Physiology and Pathology of NMDA Receptors

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Abstract: Ionotropic glutamate receptors of the N-methyl-D-aspartate (NMDA) subtype are highly expressed in the central nervous system and are involved in excitatory synaptic transmission and synaptic plasticity. Prolonged activation of NMDA receptors can lead to excitotoxicity, which is implicated in the pathogenesis of neurodegeneration occurring in various acute and chronic disorders of the central nervous system. Recent advances in understanding the function, pharmacology, genetics and structure of NMDA receptors has promoted a search for new compounds that could be therapeutically used. These compounds act on agonist binding sites, either apart from them or directly within the ion channel pore. Members of the last group are called open channel blockers, and some of them, such as memantine and ketamine, are already clinically used. Kinetic modeling of NMDA receptor activity was employed to define the effects of various groups of modulators. Quantifying the action of these substances by kinetic parameters can help us to reveal the molecular mechanism of action at the receptor and to characterize the dependence of its action on the mode of NMDA receptor activation. Two modes are considered: phasic activation, induced by synaptically released glutamate, and tonic activation, which is expected to occur under pathological conditions when low, but sustained levels of glutamate activate NMDA receptors. The aim of our review is to summarize the recent data about the structural and functional properties of NMDA receptors and their role in long-term potentiation and excitotoxicity.

Keywords: N-methyl-D-aspartate receptors – Neurosteroids – Open channel blockers – Kinetic modeling

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Introduction

The importance of the glutamate receptors for the normal function of the central nervous system is emphasized by the fact that they are responsible for the vast majority of excitatory transmission in the central nervous system and that 35% of energy expenditure in the grey matter of the brain is used to cover the needs of glutamate excitatory transmission, with an additional 3% for glutamate recycling. [1, 2].

Glutamate receptors can be divided on the basis of the mechanism of signal transduction they use. Ionotropic glutamate receptors operate as ligand-gated ion channels, formed as homo- or hetero-oligomeric assemblies of integral membrane protein subunits. Metabotropic glutamate receptors are G-protein coupled and rely on secondary messengers for signal transduction. Ionotropic glutamate receptors can be further divided in three subgroups on the basis of pharmacological criteria: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), and kainate. This review aims to focus on the pharmacological and functional properties of NMDA receptors.

Genetics of NMDA receptors

It is now well accepted that NMDA receptors are tetrameric structures. This issue was tackled from several different angles: functional, electron microscopical, and structural [3–7]. In the beginning of the previous decade, through cloning techniques based on low-stringency cDNA library screening and amplification, the genes for glutamate receptor subunits were cloned, first in the rat genotype and later in the human genotype. There are three large gene families: those for NMDA receptor subunit 1 (NR1); for NMDA receptor subunit 2 with its four subtypes, denoted A to D (NR2A-D); and for NMDA receptor subunit 3, with two subtypes, A and B (NR3A-B) [8-18].

The NR1 subunit has 938 amino acids in its primary structure, whereas NR2A-D subunits are significantly larger, having 1464, 1482, 1250 and 1323 amino acids, respectively. The overall sequence homology between NR1 and NR2s is low (~15–20%), but the homology between individual NR2 subunits is higher (~50%). On the other hand, regional homology between NR2 subunits is very high in hydrophobic regions (up to 90%), but extremely low between their C-terminal sections [15]. The NR3A subunit is 1115 amino acids long and shows only 27% and 28% homology with the NR1 and NR2 family, respectively. NR3B shares 50% of its amino acids with NR3A, but only 17–20% with other NR families [18]. When expressed as heterotrimeric NR1/NR2/NR3 receptors, both NR3A and NR3B have a dominant negative effect.

Apparently, the real receptor pool is much more diverse than what could be predicted from genetic research. There is a major post-transcriptional process, called alternative splicing, that changes the structure and properties of the primary transcripts of NR1 receptor subunits. This process produces a total of eight

functional splice variants, based on the absence or presence of three different exons in the final transcript, which are usually described by the nomenclature of Hollmann [19]. NR3A mRNA also exists in two splice variants, short and long [20].

Subunit structure

The overall properties of the NMDA receptor, owing to its modular structure, are determined by properties conferred by its individual constituent subunits, so they can be investigated in a predictable manner. The members of what is now called the Cys-loop family (nAChR, GABAAR, 5-HT3 and glycine receptors) have four transmembrane domain structures of individual subunits. On the other hand, glutamate receptor subunits have three transmembrane domains in total, and a reentrant M2 loop that makes a hairpin turn within the membrane [21–26]. A schematic representation of the NMDA receptor subunit is presented in Figure 1. The N-terminal is located extracellularly, whereas the C-terminal is intracellular. The figure shows segments S1 and S2, which are thought to create an agonist-binding site; three transmembrane segments, M1, M3 and M4; and a reentrant loop in the membrane, M2.

Activation of NMDA receptors by glutamate and glycine

A peculiarity of NMDA receptors is that when the two agonists, glutamate and glycine are bound simultaneously, two molecules of each are required for receptor activation. Glycine binds to the NR1 subunit and glutamate to the NR2 subunit [27–30]. The concept of agonist binding to glutamate receptors is supported by the "clam shell" model, in which two domains, S1 and S2, form a "pocket" that can exist in open and closed conformation. The postulated existence of this structure was later visualized [31]. S1 is formed by the region preceding M1, while S2 is formed by the loop between M3 and M4. The binding of the agonist switches the domains to the closed pocket position. This motion is

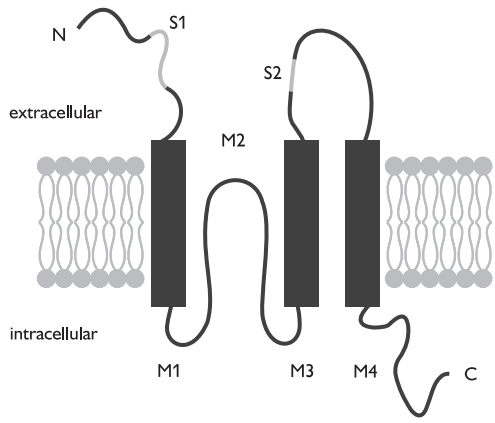

Figure 1 – The schematic structure of NMDA receptor subunit in the cellular membrane, showing the orientation of N and C terminals, pore-forming loop region (M2) and the relative position of agonist binding sites S1 and S2.

then transferred to the rest of the receptor, producing conformational changes leading to channel opening [32].

This process depends on the receptor structure and is NR2 subunit dependent. It has been shown that receptors containing NR2A subunits are the fastest and those containing NR2D are the slowest to deactivate, with the time constants of NR2B and NR2C being ~4 times longer than that of NR2A. Quite exceptionally, the deactivation time constant of NR2D is an order of magnitude longer [33, 34].

NMDA receptor desensitization

Desensitization is an agonist induced receptor state which becomes refractory to agonist application. [35]. Electrophysiologically, it is usually manifested as a time-dependent decrease of the responses during a prolonged agonist application. There are three types of molecular mechanisms that explain the diminution of NMDA receptor responses in the continuous presence of agonist: glycine-insensitive, glycine-sensitive and calcium dependent.

The first is glycine-insensitive desensitization, which occurs during prolonged whole-cell recording [36]. It has been shown that induction of this type of desensitization depends on an increase in intracellular Ca^{2+} concentration, which is irreversible over the time-course of whole-cell recording and appears to involve mechanisms that do not include only the change in the phosphorylation state of the receptors [37]. Similar results were obtained by recording the responses from outside-out patches pulled from cultured rat hippocampal neurons [38].
G-proteins play a regulatory role in glycine-insensitive desensitization. This effect arises from the direct interaction of G-proteins with NMDA receptors or proteins associated with them, but not as an indirect effect on Ca^{2+} permeability [39].
Glycine-insensitive desensitization is NR2 subunit dependent, regulated by two segments in its structure [40, 41]. One of these corresponds to a long stretch of amino acids located proximal to the S1 agonist binding domain and the other is located proximal to the first transmembrane segment. The differences in structure within these two regions could explain why this type of desensitization is more pronounced in NR2A-B than in NR2C-D subunits. However, point mutation in the highly conserved region of transmembrane segment 3 of NR1 subunits also alters the desensitization rate [42].

The glycine-sensitive "desensitization" and Ca^{2+} induced inactivation are characterized by a time-dependent diminution of the responses. However, the molecular mechanism of diminution is something other than desensitization. In glycine-sensitive changes of receptor response, although glycine itself potentiates glutamate response, the binding of glutamate reduces the affinity of glycine to its binding site [43]. This leads to a reduction of the response during prolonged glutamate exposure, as glycine reequilibrates according to its new, reduced, affinity [44]. By increasing the glycine concentration (to 10 μM), this type of "desensitization" can be overcome.

Calcium-induced inactivation is increased by elevating the intracellular Ca^{2+} concentration [37, 45]. Strictly speaking, this is not desensitization since it has been shown that inactivation results from a direct interaction of calmodulin with the C-terminal of the NR1 subunit [46].

Probability of opening

Most experimental data agree that, after stimulation by the agonist, only a fraction of the total receptor pool will be open at any moment. This is determined by the probability of receptor opening (Po), *i.e.*, the probability of a transition from a double ligand bonded receptor with closed ion channel to a conformational state with the ion channel open (RAA to O, as presented in Figure 2). The value of Po was assessed by different methods, mostly employing high-affinity voltage-dependent open channel blockers like MK-801. A very low value of 0.02% was obtained when the Po was recorded in cultured hippocampal neurons [47]. On the other hand, later studies using outside-out patches isolated from cultured hippocampal neurons produced much higher values of 30% [48]. The differences in published values of Po are a consequence of the methodological approach and the properties of the NMDA receptors [49]. When measured under the same conditions in recombinant systems, Po depends on the receptor subunit composition, which is 35% and 7% for NR1/NR2A and NR1/NR2B receptors, respectively [50].

Models of NMDA receptor activation

Several models have been proposed to describe the microscopic properties of NMDA receptors. In the first, linear model, the existence of independent and identical binding sites for glutamate is assumed [48]. By varying a number of glutamate molecules predicted to bind to the receptor, it was shown that consistently best fits are obtained for the model with two glutamate binding sites. In this model, there are three important states: a closed channel with both sites occupied, an open channel and a desensitized channel. The model produces remarkably good fits when applied on macroscopic currents, therefore correctly defining glutamate binding, desensitization and recovery from desensitization. Schematic representation of this model is presented in Figure 2A.

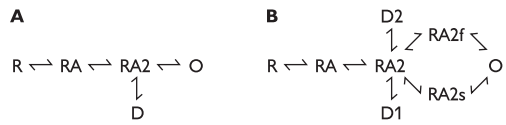

Figure 2 – The models for NMDA receptor activation. 1A A linear model, proposed by [48]. The abbreviations used represent: R – receptor with no glutamate bound; RA – receptor with one molecule of glutamate bound; RA2 – receptor with two molecules of glutamate bound, but with the closed channel; O – receptor with two molecules of glutamate bound with the open channel; D – desensitized receptor. 1B A loop model proposed by [51]. The abbreviations are the same as those used in the first model, except for RA2f and RA2s, which represent fast and slow transition states, respectively.

Nevertheless, when a more complicated analysis of single channel behaviour was introduced, this model had certain gaps. The attempt to fill them produced a different model, presented in Figure 2B [51]. There are two obvious differences. The first one is the introduction of two desensitized states, due to the double-exponential deactivation time course after prolonged agonist application. The second change was a modification of the single opening step, predicting a loop structure to assume that each subunit controls a kinetically distinct component of gating. The obligatory event that must occur before any conformational change is the preceding binding of agonist to its corresponding subunit. Supported by experimental data, this model predicts that glycine binding to NR1 evokes a rapid conformational change, whereas the binding of glutamate to NR2 produces a slower transition. Both are necessary for the transition into a state that permits a channel opening. Receptor activation modeling is important because it is the initial step in understanding not only how isolated receptors operate, but also how this process is affected by modulating substances.

Binding sites identified on NMDA receptors

A large number of structurally different groups of substances influence the activity of NMDA receptors. The three possible mechanisms by which NMDA receptors can be affected are as follows: by binding (1) to the agonist binding site (competitive antagonists), (2) apart from it (allosteric modulators) or (3) within the receptor channel (open channel blockers). The effect of these substances is often subunit dependent or even subunit specific. The major modifying sites are presented in Figure 3.

Glutamate binding site

It has been shown that glutamate EC_{50} values for all combinations of NR1/NR2 is ~1 μM [1, 52]. However, this number, which is routinely used, was

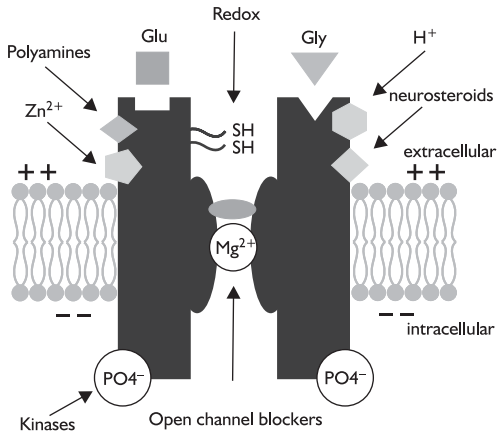

Figure 3 – A schematic representation of the sites at which NMDA receptor channel activity can be modified.

determined from dose response curves after sustained application of glutamate (order of seconds). If a more synapse-like application is used, there is a significant rightward shift to about 20 μM for NR2A and NR2B [53]. There are several groups of substances that act as competitive antagonists at the glutamate binding site. The typical competitive antagonists of this site are phosphono derivatives of short chain amino acids, such as 2-amino-phosphonovaleric acid (2-APV) and 2-amino-7-phosphonoheptanoic acid (7-APV) [54]. The effect is NR2 subunit dependent (as expected by the location of the glutamate binding site). For example, CGS 19755 (cis-4-(phosphonomethyl)piperidine-2-carboxylic acid) and CGP 39653 (D,L-(E)-2-amino-4-propyl-5-phosphono-3-pentenoic acid) display the affinity order NR1-NR2A > NR1-NR2B > NR1-NR2D > NR1-NR2C [55].

Glycine binding site

The presence of glycine is an absolute requirement for glutamate receptor activation [56]. Although the binding site for glycine is located on NR1, glycine EC_{50} is influenced by the NR2 subunit. The reported values for NR2A-D EC_{50} are as follows: NR2A> NR2B> NR2C> NR2D (approximately 1 μM) [57–59]. The measurements show that overall glycine concentration in CSF is at low micromolar levels [60]. However, glycine concentration in the synaptic microdomain is regulated by glycine transporters, which can significantly reduce glycine concentration, so receptors are not necessarily saturated [61, 62].

There are several glycine site selective antagonists; probably the best known is 5,7-dichlorokynurenic acid [63]. There are also GV196771A, 7-chloro-4-hydroxy-3-(3-phenoxyphenyl)quinolin-2(1H)-one (L-701,324), and [(+/-)-4-(trans)-2-carboxy-5,7-dichloro-4-phenyl-amino-carbonyl-amino-1,2,3,4-tetra-hydro-quinoline] L-689,560 [64-66].

H^+, Zn^{2+} and polyamine modulatory site

The proton modulatory site has emerged as the hub through which the effects of other substances, such as zinc, ifenprodil and polyamines, are mediated. This means that their inhibiting or potentiating action is exerted through changes in the proton sensitivity of NMDA receptors. The physiological pH of CNS is close to the IC_{50} of the proton effect on the most abundant NMDA subunit, NR2B, meaning that the receptors are under tonic inhibition; however, as the receptors are positioned at the steep part of the curve, the change in any direction can greatly influence the receptor function. Change of pH causes changes in the frequency of opening, but not changes in conductance or open time in single channel recording [67, 68]. In congruence with this, the site of proton action is believed to be localized on the extracellular side of the M3 transmembrane domain and the spacer region connecting it with the S2 domain in both NR1 and NR2 [42, 69]. The differences in structure within this region can explain

the difference in IC_{50} for NR2A (119 nM) and NR2C (825 nM). As mentioned previously, proton inhibition is also splice variant dependent, since the presence of exon 5 in NR1 shifts the proton curve to more acidic values [70].

Zinc is present in synaptic vesicles and can be released upon stimulation, reaching physiologically or pathologically important concentrations of up to 300 μM. Zn^{2+} can produce voltage independent inhibition at low micromolar concentrations and voltage dependent block at higher micromolar concentrations [71–73]. It has been shown that zinc reduces the frequency of channel openings, which explains the first effect. The voltage independent effect of Zn^{2+} is pronounced in the NR2A subunit, which contains a high affinity site for voltage independent block (IC_{50}~10 nM) and a low affinity site for voltage dependent block (IC_{50}~0.5 μM) [74]. For any combination of NR1/NR2B, inclusion of exon 5 in NR1 reduces sensitivity to Zn^{2+} voltage independent block [74, 75]. However, the situation becomes complicated with the analysis of both NR1 splice variants and NR2 subunits. As previously mentioned, the voltage independent Zn^{2+} inhibition via the high affinity site on NR2A is explained by the potentiation of proton inhibition. The site of Zn^{2+} binding was eventually traced to several residues in extracellular region of NR2A, the mutation of which severely perturbs Zn^{2+} effect [76, 77].

Polyamines have at least three effects on NMDA receptors: voltage dependent block, glycine-dependent potentiation and glycine-independent potentiation; the latter is mediated by the relief of tonic proton inhibition and manifests itself at saturating glycine concentrations, but in recombinant receptors it occurs only in the presence of NR2B [78, 79]. Inclusion of exon 5 in NR1 reduces glycine-independent spermine potentiation. However, even these splice variants show glycine-dependent potentiation, manifested as an apparent increase in glycine affinity [80]. A surprising finding was that a mutation in the pore region of M2 reduces proton sensitivity, thereby reducing the stimulatory spermine effect as well [70, 75].

Redox modulation

It has been shown that the current responses of the native NMDA receptors can be enhanced by reducing agents, and that this effect can be reversed and further attenuated by oxidizing agents [81, 82]. The typical substances used experimentally are the disulfide reducing agent dithiothreitol (DTT) and the sulfhydryl oxidizing agent 5,5'-dithio-bis-(2-nitro-benzoic acid) (DTNB). However, there are various endogenous substances that can produce these effects, making changes in redox state potentially physiologically important, which in turn stimulated the search for its target on the receptor [83, 84].

There was a gradual increase in the number of recognized redox sites on recombinant receptors. Initially, it was found that two cysteine residues located on NR1 (Cys 744 and Cys 798) are responsible for changes in the activity of receptors

containing NR2B, C or D [85]. However, a separate, additional site has been proposed to exist on NR2A [86]. On a single channel level, the potentiating effect of reduction is manifested by an increase in opening frequency, without changes in open time and conductance in NR2B, C or D containing receptors, but open time is prolonged in NR2A [87]. Finally, additional cysteine residues have been found to affect redox properties, making a total of 3 pairs of cysteines, two on NR1 (Cys 744 and Cys 798, Cys 79 and Cys 308) and one on NR2A (Cys 87 and Cys 320, homologous to the second pair in NR1), which are responsible for the slow/persistent, intermediate and fast/reversible component of redox modulation, respectively [88].

Open channel blockers

Open channel blockers are substances that gain access to their binding sites only after the receptors are activated and the ion channel is opened. For this reason, they are also called use-dependent inhibitors. A typical property of blockers of NMDA receptor channel is that they are positively charged, which explains why the extent of block diminishes with increasing voltage and why the recovery from block is accelerated in the presence of agonist or at positive voltage. For the same reason, it is not possible to determine unique IC_{50} values for this kind of substances, i.e. this information must be followed by the membrane potential at which it was determined and the apparent depth at which the blocker binds within the channel [89, 90].

The interaction of NMDA receptors with Mg^{2+} ions is of great physiological importance. In solutions containing this ion, after stimulation by agonists, receptor responses exhibit voltage dependent extracellular Mg^{2+} block [91, 92]. For this reason, the I–V curve is highly non-linear, having a typical J-shape. The larger the driving force of Mg^{2+} entry (*i.e.* higher concentration and/or negative potential), the larger the block. Therefore, as discussed elsewhere, for NMDA receptors to be activated under normal conditions, a sufficient previous depolarization is obligatory. The binding site for Mg^{2+} is located within the pore, at the Q/R/N site, the narrowest point of the channel pore [25, 93]. This open-channel block exists even with much lower concentrations of Mg^{2+} than those in CNS extracellular space (millimolar), and the IC_{50} for Mg^{2+} at wild type NR2A and NR2B containing receptors was determined to be ~20 μM at –70 mV [94]. However, not only is the binding of Mg^{2+} extremely fast, but Mg^{2+} can dissociate quickly from the receptors, which is manifested as a flickering block (short openings separated by brief closures) in single-channel recordings.

Open channel blockers can be divided into three groups: high affinity antagonists (dissociative anesthetic-like agents – PCP (phencyclidine), MK-801 (dizocilpine, [(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]- cyclohepten-5,10-imine maleate), ketamine), medium affinity antagonists (memantine) and low-affinity antagonists (Mg^{2+}) [47, 95, 96]. MK-801 elicits a strong channel block, dissociating from the

channel on a very slow time scale (tau ~90 min at –70 mV) [47]. Memantine antagonizes responses to NMDA in a concentration-dependent manner, with IC_{50} at hyperpolarized (–70 mV) potentials of ~2 μM and with small differences between NR2 subunits.

Neurosteroids

A recent surge in interest for these substances started long after the first observation that steroids can have rapid actions in CNS that cannot be explained by the classical nuclear transcription pathway [97]. This discovery led to a definition of neurosteroids as substances synthesized in CNS *de novo* or from circulating precursors [98]. Since then, the effects of neurosteroids have been examined on various receptors, including NMDA [99].

The two steroids we were mostly interested in were pregnenolone sulfate and 20-oxo-5-beta-pregnan-3-alpha-yl sulphate (3α5βS). Both of these substances are endogenous and their structure is presented in Figure 4. It can be seen that they differ in one double bond. The effect of pregnenolone sulfate on NMDA receptors is subunit dependent and voltage independent [100, 101]. When applied simultaneously with the agonist, it potentiates NR2A or NR2B containing receptors, while inhibiting those that contain NR2C or NR2D. However, we found that its effect also depends on the mode of application [102]. On the other hand, 3α5βS is generally an NMDAR-inhibiting neurosteroid, but its effect is stronger on NR2C or NR2D than on NR2A or NR2B containing receptors, i.e. it also shows subunit dependence [101]. Similar to the effect of PS, that of 3α5βS is also voltage independent.

Physiological roles of glutamate receptors

Glutamate receptors can be found on all neurons in CNS. Being so widely distributed in practically all pathways, it is no wonder that they play a role in a variety of different processes, ranging from vision, hormonal regulation, and higher brain function to motor and sensory control. On the other hand, a plethora of evidence indicates that glutamate receptors play a role in various pathological processes, such as Alzheimer's disease, Huntington's disease, schizophrenia, and stroke [103], a prominent common feature of all of these disorders being glutamate-induced neurodegeneration. We focus on two of these processes: long-term potentiation, which is a form of synaptic plasticity, and excitotoxicity, which is practically a synonym for NMDA receptor overactivation.

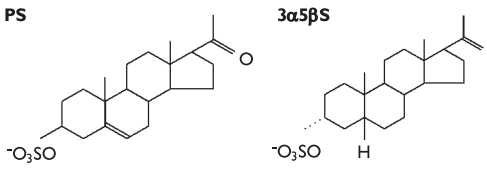

Figure 4 – The structure of pregnenolone sulphate (PS) and 20-oxo-5-beta-pregnan-3-alpha-yl sulphate (3α5βS).

Long-term potentiation

The remarkable capacity of the brain to translate transient experiences into seemingly infinite numbers of memories that can last for decades has been attributed to long-lasting, activity-dependent changes in the efficacy of synaptic communication [104], a theory initially developed by Hebb [105]. This Hebbian plasticity theory was later expanded to include any long-lasting form of synaptic modification (strengthening or weakening) that is synapse specific and depends on correlations between pre- and postsynaptic firing [106].

The NMDA-dependent form of LTP was mainly investigated in the hippocampus of rodents. LTP is experimentally induced by short high-frequency stimulation of synapses or by directly depolarizing the cell while continuing low-frequency synaptic activation (a "pairing protocol"). It is also triggered by an increase in the postsynaptic intracellular calcium concentration, which is an absolute requirement for the forming of LTP [107]. However, it is now clear that other forms of LTP exist that do not depend on NMDA receptor activation [108].

Consistent with its pivotal role in LTP induction are numerous demonstrations that genetic and pharmacological manipulations with NMDA receptors block LTP. In mice strain knocked-out for NR2A subunit, NMDA receptor activity in the hippocampal CA1 region is decreased to about half of that of wild-type mice, with a corresponding increase in thresholds for hippocampal LTP induction [109]. On the other hand, an overexpression of NR2B produces enhanced activation of NMDA receptors, facilitating synaptic potentiation by high-frequency stimulation [110].

It has been questioned whether the location of synaptic changes leading to LTP is presynaptic or postsynaptic. The presynaptic model assumes that the probability of transmitter release is initially low, so although both NMDA and AMPA receptors are co-localized in the postsynaptic membrane, synaptic failure is high [104]. LTP then occurs as a result of increased probability of transmitter release or change in the mode of transmitter release leading to an increase in the synaptic success rate [111].

However, after long debate, it seems safe to say that the mechanism of LTP can be explained through the postsynaptically localized changes in synaptic activity. It has been shown that certain synapses have only NMDA receptors localized postsynaptically with no AMPA receptors. They do not respond to presynaptic stimulation with the usual NMDA receptor excitatory postsynaptic current (EPSC) and therefore are called silent [112]. These functional data, mostly derived from LTP induction experiments in CA1 region of hippocampus, are supported by morphological studies showing that in some synapses only NMDA receptors are present, AMPA receptors being absent [113–115]. According to this model, LTP is induced by the changes in the quantitative and qualitative properties of AMPA receptors triggered by appropriate NMDA receptor activation [116]. Both of these changes occur as a consequence of calcium-calmodulin dependent kinase II

(CaMKII) induced receptor phosphorylation [108]. First, as a result of activity dependent changes in AMPA receptor trafficking, the number of AMPA receptors increases postsynaptically [117]. The insertion of AMPA receptors in the synapse is restricted owing to its subunit composition. This restriction is relieved following NMDA receptor activation [118]. At rest, AMPA receptors are inserted slowly. This process is immediately accelerated by NMDA receptor activation [119]. AMPA receptors seem to be inserted in the extrasynaptic region first and then move laterally into the synapse [120]. Second, there is a change in the electrophysiological properties of AMPA receptors, namely their conductance or peak open probability [121, 122]. Both of these changes are reversible, indicating their role in the type of changes of synaptic activity that can go in the opposite direction.

Excitotoxicity

The first documented description of this phenomenon came when Lucas and Newhouse observed that subcutaneously injected glutamate selectively damaged the inner layer of the retina (representing primarily the retinal ganglion cells) [123]. With the progress of research in this field, John Olney formulated the term "excitotoxicity" to describe this phenomenon [124].

Intracellular Ca^{2+} must be tightly regulated owing to the decisive role this ion plays in processes such as membrane excitability, exocytosis, synaptic transmission and the regulation of the activity of many enzymes. Cells can regulate Ca^{2+} influx, efflux, storage and buffering, allowing for site-directed and temporally limited Ca^{2+} signals occurring at various locations within the same cell at the same time. However, these systems have a limited capacity, and when it is exceeded there can be activation of processes that are always prepared for action but never activated in the healthy cell, eventually leading to cell death through various executioner subsystems [125].

A large variety of insults can lead to the excessive release of glutamate within the nervous system and thus excitotoxic cell death. One obvious mechanism is a severe mechanical insult, such as cranial or spinal cord injury. Acute and massive neuronal loss is not the only way this process progresses. A more subtle form of excitotoxicity is implicated in a variety of neurodegenerative disorders. Several lines of evidence indicate that Huntington's disease, Alzheimer's disease, HIV-associated dementia and amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease) have this excitotoxic component [126–132].

Further supporting the role of NMDA receptors in neurotoxicity, many *in vitro* studies have shown that blocking these receptors diminishes neuronal loss. Also, suppression of NR1 synthesis and genetic studies with NR2A knock-out animals have produced reduced effects of focal brain ischemia [133, 134]. NMDA receptors are not isolated structures in the membrane. Unlike AMPA receptors, they are a constant element of postsynaptic density, associated with various

proteins [116]. Of these, PDZ proteins (abbreviated from PSD-95, Disc large, Zonula occludens 1) are believed to be a key transmitter of downstream signals [135–137]. They remain associated with postsynaptic density even after disruption of cytoskeletal organization and at least in the early stages of excitotoxic insult [138, 139]. NMDA receptors, however, are not located only in synapses, so it has been an open question whether synaptic and extrasynaptic receptors are equally potent in evoking excitotoxicity [140, 141]. By varying the conditions under which it was initiated, it has been shown that both types are linked to the pathways that elicit neuronal damage [142].

The mechanisms by which excitotoxic damage occurs include, but are not limited to, increased NO synthesis, free radical production and activation of several groups of enzymes, such as phospholipases, calpain or DNAses, [126, 143, 144]. By reacting with reactive oxygen species, NO produces peroxynitrite, which damages DNA [145]; this leads to the activation of poly-ADP-ribose polymerase (PARP-1), an enzyme that functions as a DNA damage sensor and signaling molecule and that binds to both single- and double-stranded DNA breaks [146]. Activation of Ca^{2+}-dependent proteases leads to the destruction of the structural elements of the cell [147], whereas endonucleases cleave DNA [148].

Effects of different groups of NMDA receptor modulators

NMDA receptors possess several binding sites that, when activated by the appropriate pharmacological substances, can influence the receptors' function. This presents the opportunity for possible interventions in situations when the activation or inhibition of receptors should be modified to correct the abnormality caused by pathological process [149]. However, because of the ubiquitous involvement of these receptors in virtually every activity of the CNS, it has been suspected that pharmacological manipulations produce various side effects [143]. The task is to find a substance whose desired effects can be predicted by knowledge of the mechanism of action on the molecular level, which will not produce more harm than good in patients.

Knowledge of the molecular mechanism of action of different kinds of modulators can have a predictive power. Some effects, such as neuroprotection, are common for all types of modulators. They behave in a way that correlates with their effect on NMDA receptors, *i.e*. inhibitors produce protective results and stimulators have aggravating results. However, these studies are based on *in vitro* studies or studies in animal models. There is only a limited amount of data that concern humans. Competitive antagonists on both the glutamate and glycine binding site have a neuroprotective effect. APV has a protective effect on cultured cortical neurons, 7-chlorokynurenate on hippocampal and cortical cultures and glycine site antagonists in an animal model *in vivo* [150–153]. Neuroprotective activity is also a property of a strong open channel blocker, MK-801, both *in vivo* and *in vitro* [154]. It has been shown that memantine, a low-affinity open channel

blocker, has a neuroprotective effect on cultured hippocampal neurons exposed to glutamate or NMDA [155, 156]. It also has the same effect *in vivo* after ischemic insult [157]. In some studies, polyamines protected against excitotoxicity as well [158, 159]. By potentiating NMDA receptor function, DTT also potentiates excitotoxicity [160]. Neurosteroids 3α5βHS and 17-beta-estradiol, both inhibitors of NMDARs, protect against neurotoxicity *in vitro* (rat hippocampal cultures) and *in vivo*. On the other hand, pregnenolone sulfate, a potentiating agent, augments neuronal death [161–164]. Although these effects are in congruence with what could be theoretically predicted, more data are necessary to estimate the action of the drug in a living organism. Sometimes, *in vitro* models are designed to selectively test one of its effects, neglecting or simply being unable to test other effects simultaneously.

Open channel blockers have a neuroprotective effect, as expected by the fact that they act on activated receptors. However, when tested in human subjects, they have serious psychotomimetic side effects. These effects seem to be the exclusive property of open channel blockers and not other classes of NMDA receptor inhibitors. They are well known for phencyclidine (PCP), so much so that it became the standard model of schizophrenia in rats [165]. Ketamine, another open channel blocker, induces schizophrenia-like and dissociative symptoms in drug addicts, as well as (dose-dependently) in healthy volunteers [166, 167]. Even more importantly, ketamine induces a dose-related worsening in the mental status of schizophrenic patients [168]. On the other hand, although it belongs to the same group of open channel blockers, memantine shows far less of this type of adverse effect [169]. Finally, Mg^{2+}, which acts as an endogenous open channel blocker, does not produce these side effects at all. Attempts have been made to correlate the psychotomimetic effects of various NMDA receptor modulators in animal models with those in human subjects. In general, the rule is that the risk of hallucinations depends on the mechanism of action (open channel blocker) and the affinity of its binding. In correlation with this rule, there is a conspicuous

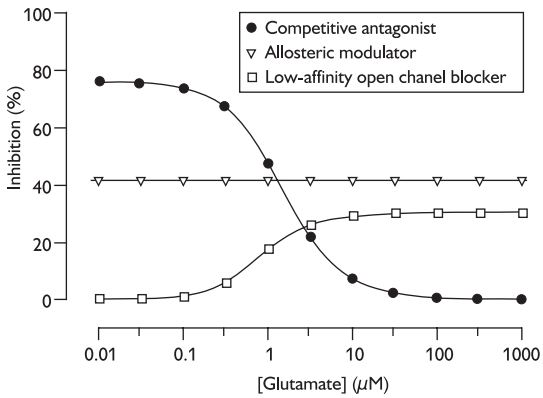

Figure 5 – The effects of different types of blockers on NMDA receptors tonically activated by various glutamate concentrations.

absence of these effects in glutamate and glycine site antagonists [170, 171], as well as in ifenprodil congeners [172]. We have recently used kinetic models of competitive, allosteric and open channel blocker action to show what effect these compounds have on tonically and phasically activated NMDA receptors [173].

Figure 5 shows the effects of different classes of NMDA receptor inhibitors on NMDA receptor responses induced by tonically elevated glutamate. By raising glutamate concentration, the degree of inhibition by the competitive antagonist falls, since it is overcome by agonist. On the other hand, the more receptors are activated, the higher is the effect of open channel blockers, since they are allowed to approach their binding site within the channel. For allosteric inhibitors, the effect is the same regardless of agonist concentration. The conditions of tonically elevated glutamate are similar to those believed to exist during excitotoxicity, indicating the potential use of open channel blockers.

On the other hand, the model shows that the opposite occurs when these same substances, in the same concentration, are applied to phasically activated NMDA receptors, which mimics their synaptic activation. Figure 6 shows that competitive antagonist has an effect which is higher than that during tonic activation, since the slow dissociation rate of competitive antagonist does not allow synaptic glutamate to compete with the inhibitor during a short (~1 ms) glutamate presence in the synaptic cleft. Allosteric inhibitors have the same effect that they have under conditions of tonic glutamate application, since their binding is not related to the agonist presence. The effect of open channel blocker will strongly depend on their binding and unbinding rates. On the basis of these parameters, it can be shown that the inhibition will range from that close to competitive inhibitors to no inhibition [169].

Experimental data, supported by the kinetic modeling of inhibitor action on NMDA receptor function, show that inhibitory effect depends on whether the receptors are tonically or phasically activated. These conclusions, which are supported by experimental and clinical data, indicate that detailed qualitative

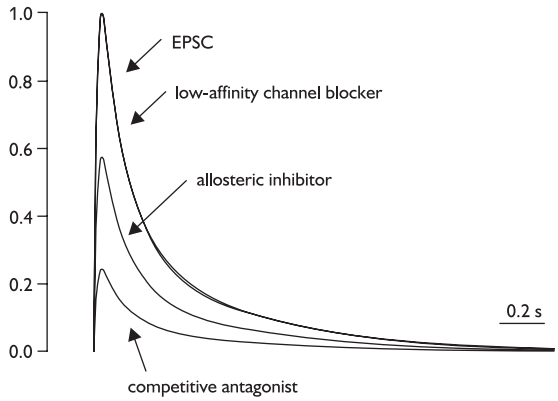

Figure 6 – Effects of competitive, allosteric and low-affinity open channel blocker under conditions that imitate synaptic transmission.

understanding of the molecular action of the modulating substance on the NMDA receptor, as well as its quantification by kinetic parameters, can be of enormous help in assessing its activity.

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