Clinical Perspective

The N-Methyl-D-Aspartate Receptor in Cerebral Ischemia

Terence Tan, Mitchell Albert, PhD, Yanping Sun, PhD

Abstract

Excitotoxicity is a major mechanism of neuronal damage in cerebral ischemia and is mediated largely by neuronal N-methyl-D-aspartate receptors (NMDAR). This review presents the structural and functional alterations in NMDAR after an ischemic insult, including the antagonistic roles different NMDAR sub-types play in the post-ischemic period. By elucidating these molecular changes and the effects they have on downstream cellular signaling, rational therapy can be devised in an attempt to mitigate the effects of cerebral ischemia.


Introduction

In 2005, 2.7% of men and 2.5% of women above 18 years old in the U.S. had at least one episode of stroke. This corresponds to an incidence of 158 per 100,000 people, a figure that translates to one stroke occurring every 40 seconds. Furthermore, stroke is the 3rd most common cause of death after heart disease and cancer, and it is the 6th most common cause of reduced disability-adjusted life years (DALY). As the population in developed countries ages and the number of stroke cases increases, this burden of morbidity will increase. Myocardial infarction, with an incidence of 200-250 per 100,000 and a fatality rate of 9-16%, is also a major cause of global cerebral ischemia.

Several novel research studies have been done to elucidate the pathophysiology of stroke and global ischemia. Glutamate-mediated excitotoxicity has been implicated as one of the main processes in the evolution of stroke injury. During neuronal excitotoxicity, massive amount of glutamate, the principal excitatory neurotransmitter of the central nervous system (CNS), is released by ischemic nerve endings and glial cells into the extra-cellular space. Glutamate then binds to N-methyl-D-aspartate receptors (NMDAR) on surrounding neuronal and glial cells, activating the receptor and opening its channel pore. As a cationic channel, NMDAR allows Na$, and more importantly, Ca$^{2+}$, into the post-synaptic neuron. Accumulation of Ca$^{2+}$ in the cytosol is particularly detrimental to neuronal survival, as it can lead to the generation of reactive oxygen species, mitochondrial dysfunction, proteolytic cleavage of cellular proteins and eventually cell death. This review will begin by providing a background of the structure and function of NMDAR, followed by an introduction to the sub-membranous microenvironment of NMDAR known as the post-synaptic density (PSD). We will detail factors mediating the localization and trafficking of NMDAR, and we will review molecular and functional changes in NMDAR under ischemic conditions. Finally, we will present the opposing roles played by different NMDAR receptor sub-types in ischemia.

Structure and Function of NMDAR

The NMDAR is a hetero-tetrameric receptor made up of a combination of sub-units denoted as NR1 and NR2. There are eight variants of the NR1 sub-unit as a result of alternative mRNA splicing, and NR1 is an obligate component of the NMDAR. The NR2 sub-unit is comprised of four different isoforms: NR2A, NR2B, NR2C, and NR2D. These isoforms are expressed by different alleles in the genome. Depending on the availability of NR2 sub-units and the brain region involved, different sub-types of NMDAR can be found. While dihetero-tetrameric (e.g. NR1/NR2A) receptor sub-types are most commonly found in the brain, trihetero-tetrameric (e.g. NR1/NR2A/NR2B) receptor sub-types have also been found endogenously. Of the dihetero-tetrameric receptors, NR1/NR2A and NR1/NR2B are the predominant sub-types found within the forebrain (see below for discussion of the distribution of these two receptor sub-types). Current research is starting to unveil the different, and mostly opposing, functional differences between NR2A and NR2B sub-units. Consequently, this will allow researchers to more accurately target disease processes found in stroke, Parkinson’s, and dementia. In addition to NR1 and NR2, there also exists a less common NR3 sub-unit, which has two isoforms denoted NR3A and NR3B. With regards to NR3, trihetero-tetrameric NR1/NR2/NR3 have been found endogenously, although their relative contribution to NMDAR signaling is unresolved.

The quaternary structure of NMDAR creates an ionic channel that is physiologically blocked by Mg$^{2+}$. This is relieved upon membrane depolarization, effectively producing a voltage-dependent block. In addition to Mg$^{2+}$, chemicals such as MK-801 (dizocilpine), phencyclidine, and ketamine can bind in a location within the channel of the NMDAR. These channel-blockers produce a voltage-dependent blockade that is most profound when the receptor is activated - i.e., a use-dependent blockade. The NMDAR has multiple sites for

Terence Tan, Mitchell Albert, PhD, Yanping Sun, PhD
All from Department of Radiology, University of Massachusetts Medical School, Worcester, MA
agonists and antagonist binding. The NR1 sub-unit binds the co-agonist glycine, without which the NMDAR receptor will not be activated. Acute, transient exposure to glycine in the presence of glutamate is known to potentiate the NMDAR-derived whole-cell neuronal current. Further, conditioning or sustained exposure (2-3 minutes) of glycine in the presence of glutamate permanently reduces the whole-cell current via clathrin-dependent endocytosis of the NMDAR. Presumably, this serves as a protective response to attenuate NMDAR overactivation in periods of pathological neurotransmitter release, as occurs in cerebral ischemia. Physiologically, Zn$^{2+}$ serves as an endogenous modulator of NMDAR function, binding to an allosteric site found on NR2A and NR2B sub-units. This serves to elicit a glycine-independent down-regulation of NMDAR activity. Pharmacologists have exploited this finding by synthesizing molecules that are selective for either NR2A or NR2B, thus allowing the different functions of these sub-units to be explored under both physiological and pathological conditions.

During excitatory synaptic transmission, whole-cell voltage recordings show two typical stages: a fast excitatory post-synaptic current (EPSC) and a slow EPSC. The fast EPSC is mediated by the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor, a separate class of glutamate receptor with fast activation and deactivation kinetics. The AMPA receptor (AMPAR) has also been shown to be involved in cerebral excitotoxicity, and its role in cerebral ischemia will have to be considered along with the contribution of the NMDAR. The slow EPSC is mediated by the NMDAR, with activation and deactivation time constants comparably longer than that of the AMPAR. Different NMDAR sub-types display different deactivation kinetics that will affect the total amount of Ca$^{2+}$ diffusing from the synaptic cleft into post-synaptic terminals. NR2A-containing NMDAR deactivates twice as fast as NR2B and NR2C-containing receptors, whereas NR2D-containing receptors have the longest deactivation times. As expected, mixed NR1/NR2A/NR2B receptors display kinetics that are intermediate between NR2A- and NR2B-containing receptors.

Developmental Profile, Sub-unit Expression, and Localization of NMDAR

Embryologically, neurons express only one kind of NR2 sub-unit in addition to the obligatory NR1 sub-unit. This NR2 sub-unit is either NR2B or NR2D, with NR2B found in widespread regions of the embryonic brain, whereas NR2D is confined to the brainstem and the diencephalon (thalamus and hypothalamus). During the perinatal period, neurons start to express a second NR2 allele, resulting in the expression of NR2A or NR2C sub-units in specific regions of the brain. By adulthood, expression of NR2A is ubiquitous in the brain, whereas NR2C is mainly confined to the cerebellum. As mentioned above, NR2A-containing NMDAR exhibits fast deactivation kinetics, thus shortening the EPSC of many excitatory neurons during brain development. This phenomenon has been implicated in the synaptic pruning that occurs in experience-dependent synaptic plasticity during brain development. Furthermore, there is a simultaneous decrease in NR2B as the expression of NR2A increases in the brain perinatally; by adulthood, NR2B is found only in the forebrain.

On a cellular level, different types of neurons express a distinct complement of NR2 sub-units, often a combination of NR2A and NR2B together with NR2D sub-units. This results in the possibility of different NMDAR sub-types occurring in different parts of the same neuron (e.g., NR1/NR2A, NR1/NR2A/NR2B, NR1/NR2A/NR2D). However, NR2D-containing receptors have thus far only been found in extra-synaptic locations as dithetero-tetrameric NR1/NR2D NMDAR, and are not believed to partake in synaptic neurotransmission. Current consensus is of the view that synaptic NMDAR is composed mainly of a mixture of NR1/NR2A and NR1/NR2B receptors together with a relatively smaller population of NR1/NR2A/NR2B receptors. Extra-synaptic NMDAR, which is activated by synaptic transmissions only when there is a spillover effect of glutamate from the synapse (as occurs in excitotoxicity), is composed mainly of NR1/NR2B receptors complemented by a smaller NR1/NR2A receptor population.

Whereas NMDAR populations in neuronal membranes are known to be relatively stable and exhibit low-turnover rates and little variations in receptor numbers, it has been shown that internalization and changes in sub-unit composition of NMDAR does occur at synaptic and extra-synaptic locations. In vivo, NR1 sub-units are found in both the membrane surface (as functional receptors) and as unassembled free NR1 sub-units within the neuronal cell. NR2 sub-units, on the other hand, are mostly found on the neuronal membrane incorporated as functional receptors or sequestered within the endoplasmic reticulum (ER) awaiting final modification prior to incorporation into a functional receptor. In studies where expression of NR2A and NR2B was increased in neurons by transfection, there was an increase in the number of cell-surface NMDAR, suggesting that NR2 sub-unit synthesis and ER processing is a rate-limiting step in the expression of functional NMDAR on the post-synaptic membrane. Further, it has also been found that increased NR2A sub-unit expression and spontaneous excitatory synaptic transmission can increase the expression of NR2A-containing receptors at the cell surface. This phenomenon is not observed for NR2B sub-units. As mentioned above, glycine binding tags the NMDAR for internalization by a clathrin-dependent mechanism. This effect is mediated by a site at the C terminus of NR2B (Tyr-1472), which is believed to interact with AP-2 to cause endocytosis. A similar site and effect exist in NR2A sub-units.

Another factor that affects the localization and function of NMDAR is the intra-cellular microenvironment in which the receptor is located, or the PSD. The PSD contains multiple PDZ proteins, including the abundant PSD-95, Synapse-
associated protein (SAP) 107 and PSD-93. Each PDZ protein is made up of three PDZ domains, one SH domain, and one guanylate kinase-like (GK) domain.28 PDZ proteins, like PSD-95, serve two main functions. Firstly, it is believed to stabilize receptors on the neuronal membrane. A PDZ-binding domain exists on the C terminus of NR2 sub-units, and this allows direct interaction of the NMDAR to the PSD.29,30 It has been found that mutation of this PDZ-binding domain in NR2B sub-units effectively excludes NR2B-containing NMDAR to be expressed on the neuronal cell surface, affirming the role that PSD-95 plays in anchoring NR2B-containing NMDAR. However, the same effect was not observed for NR2A sub-units. This suggests different mechanisms regulating the anchoring of NR2A and NR2B-containing NMDAR at the cell surface, possibly via binding at a different site or via an intermediary protein found in the PSD. Secondly, PSD-95 serves to bring together, both physically and functionally, NMDAR to the intra-cellular signaling machinery.31 For example, neuronal nitric oxide synthase (nNOS) contains a PDZ-binding domain, and it has been found in the PSD associated with PSD-95. This physical association forms a NMDAR-PSD95-nNOS signaling complex that effectively couples NMDAR activation to the production of nitric oxide.28

**Ischemia-induced Alterations in NMDAR Structure and Function**

**Changes in sub-unit expression**

As a key player in glutamate-mediated excitotoxicity, much attention has been given to changes in the sub-unit expression of NMDAR under experimental ischemic conditions. In hippocampal slices where neurons were transiently (5 minutes) deprived of oxygen and glucose there was a significant increase in NR1 sub-units found in the neuronal membranes 1 hour after the insult.34 This acute response to ischemia is postulated to be one of the mechanisms by which NMDAR exhibits a hyperactive response to stimulation in the acute phase of excitotoxicity. However, this increase in NR1 sub-unit concentration is short-lived, and disappears within 6–24 hours.35 When the duration of ischemia is prolonged and severe, as occurs in stroke and global cerebral ischemia due to cardiac arrest, the NR1 sub-unit has been shown to undergo a time-dependent decrease in surface-receptor expression, with severely decreased levels occurring after an hour-long ischemic period. This is believed to occur via a decrease in NR1 sub-unit mRNA expression, and is dependent upon a NR2B-mediated transcriptional inhibition mechanism.36 Because NR1 is the obligatory sub-unit of all NMDAR, any decrease in NR1 transcription (and translation) will eventually result in a decrease in the number of NMDAR present on the neuronal post-synaptic membrane.

There is also evidence suggesting that NR2A and NR2B sub-units are transcriptionally down-regulated during a transient, severe ischemic insult. Here the down-regulation is non-uniform in different brain regions experiencing the same ischemic severity, as shown by the different temporal decreases in NR2A and NR2B sub-unit mRNA expression in hippocampal CA1 and CA3 sub-fields. The CA1 region is known to be selectively vulnerable to ischemia, whilst the CA3 subfield is selectively resistant. After a transient ischemic insult, NR2 mRNA decreases in the CA3 region more quickly than in the CA1 region.35 It is possible that the down-regulation of NMDAR activity via decreased NR2 transcription serves to limit the excitotoxic damage caused by ischemia and contributes to preferential CA3 neuronal survival during ischemia.

Whereas a high concentration of NMDAR agonist will cause excitotoxicity and irreversible neuronal damage, a sub-lethal exposure of NMDAR agonist or Potassium Cyanide (KCN, which causes an anoxic-type cell injury) results in enhanced neuroprotection against future ischemic insults, a phenomenon known as ischemic pre-conditioning.37 Note that whereas NR2 sub-unit mRNA is decreased following transient ischemia, as mentioned above, a sub-lethal preconditioning stimulus causes no change in NR2 sub-unit levels.38

**Changes in NMDAR kinetics**

After a transient ischemic insult, NMDAR show a biphasic response. Early in the post-ischemic phase, the NMDAR-mediated EPSC component shows a sustained increase.34 This corresponds to an increased amount of Ca²⁺ entering the intra-cellular compartment, and probably contributes to the delayed neuronal damage experienced by cells after transient cerebral ischemia. Later in the post-ischemic phase (around 18-24 hours), there is a progressive negative modulation of receptor kinetics, eventually resulting in drastically reduced receptor currents mediated by NMDAR.35 This change in receptor kinetics parallels the decrease in NR1 and NR2 sub-unit mRNA expression. It should, however, be noted that while these receptor kinetics may be true in conditions of transient ischemia, they may not reflect the kinetics of NMDAR in focal cerebral ischemia, where glutamate levels are sustained at high levels throughout a longer period of time.39,40 Transient ischemic insults also result in the appearance of a different subset of NMDAR with small amplitude channel conductances.35 This is probably due to the insertion of NR2C-containing NMDAR into the postsynaptic membrane.41 NR2C-containing receptors have an intrinsically lower conductance than NR2A and NR2B-containing receptors, and thus allow a smaller amount of Ca²⁺ into the cell.42 This may yet represent another mechanism whereby neurons limit excitotoxic damage.

**Phosphorylation and cleavage of NMDAR signaling complex**

Ischemia induces significant changes to the chemical structure and configuration of the NMDAR and its associated proteins. These changes are characteristically quick to occur, as they employ substrates and enzymes already available within the PSD and cytosol. They thus represent an expedient response to the ischemic stimulus. The NR1 sub-unit of the NMDAR is phosphorylated by both protein kinase A (PKA) and protein kinase C (PKC) early after transient global ischemia.43 This is partially mediated by an increased translocation of both Ca²⁺-dependent and Ca²⁺-independent
PKC isoforms to the PSD. Similarly, NR2A and NR2B subunits are phosphorylated by PKC in the same time window as NR1. NR2A and NR2B phosphorylation by PKC in transient global ischemia is dependent upon the activation and increased translocation of the Src-family kinase (SFK) Src and Fyn, and also the tyrosine kinase Pyk2 (Proline-rich kinase 2). As phosphorylation of NMDAR potentiates the activity of the receptor, such an effect during cerebral ischemia will increase deleterious downstream signaling mediated by NMDAR, at least during the earlier phases of transient global ischemia when receptor activity is enhanced via receptor tyrosine phosphorylation.

Besides phosphorylation, another aspect of ischemia-induced NMDAR modification is the structural changes that occur with respect to its association with the PSD. Significant modifications to the NMDAR signaling complex with ischemia have been demonstrated, with NR2 sub-unit cleavage being the most investigated to date. Transient ischemia causes a degradation of both NR2A and NR2B sub-units, and this process is dependent upon the Ca2+-dependent enzyme calpain. This degradation of NR2A and NR2B is mediated by activation of NR2B receptors (and not NR2A), as it can be blocked by the NR2B-selective antagonist ifenprodil if administered before the ischemic insult. In addition, the processing of the NR2 sub-units is extensive, involving residues in both the C-terminus and N-terminus. After processing by calpain, the remaining, truncated NR2A or NR2B sub-unit remains localized to the post-synaptic membrane as a hetero-oligomer together with NR1 sub-units. The functional significance, if any, of this oligomer is unknown, but downstream signaling mediated by this truncated NMDAR will be expected to be defective due to a physical uncoupling of the receptor with the underlying PSD and signaling complex.

It is evident that ischemia results in a gamut of NMDAR modulation at multiple levels from transcriptional repression to chemical modifications to the receptor and the PSD. Whereas ischemia shows a trend towards a reduction in NMDAR function, especially in the 24 hours after transient ischemia, care must be taken when interpreting these data because the nature, severity, and duration of the ischemic insult will play a big role in determining the changes that occur to the NMDAR.

Differential Roles of NR2A and NR2B in Cerebral Ischemia

Excitatory neurotransmission mediated by NMDAR have both a physiological and pathological role. The brain’s dependency on NMDAR signaling is often described as resembling a bell curve: too little NMDAR activation and brain cells risk apoptosis, necrosis and increased vulnerability to insults, whereas too much activation results in excitotoxicity and neuronal death. The optimal amount of NMDAR stimulation ensures neuronal survival. Upon further investigation of the factors affecting excitotoxonic signaling, it was found that activation of extra-synaptic NMDAR led to neuronal death whereas activation of synaptic NMDAR led to enhanced neuronal survival under ischemic conditions. The pro-survival effect after synaptic NMDAR stimulation is mediated by activation of cyclic AMP response element binding protein (CREB), which switches on the transcription of various pro-survival genes. Stimulation of extra-synaptic NMDAR switches off this CREB pathway and leads to eventual cell death. As mentioned above, synaptic NMDAR is composed of a mixture of NR1/NR2A, NR1/NR2B, and NR1/NR2A/NR2B receptors, whereas extra-synaptic NMDAR is primarily NR1/NR2B receptors, with a smaller contribution of NR1/NR2A NMDAR. In view of these results, some researchers have postulated that synaptic and extra-synaptic NMDAR, regardless of their sub-unit composition, induce a pro-survival and pro-death signaling cascade respectively. However, a string of carefully conducted studies have supported the hypothesis that it is the specific NMDAR sub-unit composition, and not the location of NMDAR (synaptic or extra-synaptic) that determines cell survival or death in ischemia.

In a comprehensive set of experiments by Liu et al, blockade of NR2B-mediated NMDAR activity consistently decreased the severity and extent of excitotoxic-induced apoptosis and necrosis in brain slices. Instead of attenuating ischemic damage, blockade of NR2A-mediated NMDAR activity exacerbated ischemic neuronal cell death. To determine if it was the location of the NMDAR or the sub-unit composition that affected ischemic damage, excitotoxic stimulation of NMDAR in cortical neuronal cultures was restricted to either synaptic or extra-synaptic locales. At each locale, sub-type-specific NMDAR activity was then blocked with either the NR2A-selective antagonist NVP-AAM077 or the NR2B-selective antagonist Ro 25-6981. It was found that at both synaptic and extra-synaptic sites, blockade with the NR2A-selective antagonist increased the amount of ischemic damage, whereas the opposite was observed when NR2B sub-units were blocked. The reduction of damage found in NR2B-blockade might be due to a decrease in deleterious post-ischemic LTP, an occurrence that has been implicated in post-ischemic neuronal death. In addition, these results were replicated in vivo using a rat model of focal stroke. Of clinical significance, it was found that selective activation of NR2A-containing NMDAR has a wide window of therapeutic efficacy (4.5h). The authors suggested that it might be useful to pursue NR2A activation as a treatment for stroke because it theoretically has no limiting therapeutic window, given that activation of NR2A-containing receptors induces a pro-survival signaling cascade. This is in contrast to the numerous NMDAR antagonists that suffer from narrow treatment windows, all of which have thus far proved ineffective in clinical trials. In a separate study, these results were replicated in an animal model of global cerebral ischemia, where it was found that blocking of NR2A-signaling abolished the phenomenon of ischemic tolerance mentioned earlier, whereas NR2B blockade enhanced the ischemic pre-conditioning effect. Thus, NR2A receptor-mediated signaling is involved not only in pro-survival
signaling after ischemic insult, but also contributes to the phenomenon of ischemic pre-conditioning. Whether these two neuroprotective processes are part of the biological pathway remains to be investigated.

Conclusion
Excitotoxicity is one of the central processes in cerebral ischemia, and is of great importance in the pathophysiology of both stroke and global ischemia. In this review, we have attempted to tackle the structural and functional changes to NMDAR after ischemia and explore the different roles NMDAR sub-units play in cerebral ischemia. Other associated pathologic processes, such as inflammation, apoptosis, and necrosis, will undoubtedly influence and overlap with excitotoxic pathways, and may result in further alterations to NMDAR structure and mechanisms. As research illuminates the pathway to NMDAR-mediated excitotoxic damage and neuroprotection, we hope that this will be followed by emergence of treatment of important conditions like stroke. Already, attempts have been made to prevent pro-death cascades by inhibiting NMDAR signaling at the level of the PSD, thus sparing any neuroprotective action stimulated by receptor activation.60 We hope that these and other therapies will prove that the elusive concept of neuroprotection is not a myth.61

References