is unusually slow to rise, reaching maximal levels ~10 ms after the synaptic glutamate has been cleared from the cleft [10–12]. Second, after binding glutamate the receptors can remain active for remarkably long times (50 ms–2 s) [13–15]. Last, only a fraction (3–30 %) of the active receptors contribute to the peak of the synaptic current [16, 17].

The time course with which NR-mediated currents decay is a critical feature of the electrical signals generated by NRs. At resting membrane potentials, NR currents are largely blocked by voltage-dependent binding of Mg^{2+} in the channel pore, which makes NRs into veritable coincidence detectors [18–20]. The time window within which pre-synaptic activity and post-synaptic depolarization are, in effect, coincidental is set by the time course for NR current decay. The NR current decay also determines the period within which contemporaneous NR activations will be integrated across multiple synapses along a dendritic tree. Electrical signals generated by NRs participate in fast synaptic transmission, synaptic integration and contribute to the computational power of the dendritic tree.

NRs have substantial calcium permeability, and thus their activation also generates powerful biochemical signals in the post-synaptic cell in the form of calcium influx [21–24]. Although calcium buffering systems and pumps also participate in shaping the intracellular calcium transient generated by NR activation, in the small volumes of diffusionally restricted spines, NR response amplitudes in the picoampere range translate directly into micromolar post-synaptic Ca^{2+} concentrations [25, 26].

At many synapses, NR-mediated Ca^{2+} influx initiates in a concentration-dependent manner the necessary physiological processes of long-term synaptic plasticity and is required for synapse formation, synaptic maintenance and physiological pruning during development. Unfortunately, this influx may also underlie pathological plasticity phenomena associated with some forms of addiction and chronic pain. In addition, excessive concentrations of intracellular Ca^{2+}, admitted specifically through NRs, trigger excitotoxic pathways that lead to pathologic neurodegeneration [8, 27]. Thus, the amplitude of the NR-mediated current may be the most relevant feature of the NR biochemical signal.

Although research on NR-mediated intercellular signaling has focused overwhelmingly on the ability of NRs to act as highly regulated ionic valves, recent reports suggest that they may also have a metabotropic function, transducing signals across the neuronal membrane in a flux-independent manner. In this scenario, agonist binding to NRs is relayed to intracellular receptor regions as changes in conformation which can couple the receptor to distinct intracellular pathways [28, 29]. The physiologic significance of NR flux-independent signaling is insufficiently understood.

NR responses are physiologically controlled by multiple mechanisms

Many endogenous and synthetic agents have been identified that modify NR responses by binding with characteristic affinity to NRs [1, 30, 31]. These ligands have specific effects on receptor function according to the location of their binding sites: i) blockers bind in the channel pore and impede ionic flux in a voltage-dependent manner; ii) orthosteric ligands bind at sites on the protein which overlap topologically with the agonist-binding sites and substitute for or compete with the physiologic agonists; and iii) allosteric modulators bind at locations that are separate from the agonist-binding sites and modify the kinetics of the receptor's response to physiologic or pathologic activation.

Ligands that modify receptor responses with an allosteric mechanism present several advantages over channel blockers and orthosteric modulators. First, they don't compete with the physiologic ligands whose concentration varies greatly during synaptic transmission; thus they are active at low, intrinsically defined concentrations and remain safe even at high doses. Second, allosteric modulators only affect receptors activated by the endogenous neurotransmitter, and thus this intervention preserves the physiologic, pulsatile pattern of synaptic transmission. Third, allosteric sites are often differentially represented on receptor isoforms, and thus target specifically distinct receptor populations [32].

Allosteric modulators may represent a particularly effective approach to control rationally NR activations. First, NRs are naturally rich with allosteric sites many of which are isoform-specific; second, allosteric modulators act by altering a critical feature of the NR function, its kinetics; and last, NRs activate with a multi-step reaction mechanism, and allosteric modulators may represent state-dependent ligands with high potential for differential regulation of NR signals.

NRs are rich with nonoverlapping regulatory sites

NR activity can be altered quickly, bi-directionally and reversibly by endogenous modulators acting at distinct allosteric locations. The binding of several small ions and molecules, interactions with specific synaptic proteins and covalent modification of NR residues result in NR responses with altered kinetics. NR residues required for these modulatory effects can be mapped on a putative receptor architecture at several non-overlapping locations, as illustrated in figure 1.

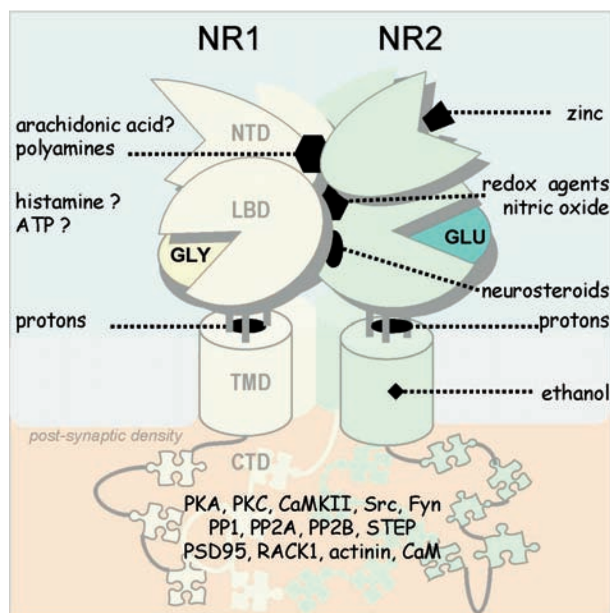


Figure 1. Allosteric sites mapped on the proposed NR architecture. Two NR1 (yellow) and two NR2 subunits (green) interact extensively to form a heterotetrameric receptor. Each subunit has modular structure with extracellular, globular N-terminal (NTD) and agonist-binding (LBD) domains, a pore-forming, transmembrane domain (TMD) and a large cytoplasmic C-terminal domain (CTD). The agonists glycine (GLY) and glutamate (GLU), as well as a number of endogenous allosteric ligands (black symbols) bind at the interface between mobile receptor units, promote changes in receptor conformation and modify the receptor's overall intramolecular dynamics. The CTD serves to anchor the receptor in the post-synaptic density, hosts residues which are target for covalent modification by a number of kinases and phosphatases, and interacts directly with signaling, adaptor and structural proteins.

Physiologic concentrations of endogenous ions, including Zn^{2+} and H^{+}, bind at extracellular sites, reduce receptor activity and may serve to keep NRs under tonic inhibition [33–36]. Polyamines, arachidonic acid, ATP, nitric oxide, sufhydryl redox agents, neurosteroids and ethanol also modulate NRs with an allosteric mechanism [37–41]. These extracellular regulatory pathways may be used in a combinatorial manner to adjust NR signals during particular physiological states or to locally coordinate synaptic transmission. Multiple intracellular regulatory pathways converge on NRs and modulate their activities. Several protein kinases (PKA, PKC, CaMKII, Src, Fyn), protein phosphatases (PP1, PP2A, PP2B, STEP) and signaling, adaptor or structural proteins (PSD95, actinin, calmodulin) directly modulate NR activity [42]. Many of these interactions and regulatory effects are isoform-specific and can be potentially exploited for rational design of pharmacologic agents with specific temporal, regional and functional efficacy profiles.

NRs are tetrameric proteins with modular architecture

NRs are expressed throughout the CNS in a multiplicity of isoforms which differ in molecular composition, temporal and spatial expression, intracellular location and trafficking, and have distinct kinetic, pharmacologic and functional properties [43, 44]. Although the exact subunit makeup of native NRs is not known, it is widely accepted that NRs assemble as hetero-tetramers of two obligatory NR1-type subunits and two regionally localized NR2-type subunits [45–47]. Receptor isoforms result from incorporating more than eight alternatively spliced variants (a–h) of the NR1 gene product and polypeptides encoded by four separate NR2 genes (A–D). In restricted brain regions and developmental periods, one or both of the NR2 subunits may be replaced by NR3-type subunits with an inhibitory effect on channel activity [48–50].

The structure of NRs at atomic resolution remains unknown, yet valuable information about subunit topology and the location of potential regulatory sites exists [31, 40]. Functional studies have demonstrated wide-ranging communication among discrete allosteric sites and with the agonist-binding sites [41, 51–55]. NR subunits have modular design, and structural modules also represent functional units [56, 57]. Each subunit has two extracellular, globular domains (NTD and LBD), a transmembrane domain (TMD) and a cytoplasmic, C-terminal domain (CTD). Most allosteric modulators bind at the interface between mobile receptor units, and perhaps promote or impede intramolecular motions associated with receptor activation (fig. 1) [58].

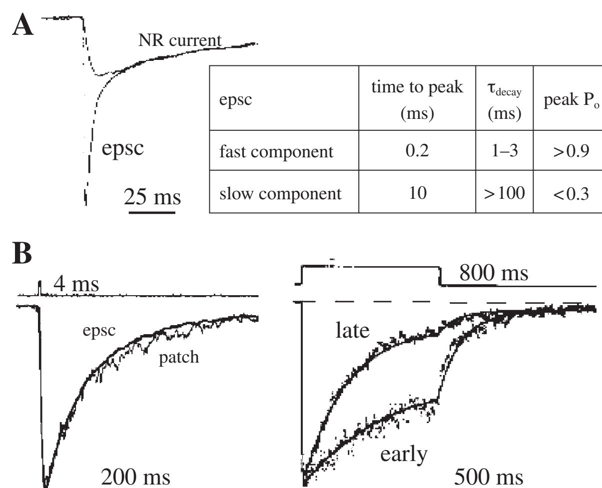
On each subunit, the N-terminal domain (NTD) and the ligand-binding domain (LBD) are homologous and share

an overall bilobate structure. Residues in the LBD form the physical binding sites for the physiological agonists. NR1 and NR3 subunits bind the required co-agonist glycine, and the NR2 subunits bind the neurotransmitter glutamate. The structure of the NR1 LBD expressed as a soluble protein has been solved at atomic resolution [59]. These studies have confirmed that glycine binds in the crevice between the two lobes and forces these to move closer to each other. It is believed that lobe movements induced by glycine and glutamate at their respective binding sites are requisite for receptor activation [60, 61]. Given the extensive homology between NTDs and LBDs, ligands that bind in the NTD crevice are also expected to induce rigid-body motions of the NTD lobes and thus influence agonist-induced motions in the LBD and/or the ability of agonist-induced motions to elicit gating. Additionally, allosteric sites have been identified at receptor interfaces between globular domains. NTDs and LBDs interact extensively with each other on the same subunit and with their respective counterparts across subunits. Perturbations at these sites induced by ionic or covalent interactions with ligands may alter NR responses by changing the dynamics of intramolecular motions that underlie receptor activation. It is not known how this large collection of extracellular and intracellular signals is integrated to change synaptic NR responses and how this change affects synaptic transmission and physiology. Individually, however, modulators that act through allosteric mechanisms alter the kinetics of NR activation.

The time course of NR response is critical for biological function and is physiologically modulated

NR-mediated responses at central glutamatergic synapses were initially identified as depolarizing currents with characteristically slow kinetics, high Ca^{2+} content and voltage-dependent block by Mg^{2+} (fig. 2A) [18, 19, 21, 62, 63]. It was demonstrated that currents with similar kinetics and pharmacology as the NR-mediated epsc can be elicited by brief (4 ms) applications of glutamate onto excised neuronal membrane patches (fig. 2B, left panel). Thus, the characteristic time course of the NR-mediated epsc reflects properties intrinsic to the channel protein rather than slow decline in the synaptic glutamate transient. The time course in the NR current varies with the developmental stage and brain region investigated and is altered in some neuropathologies [64–66]. In addition, time-dependent changes in NR response kinetics suggest that changes in environment and/or in the receptor's metabolic state also influence NR activation (fig. 2B, right panel).

The decay time of the NR response is developmentally and regionally controlled by regulated expression of distinct NR isoforms. The kinetic and pharmacologic



epsc	time to peak (ms)	τ_{decay} (ms)	peak P_o
fast component	0.2	1–3	>0.9
slow component	10	>100	<0.3

Figure 2. NRs generate currents with distinct kinetics. (**A**), Superimposed synaptic currents recorded in control medium (epsc) and in the presence of a selective inhibitor of the fast component reveal a slow, NR-mediated current (reproduced with permission from [15]). The inset shows kinetic parameters of the fast and slow components of the epsc [1]. (**B**), Superimposed NR currents elicited by synaptic stimulation (epsc) or by 200 μM glutamate (left, 4 ms, right 800 ms) onto NRs residing in excised membrane patches. NR kinetics varies with time since patch excision; the response from 'late' patches (right) resembles the synaptic currents (reproduced with permission from [81]).

properties of NRs with defined subunit composition were determined for recombinant proteins expressed in HEK cells and were found to mirror changes in isoform expression during synaptic development *in vivo* and *in vitro* [64, 67–71]. NRs differ markedly in their deactivation time course, with the NR1/2A isoform having the fastest kinetics and the NR1/2D isoform the slowest (fig. 3) [72].

It is abundantly clear that many endogenous and synthetic agents modify NR response kinetics, but the mechanisms by which this modulation occurs are poorly delineated. Understanding how NR responses are shaped by perturbations in channel structure at allosteric locations requires the ability to trace observed changes in macroscopic kinetic parameters to the underlying changes in receptor activation dynamics. To identify and measure modulator-induced changes in the rates with which the receptor switches between inactive and active conformations, it is necessary to understand in some detail the process by which NRs become active.

A two-state gating model accounts for the NR macroscopic waveform

Early kinetic studies have shown that two molecules of glycine and two molecules of the neurotransmitter glutamate must bind to NRs before the channel can open and that NR-mediated currents desensitize in the

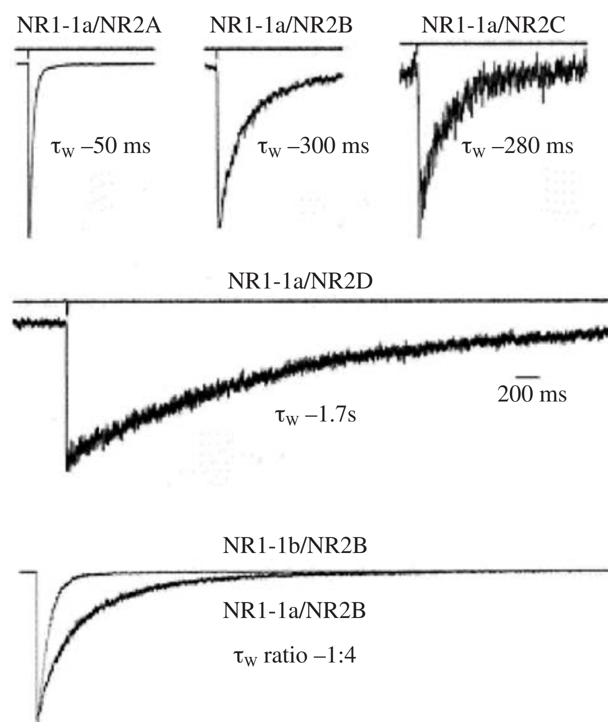


Figure 3. NR kinetics depend on subunit composition. Macroscopic currents elicited from recombinant NRs residing in excised membrane patches (HEK293 cells) by brief (1-ms) applications of glutamate (1 mM) decay with distinct time courses; t_w represents the weighted deactivation time constant calculated from a fit to a double exponential function (data from [72] and 72b, reproduced with permission from [43]).

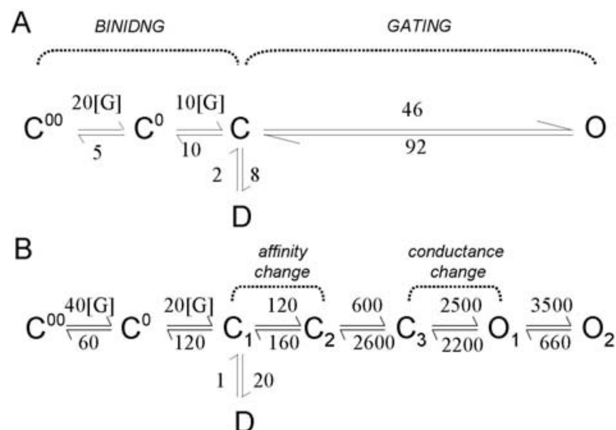


Figure 4. Kinetic models for NR activation. (**A**), Minimal functional model describes gating as a one-step, concerted transition (adapted from [81]). (**B**), Statistical multi-state model postulates that gating involves four kinetically distinct transitions. The model distinguishes between transitions that change the receptor's affinity for glutamate (C1→C2, agonist trapping) and those that result in a change in receptor conductance (C3→O1, pore opening). Rates are for L-mode NR1/2A receptors (from [98]). All rate constants are in s^{-1} except for the glutamate binding rate constants, which are in s^{-1} mM^{-1}; [G], glutamate concentration in mM.

continued presence of glutamate [73–80]. To account for the molecular events that take place during channel activation, these functional observations were incorporated into a scheme which postulates that after binding two molecules of glutamate ($C^{00}\rightarrow C^{0}$ and $C^{0}\rightarrow C$), fully liganded NRs can open (C→O) or desensitize (C→D) (Fig. 4A) [81]. In this model, both the gating and the desensitization reactions are represented as single-step, concerted conformational changes. Despite experimental evidence that NRs follow more complex activation pathways (see more below), this simplified two-state gating model describes remarkably well the time course of the NR-mediated epsc.

Fitting macroscopic NR responses by the two-state model allowed estimation of microscopic rate constants for the postulated microscopic transitions and led to specific predictions regarding the mechanisms responsible for the macroscopically observed receptor behaviors. The model accounts for the high affinity of NRs for glutamate by estimating a slow dissociation rate constant from closed receptors (5 s^{-1}); the slow rise in current mirrors slow channel opening (40 s^{-1}); and the long biphasic deactivation reflects the distinct kinetics of agonist dissociation and channel desensitization. Within the conceptual framework of the two-state gating model the mechanism by which an allosteric ligand modifies channel function can be accounted for by differential binding of the modulatory ligand to resting, open or desensitized receptor states [82]. Binding with preferential affinity to C, O and D states forces a redistribution of receptors among these states, is reflected in modified microscopic rate constants, and underlies the observed changes in macroscopic kinetic parameters.

However, examination of current patterns generated by *individual* receptors reveals that during activation any NR can occupy a much larger number of kinetically distinct states than those postulated in the two-state gating model. This observation suggests that the repertoire of distinct states to which allosteric ligands can bind with differential affinity may be far richer than that indicated by the two-state model. To learn how state-dependent modulators may affect receptor responses and to delineate the mechanism by which specific modulators act to modify receptor function requires that we understand how the multiple conformations adopted by NRs relate to one another.

Single-channels reveal complex gating mechanism

Individual NRs generate complex current patterns that reflect elaborate sequences of channel openings and closures. Individual opening and closing events and their associated durations can be visualized and measured directly from single-channel current records. Careful statistical analysis of large numbers of open and closed intervals originating from individual NR activations has shown

unequivocally that NRs can reside in multiple open and closed states each characterized by distinct mean durations and occupancies [83–85]. The presence of allosteric modulators changes the structure of single-channel currents by changing mean durations for the open and closed components or/and component occupancies [73, 86–88]. Neuronal NRs originating from distinct brain regions, normal vs. pathologic preparations and recombinant NR isoforms expressed in heterologous systems also differ in the structure of their single-channel current pattern [89–93]. In the absence of a reaction mechanism that accounts for the multiplicity of states that NRs can occupy it is not possible to determine how changes in the single-channel activity patterns relate to macroscopically observed changes in current waveform.

Observing currents generated by individual NRs for prolonged periods has revealed that NR1/NR2A receptors expressed in HEK cells can generate three distinct patterns of currents that can be identified and sorted by the mean time the channel spends in open conformations (fig. 5A, B). In each gating mode, channels cycle on a millisecond timescale between five fully liganded conformations: three that are nonconducting or closed (3C) and two that are active or open (2O). The switch in gating pattern reflects a change in the stability of the open states and occurs on a minute time scale. Importantly, irrespective of mode, NRs gate with the same 3C2O core mechanism (fig. 4B) [94–97].

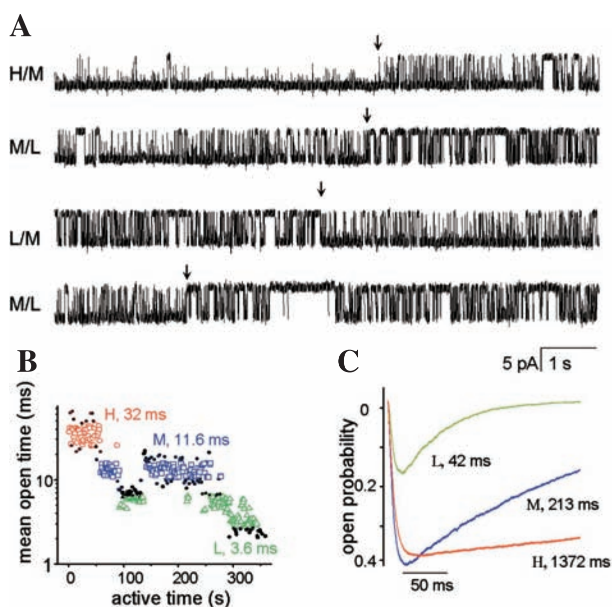


Figure 5. NRs can gate with three gating modes. (**A**), Four single-channel current traces (10 s each) recorded from NR1/2A receptors residing in cell-attached patches (HEK cells) illustrate shifts in current pattern (arrows); open is down, (**B**), Segments (1 s each) of NR active time sorted by their mean open time fall into three categories and mirror the four switches in current pattern illustrated in **A**. Numbers indicate the mean for each population. (**C**) Predicted current waveform elicited by a 1-ms pulse of 1 mM glutamate from NRs in each mode. For channels in each mode, peak P_o is approximately half the equilibrium P_o. Numbers indicate time constants of single exponential fits to the decay phase of the currents [98]. Red, high (H); blue, medium (M); green, low (L); filled symbols, excluded.

NRs switch between gating modes in a stochastic manner such that at any time experimentally recorded macroscopic responses reflect the average behavior of many channels whose individual gating mode is not known. However, once the microscopic kinetic parameters are determined for receptors gating in each mode, predictions can be made for responses from populations of receptors with homogeneous and/or mixed kinetics. Macroscopic responses simulated with the rate constants determined by fitting the 3C2O model to bursts of activity recorded from single NR1/2A receptors in each gating mode predict that a switch in gating mode substantially changes the NR macroscopic waveform (fig. 5C). Comparing the decay kinetics of the simulated macroscopic currents for each mode (42 ms, 213 ms and 1.3 s for L, M and H receptors, respectively) with those reported for NR1/2A whole-cell responses (54 ± 24 ms, fig. 3) suggests that whole-cell currents reflect mostly the activity of L-mode receptors [72, 94]. Also, L-mode kinetics most closely reproduced the measured decay of epsc at mature synapses [98, 99]. The peak open probability predicted for L-channels (0.2; for comparison, the values are 0.4 and 0.45 for M- and H-modes, respectively) is also consistent with that determined for neuronal somatic channels (0.3) but much higher than the open probability of synaptic NRs (0.04) [16, 17].

It is not known what causes channels to switch gating mode and whether mode switching represents a physiological mechanism for NR activity modulation. It has been suggested that modal switches result from perturbations in channel structure that occur at intracellular locations [94]. Many cellular components interact with NRs and modulate their activity. Significantly, several of these affect channel function by changing the receptors' mean open time [100–102]. The level and time course of NR activity is controlled by many dynamically regulated cellular pathways but the precise mechanisms by which these pathways impose changes in NR kinetics remain to be determined.

Statistical multi-state gating models

Within each gating mode, single-channel activity recorded from NRs consists of bursts of activity interrupted by long silent periods. The pattern of openings and closures within each burst of activity is statistically best described by a sequential activation model which postulates that after binding two molecules of glutamate, NRs change conformation several times along a linear reaction pathway consisting of three closed and two open states (fig. 4B).

A complete statistical description of NR single-channel currents within bursts was made possible by i) observing activity from individual channels for long periods of time under conditions when channels gate with maximal activity and populate with high probability a minimum number of states; and ii) by fitting putative models with various topologies directly to the experimentally observed sequences of open and closed intervals rather than to distributions of interval durations separated by class. This approach has led to a high-resolution description of the gating reaction as a sequential multi-step pathway of conformational change. Notably, the model predicts that a conformational change that increases the receptor's affinity for glutamate ($C_1 \rightarrow C_2$) occurs before the conformational change that changes the channel's conductance ($C_2 \rightarrow C_3$) and estimates discrete rate constants for each transition. In addition, the model postulates two separate conformational changes (C_2-C_3 and O_1-O_2) whose functional correlates are not known. The model with rate constants determined for NR1/2A receptors from single-channel currents accounts well for previously reported kinetic characteristics of the macroscopic NR responses and has predicted novel NR behaviors which were later demonstrated experimentally [98].

The statistical multi-state model correctly predicts NR macroscopic behaviors

Radioligand binding and antagonist competition assays have estimated that neuronal NRs have high affinity for glutamate (K_D = 0.9 μM) [75, 103]. Fitting NR single-channel activity across several glutamate concentrations by the statistical multi-state gating model estimates that, in either mode, glutamate binds with a rate constant $k_+ =$ 20 $\mu M^{-1}s^{-1}$ and dissociates with a rate constant $k_- = 60\ s^{-1}$ (fig. 4B), predicting a microscopic affinity for glutamate K_d = 3 μM. These values are in excellent agreement with macroscopic measurements for the same receptor preparation, EC_{50} (median effective concentration) = 2.9 μM [104].

Although the microscopic affinity of resting receptors for glutamate does not change when receptors switch mode, the model predicts that the measured EC_{50} will (fig. 6A). The phenomenon illustrates the theoretical principle that any macroscopic kinetic parameter, including K_D and EC_{50}, depends on the microscopic rates for all transitions experienced by the receptor [105]. In this case, although a modal transition changes mainly the mean time the receptors spend in open states and does not affect the mean lifetimes of closed states, inevitably, the fractional occupancies of all visited states change. Thus, although clearly the kinetics of glutamate binding and dissociation are mode-independent, changes in the relative occupancy of the state C_1, the only state from which glutamate can dissociate with measurable probability, result in distinct, mode-dependent frequencies of agonist dissociation events.

Moreover, the model predicts that independent of gating mode, the glutamate EC_{50} will vary with the duration of the applied pulse (fig. 6B). This predicted response has been observed experimentally for both neuronal and recombinant NR preparations (fig. 6C) [106]. In the absence of a reaction mechanism, and relying only on macroscopic measurements, it was difficult to infer the mechanism underlying this phenomenon. The multi-state statistical model explains this behavior by slow and equal rates for agonist dissociation ($C^0 \leftarrow C_1$) and for the isomerisation that traps the ligand ($C_1 \rightarrow C_2$). This reaction mechanism ensures that for agonist pulses that are shorter than the time spent by receptors in state C_1 (~4 ms), even though all the receptor binding sites become occupied within the first 75 μs of a 1-mM glutamate pulse, significant glutamate dissociation occurs before the $C_1 \rightarrow C_2$ transition takes place. This failure to effectively trap the agonist results in responses with submaximal amplitudes.

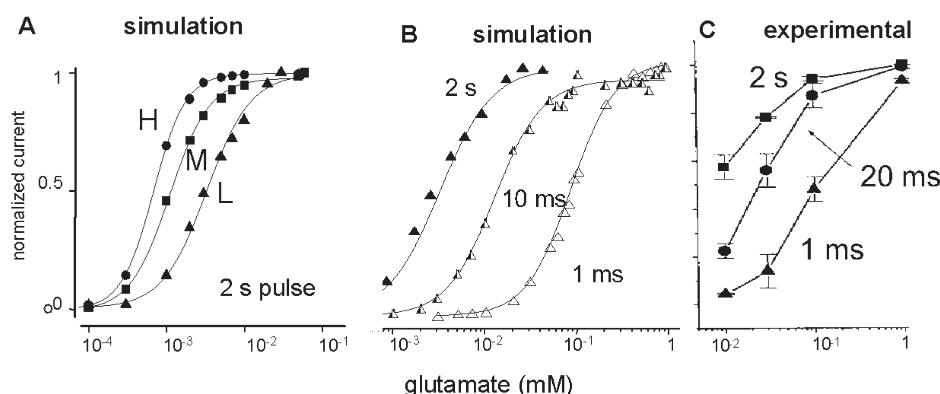


Figure 6. NR glutamate dose-response curves. The multi-state statistical model predicts that EC_{50} varies with gating mode (**A**) and with pulse duration (**B**). Simulations were done with the model in figure 4B, and the rates estimated form single-channel records of NR1/2A receptors gating in each mode: H (circles), M (squares), L (triangles); [98]. (**B**) Experimental dose response curves for NRs native to neocortical neurons show a decrease in EC_{50} with pulse duration (reproduced with permission from [106]).

Put another way, although a 1-ms glutamate pulse saturates all receptor binding sites, this pulse activates only ~50% of the receptors. This means that further increasing glutamate concentration will not result in increased current. In contrast, increasing the duration of the pulse or repeating the pulse at short intervals will engage more receptors and thus will, in fact, produce more current as a result (fig. 7A). As the model predicts, synaptic and recombinant NRs do show increased response following repetitive stimulation (Fig. 7B, C).

Concurrent single-dendrite calcium imaging and post-synaptic current recording have convincingly shown that the amplitude of the NR-mediated epsc increases when a second stimulus is applied at the peak of the response to the first pulse (fig. 7B) [107]. An increase in current following repetitive stimulation could indicate that synaptic NRs are not saturated by a single synaptic pulse. It is widely believed that at central excitatory synapses the glutamate concentration transient experienced by synaptic receptors peaks in the 1–5 mM range, and decays with biphasic time course (100 μs and 2 ms) [12]. This waveform together with the high affinity of NRs for glutamate would suggest that a single synaptic pulse fully saturates NRs. Still, this issue is not yet fully resolved, as the glutamate transient at particular synapses can vary according to the geometry and size of the cleft, the size of the released vesicle, and the number and location of glutamate transporters. Even so, the multi-step statistical model for NR activation predicts that high-frequency repetitive stimulation will amplify NR currents even when the glutamate released by each pulse saturates all NR-glutamate binding sites.

This phenomenon can be verified by applying glutamate onto NRs residing in excised membrane patches. In this experimental setup, the glutamate concentration and the pulse duration experienced by receptors can be controlled to approximate the stereotypical synaptic stimulus. Currents from NR1/2A receptors residing in membrane patches are amplified by repetitive stimulation with 1-ms pulses of high glutamate concentrations (1-mM) when pulses are applied at 10-ms intervals (fig. 7C) [96]. These experiments fully validate the topology and rate constants postulated by the multi-state statistical model and suggest that NRs are sensitive to pulse frequency rather than pulse concentration. Thus, the receptor's activation mechanism determines not only the shape of the receptor's synaptic response but also controls the frequency-dependent integration of information encoded in pulse trains.

The multi-state statistical model fully describes single-channel NR currents, provides a mechanistic interpretation of the receptor's macroscopic behaviors and can help predict receptor responses in circumstances that are difficult to access experimentally. The model also represents a valuable instrument with which to measure directly from single-channel records the rates with which NRs change conformations; to investigate the physical nature of the receptor's moving parts; and to identify and quantify the effects of specific allosteric modulators.

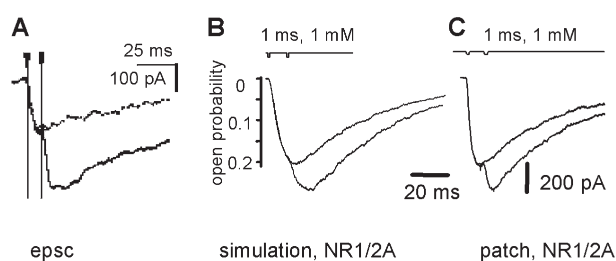


Figure 7. Repetitive stimulation potentiates NR currents. (**A**) Superimposed averages of NR-mediated epsc in a single spine (hippocampal slice) following a single stimulus and a pair of stimuli delivered at 10-ms interval; lines indicate the time of stimulation (reproduced with permission from [107]). (**B**) The multi-state statistical model predicts that repetitive stimulation with pulses of 1 mM glutamate at 100 Hz amplifies NR currents. (**C**) Superimposed currents recorded from NR1/2A receptors (excised patch, HEK cell) in response to a single 1-ms pulse of 1 mM glutamate and a pair of pulses spaced at 10 ms [96].

Structural interpretations of multi-state models

Although the structure of NRs at atomic resolution is not known, homology with proteins of known structure have helped formulate valuable hypotheses regarding structural correlates of observed receptor functions [59, 108–111]. The reader is referred to several excellent recent reviews that discuss structure-function relationships in glutamate-activated ion channels [60, 61, 112–114]. A significant advance was facilitated by the observation that the identity of the agonist at each NR subunit correlates with shifts in distinct components of the single-channel closed interval structure [115].

Kinetic analyses of single channel currents have led to the consensus that during gating, fully liganded NRs can adopt three closed conformations as indicated by the three exponential components present in the closed-time interval durations of fully liganded receptors [94, 97, 98, 115]. When channels are activated with partial agonists either at the glycine-binding sites on NR1 subunits or at the glutamate-binding sites on NR2 subunits, the changes observed in the structure of the single-channel closed-interval durations correlate with the identity of the site perturbed. Thus, the slowest component becomes even slower in the presence of glutamate-site partial agonists and is not affected by glycine-site partial agonists. In contrast, the middle-range component is sensitive to the identity of the ligand at the glycine site but not at the glutamate site. The duration of the shortest component is independent of the agonist used to activate the channel [115]. This observed correlation prompted the hypothesis that following agonist binding, each subunit type switches

independently ($C_1 \rightarrow C_2$ and $C_2 \rightarrow C_3$) into a conformation that is permissive for gating, and once all subunits have been activated, rapid, agonist-independent fluctuations (C_3-O_1), perhaps in the pore structure, allow intermittent ionic flow. This extended functional model proposes that during gating, fully liganded receptors undergo agonist-dependent intra-subunit movements in each subunit type, which may occur in any order but must precede the fast, agonist-independent switches. No functional or structural correlates have been proposed for the O_1-O_2 transition postulated by the multi-state statistical model.

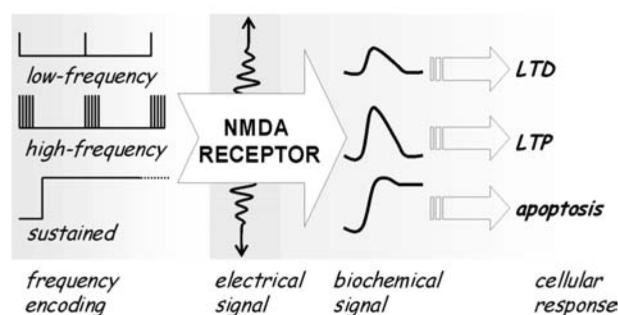


Figure 8. NRs as frequency discriminators. NRs recognize the information encoded in the frequency of the arriving stimulus (FM) and respond by generating currents with distinct amplitudes (AM). The resulting levels of intracellular Ca^{2+} initiate particular signaling cascades, which lead to specific cellular responses.

Implications for physiology and modulation

The recently proposed statistical and functional multi-state models for the NR gating reaction powerfully portray NRs as dynamic molecules whose macroscopic behaviors depend intrinsically on the rates with which agonist-induced conformational changes take place. These models attest to the multiplicity of intramolecular movements that shape NR functions and are fully compatible with the structural view of glutamate receptors as machines with many interacting mobile parts. In addition to revealing a previously unsuspected ability of NRs to discriminate stimulation frequency, the statistical model may provide additional insights into the physiological roles of NRs at synapses and the possible consequences of NR activity modulation.

First, the NR kinetic scheme for NR activation postulates that the amplitude of the NR response varies with stimulation frequency. This behavior may be of particular importance at central excitatory synapses onto spiny neurons where NR-mediated Ca^{2+} influx initiates, in a concentration-dependent manner, distinct intracellular signaling pathways. It was shown that discrete levels of dendritic Ca^{2+} transients above a threshold determine whether a synapse will become weakened or strengthened in response to synaptic activity [116, 117]. Additionally, in most systems, the direction of the ensuing activity-dependent synaptic modification correlates with stimulation frequency, with high-frequency stimulation resulting in long-term potentiation and tonic, low-frequency stimulation initiating long-term depression [118, 119]. Thus it is particularly intriguing to consider the possibility that synaptic NRs, in addition to their well-established roles as coincidence detectors, also represent frequency discriminators, potentially translating the information encoded in the pattern of neurotransmitter release into distinct current amplitudes and thus distinct dendritic Ca^{2+} concentrations (fig. 8). With this in mind, it is essential that potential pharmacologic agents be tested not only for their effects on the NR response waveform and on total charge transfer but also with respect to their consequences on the frequency-discrimination properties of NRs.

Second, the demonstration that NRs populate multiple kinetic states, which are also functionally distinct, suggests an additional layer of specific modulation of NR responses. A multi-step gating pathway raises the possibility that individual allosteric modulators may target specific receptor transitions and thus may have distinct efficacies in controlling separate receptor functions [120]. For example, NR current potentiation by high-frequency stimulation depends largely on only two rate constants ($C^0 \leftarrow C_1$ and $C_1 \rightarrow C_2$), and thus modulators affecting rates for any other transitions, while changing the total charge transfer and the kinetics of the NR macroscopic response, will leave the receptor's frequency discrimination properties intact. The perturbation that causes modal shifts illustrates this scenario. Rate constants determined for the reaction mechanism of NR1/2A receptors gating in each mode indicate that modal switches drastically change the decay kinetics of the predicted response and the total charge transferred but do not affect glutamate binding kinetics or the receptor's response to repetitive stimulation. It is particularly intriguing to consider that individual allosteric sites may have distinct efficacies in modulating separate receptor functions. It is thus essential to determine the mechanism by which individual sites affect receptor function and how combinatorial effects may be harnessed to elicit specific functional phenotypes.

Last, when considering NRs as complex allosteric proteins, it is particularly relevant to point out that agonist-activated NRs remain competent intercellular signal transducers even in the absence of ionic conduction. Although the physiologic significance of this recently reported metabotropic activity is still unclear, evidence is accumulating for a complex participation of NRs in the physiology of central excitatory synapses. The specific conformations (states) involved with particular interactions are not known, but it is conceivable that each conformation exposes a particular set of epitopes possibly coupling NRs to specific cellular machineries.

Despite the modest success that NR-targeted therapies have yielded so far, recent insights into the NR activation mechanism offer new optimism in the quest to control harmful NR functions while sparing its necessary activities. At the same time, these novel findings indicate that the macroscopic assessment of drug effects at NRs represents only the first of many functional tests needed to identify pharmacologic interventions whose benefit-to-risk ratios can be predicted with some confidence.

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