Synaptic and Extrasynaptic Glutamate Signaling in Ischemic Stroke

Naijian Chao[#] and Sheng-Tian Li[*]

Bio-X Institutes, Shanghai Jiao Tong University, Shanghai, P.R. China

Abstract: Stroke is a leading cause of human mortality and disability where most cases of stroke are ischemic. The central nervous system (CNS) is extremely vulnerable to ischemic stroke particularly due to its unique ability: synaptic transmission. Not only does elaborate synaptic transmission consume extravagant energy that constrains neuronal viability under ischemic conditions, but glutamate, the most predominant neurotransmitter in the CNS, also triggers several catastrophic signaling cascades at both synaptic and extrasynaptic sites when excessively released. These signaling cascades accelerate neuronal death and exacerbate cerebral injuries during ischemic stroke. In this review, we discuss the complete picture of synaptic and extrasynaptic glutamate signaling in ischemic stroke. We hope to provide substantial insights into potential therapies by reviewing recent discoveries that have advanced our understanding of the complex glutamate signaling mechanisms in ischemic stroke.

Keywords: Astrocyte, excitotoxicity, extrasynaptic, glutamate, ischemic stroke, N-methyl-D-aspartate (NMDA) receptor, subunit.

INTRODUCTION

Glutamate signaling has played a critical role in accelerating neuronal death and exacerbating cerebral injury during ischemic stroke [1, 2]. Within the mammalian central nervous system (CNS), glutamatergic transmission accounts for roughly 90% of the total synaptic transmission [3], making glutamate the most predominant excitatory neurotransmitter in the CNS. Despite the pivotal roles of glutamate in neurophysiology, it has also been well established for the past 30 years that high concentrations of this excitatory transmitter is neurotoxic [4] and an excessive amount of glutamate is released during ischemic stroke due to impaired synaptic transmission [5, 6].

The theory of "excitotoxicity" was developed based on several early observations showing that neurons could be destroyed rapidly during exposure to toxic levels of glutamate, and such toxic exposure is self-propagating (*i.e.* an initial toxic exposure would trigger further release of excessive glutamate from endogenous glutamate stores, causing more neurons to be exposed to toxic levels of glutamate)[7, 8]. Early studies concerning the mechanism of excitotoxicity stressed the toxic influx of extracellular calcium through N-methyl-D-aspartate (NMDA) receptors [9], a major type of glutamate receptor permeable to Ca^{2+} when activated (to be discussed later in detail), as well as implicated its role in the pathology of ischemic stroke [10, 11]. However, the subsequently devised NMDA receptor antagonists have failed in clinical treatment [12, 13], emphasizing the urgency of a more thorough investigation into the glutamate signaling mechanism in ischemic stroke [14, 15].

After nearly 15 years of immense effort, the complete picture of toxic glutamate signaling has gradually become more and more clear. In the present review, we address recent progress that has advanced our understanding of the mechanism of glutamate signaling in ischemic stroke. We start with an overview of recent discoveries on the glutamate receptors with an emphasis on the role of NMDA receptors in ischemic stroke, elucidating many of the sophisticated mechanisms underlying this central player of glutamate signaling that have been substantiated recently. We then discuss the integrated glutamate signaling pathways in detail, setting our scope at both synaptic and extrasynaptic locations, as extrasynaptic glutamate signaling has recently been revealed as a new element in glutamate signaling, and has been associated with several devastating effects under pathological conditions. Furthermore, we briefly review the role of astrocytes in extrasynaptic glutamate signaling, highlighting their potential in regulating glutamatergic processes.

GLUTAMATE RECEPTORS

Glutamate receptors are the key components of glutamatergic signaling, and can be categorized into two receptor families: the ionotropic receptors and the metabotropic receptors. The ionotropic receptors are linked to membrane ion channels and can be further classified into three different types according to their specific agonist, which is the NMDA receptor, the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor and the kainate (KA) receptor. Upon glutamate activation, ionotropic receptors open and induce ion flux passing through the postsynaptic membrane [16], whereas metabotropic receptors generate downstream effects mainly by interacting with their coupling G-proteins [17].

*Address correspondence to this author at the Bio-X Institutes, Shanghai Jiao Tong University, Dongchuan Road 800, Shanghai, 200240, P.R. China; Tel: +86-21-34204287; Fax: +86-21-34204270; E-mail: lstian@sjtu.edu.cn

#*Current Address: School of Life Sciences, Swiss Federal Institute of Technology Lausanne (EPFL), Lausanne, Switzerland*

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NMDA Receptor

The NMDA receptor has been the focus of extensive amounts of studies regarding ischemic stroke throughout many years. Although virtually all members of the glutamate receptors are involved in mediating excitotoxicity, the NMDA receptor has been suggested to play a dominant role. Not only because the NMDA receptor is the major Ca^{2+} permeable iontotropic glutamate receptor [18], but also due to the fact that NMDA receptors associate with abundant intracellular proteins that could convert synaptic activity into genetic signaling and govern the fate of the cell [19] (Fig. **1**). Nonetheless, NMDA receptors exhibit a broad range of functional diversity with differences in subunit composition, subcellular localization and intracellular coupling [20], all of which contribute to differentially activating the various signaling pathways triggered by NMDA receptors. During recent years, knowledge has advanced substantially with regard to the role the NMDA receptor plays during ischemic stroke, revealing many complicated mechanisms. Therefore, we felt of great significance to invest certain lengths on discussing this important receptor.

Basic Properties of the NMDA Receptor

In the CNS, NMDA receptors are postsynaptic heterotetramers composed of two obligatory NR1 (newly renamed GluN1) subunits and two regulatory subunits [21]. The regulatory subunits include four types of NR2 (newly renamed GluN2) subunits: NR2A-NR2D (GluN2A-GluN2D), and two types of NR3 (newly renamed GluN3) subunits: NR3A-NR3B (GluN3A-GluN3B)[22]. The binding sites in these NMDA receptor subunits involve glutamate, glycine, D-serine, Zn^{2+} and polyamine [23]. As a co-agonist of the NMDA receptor, D-serine has recently been suggested to act as an endogenous ligand that modulates glutamate activation of NMDA receptors, and is released mainly from glial cells [24]. Specifically, the pore of the NMDA receptor channel is blocked by Mg^{2+} in a voltage dependent manner to prevent transmembrane ion flux when the receptor is not activated [25]. Therefore, NMDA receptors are gated in two ways: the binding of appropriate chemical ligands(*e.g.*, glycine and glutamate), as well as the coincidental membrane depolarization to remove the Mg^{2+} blockade. Under physiological conditions, glutamate released from the presynaptic site firstly activates postsynaptic AMPA receptors, causing AMPA receptor-mediated Na^{+} influx and partial membrane depolari-

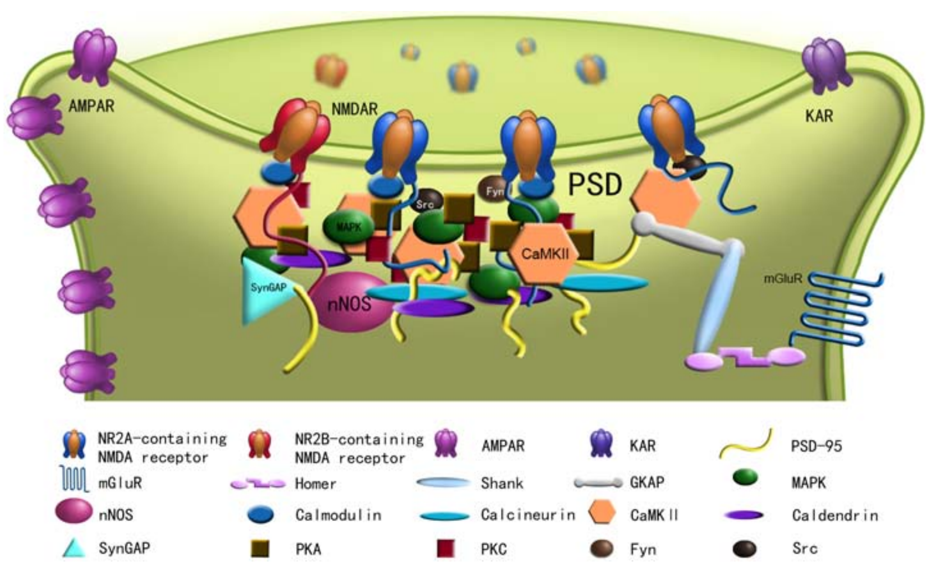

Fig. (1). The postsynaptic signaling mechanism of a glutamatergic synapse.

On the postsynaptic membrane of a glutamatergic synapse, N-methyl-D-aspartate (NMDA) receptors are generally sitting at the center, whereas α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (AMPAR) and kainate (KA) receptors (KAR) are more peripheral. Upon glutamate activation, AMPA/KA receptors are activated at first, allowing Na^{+} influx and partial membrane depolarization to relieve the Mg^{2+} blockade (not shown) in NMDA receptors. Once activated, NMDA receptors allow Ca^{2+} influx, triggering interactions with abundant postsynaptic density (PSD) proteins through their C-terminus (the tails stretching out from the NMDA receptors, blue for NR2A C-terminals and red for NR2B C-terminals), as well as through their association with scaffolding proteins (PSD-95 family proteins, illustrated in yellow stripes). These PSD proteins include tyrosine kinases (Src/Fyn), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), protein kinase A (PKA), PKC, mitogen activated protein kinase (MAPK, such as ERK1/2), synaptic Ras GTPase activating protein (SynGAP), calmodulin, caldendrin, calcineurin, and neuronal nitric oxide synthase (nNOS). Synergistically, these PSD proteins may modulate the signaling properties of glutamate receptors, transmit glutamate receptor activity, and also regulate downstream signaling by carrying out specific phosphorylation events. Although clear differences between NR2A and NR2B subunit signaling complexes are still hard to distinguish so far, it has been suggested that SynGAP is selectively associated with the NR2B subunit [66], and the NR2B C-terminal exhibits stronger coupling to the PSD-95-nNOS pathway [281], which has been determined to be a more significant involvement of the NR2B subunit in mediating neuronal death. Metabotropic glutamate receptors (mGluRs) are located at the adjacent membrane, while they are also connected to the NMDA receptor signaling complex through scaffolding proteins (guanylate kinase-associated protein (GKAP), Homer and Shank)[85].

zation that is sufficient to relieve the block of Mg^{2+} in NMDA receptors. When activated, NMDA receptors permit Ca^{2+} influx as well as a slight influx of Na^{+}. This Ca^{2+} influx mediated by NMDA receptors triggers several downstream signaling pathways through intracellularly-coupled proteins [26-28], which play fundamental roles in regulating neuronal functions, such as neurotransmission [29], synaptic plasticity [30], neurogenesis [31] and learning and memory [32], and even ultimately in regulating cellular viability [33].

NMDA Receptors of Different Subunit Compositions

In recent years, the discovery of the various signaling pathways triggered by NMDA receptors of different subunit compositions has been intensively studied [34-36]. As mentioned above, NMDA receptors are genetically comprised of seven types of subunits. The majority of NMDA receptors in the CNS are hetero-tetramers containing two NR1 subunits and two NR2 subunits [22], though tri-heteromeric receptors (NR1/NR2/NR3) also exist among glial cells [37, 38].

The presence of two NR1 subunits enables the obligatory functional properties of the NMDA receptor, such as affinity to agonists and antagonists [39], permeability to Ca^{2+}, intracellular trafficking [22], and regulation of downstream genes [21]. The NR1 subunit contains three splicing boxes (the N1 box at the N-terminal and the C1 and C2 boxes at the C-terminal), thus producing eight types of splicing variants [40]. NR1 subunits display distinct characteristics within themselves as a result of these different splicing variants [40-42]. The C-terminal of the NR1 subunit contains several phosphorylation sites as well as many binding sites for regulatory proteins or molecules [43, 44]. However, in contrast to the large C-terminal of NR2 subunits, the NR1 subunit possesses a relatively small C-terminal [45, 46]. The ER retention signal motif in the C1 box generally regulates the expression, assembly and trafficking of NMDA receptors, as different NR2 subunit compositions will promote specific membrane targeting [22, 47]. The interaction between the C0 section, which precedes the C1 section, and α-actin mediates the binding of the NR1 subunit to the cytoskeleton [43]. The C2 section includes a threonine/serine-X-valine-COOH (T/SXV) motif, which can regulate the location and expression of receptors in the postsynaptic density [48]. Furthermore, both the C0 and C1 sections contain binding sites for calmodulin, which may participate in the calcium-dependent inactivation effect of NMDA receptors, a process highly enhanced during ischemic episodes [49]. However, though NR1 subunits display various properties, the direct involvement of NR1 subunits in mediating excitotoxic damage is considered less significant than the NR2 subunits. It has also been postulated that the effect of NR1 subunits in ischemic stroke may be due to the differential response to glutamate activation from altered splicing, and furthermore, the resultant differentiation in subunit composition [50].

The regulatory composition of NR2 subunits has provided the NMDA receptor with remarkable functional diversity, especially the NR2A subunit and the NR2B subunit, as their involvement in ischemic stroke has been addressed extensively during recent years. NMDA receptors consisting of distinct NR2 subunits differ greatly in the following aspects: i) Channel kinetics: NR2A-containing NMDA receptors display faster channel kinetics with a shorter decay time in comparison with NR2B-containing NMDA receptors [51, 52]. ii) Affinity to agonists: Compared with NR2C- and NR2D-containing NMDA receptors, NR2A- and NR2B-containing NMDA receptors have lower affinity for glutamate and glycine [53, 54], whereas NR2B-containing NMDA receptors show even lower affinity to glutamate than NR2A-containing NMDA receptors [55]. Interestingly, NR2C- and NR2D-containing NMDA receptors have much lower opening probabilities compared to NR2A- and NR2B-containing NMDA receptors [56, 57]. iii) Sensitivity to the blockade of Mg^{2+} and antagonists: Sensitivity to the blockade of Mg^{2+} and antagonists differ dramatically among NMDA receptor subunits. While NR2A- and NR2B-containing NMDA receptors show a high sensitivity to Mg^{2+} blockade, NR2C- and NR2D-containing NMDA receptors present a relatively modest sensitivity [53, 54]. The NR3-containing NMDA receptors possess the lowest sensitivity to Mg^{2+} blockade as they can pass Ca^{2+} current even in the presence of magnesium [58]. iv) Intracellular signaling: The distinct intracellular signaling pathways triggered by different subunit compositions are the most important determinants for the NMDA receptor's involvement in a wide range of physiological and pathological functions [20]. Particularly, NR2A subunits as well as NR2B subunits may trigger several divergent intracellular signaling pathways respectively. Among these signaling pathways, many of them may even be opposing to each other [33, 59, 60].

In ischemic stroke, the involvement of distinct signaling from NR2A and NR2B subunits has been extensively addressed in recent years. For instance, in a study using a four-vessel occlusion model of transient global ischemia in rats, it has been demonstrated that blocking NR2A-containing NMDA receptors enhances neuronal death and abolishes the induction of ischemic tolerance, whereas inhibiting NR2B-containing NMDA receptors attenuates ischemic cell death and enhances preconditioning-induced neuroprotection [61]. However, compared to the clearly specified role of NMDA receptor subunits in modulating synaptic plasticity [20, 59, 62], there is still some debate regarding whether distinguishable effects have been actually caused by NR2A and NR2B subunits through their distinct signaling, particularly during ischemic stroke. For example, it has been suggested that both NR2A- and NR2B-containing NMDA receptors could mediate excitotoxicity in cultured cortical neurons [63]. In addition, other studies have demonstrated that mice deficient in the NR2A subunit of NMDA receptors induces attenuation of focal ischemic brain injury, providing evidence for the involvement of NR2A in excitotoxic mechanisms [64].

NMDA Receptors of Different Subcellular Localizations

A novel hypothesis describing the dichotomy of NMDA receptor signaling has been proposed recently, which focuses on the localization rather than the subunit composition of the NMDA receptor [65]. This hypothesis has suggested that NMDA receptors could be classified according to whether they are located inside the dendritic spine or not, to be either synaptic or extrasynaptic. NMDA receptors on the peripheral membrane surface of the spine neck have been subsequently noted as perisynaptic. This hypothesis is primarily based on numerous reports showing that synaptic NMDA receptors, not necessarily restricted to any subunit type, could promote neuronal survival mainly through activating cyclic-AMP response element binding protein (CREB) function as well as other additional pathways [66, 67]. On the contrary, extra-synaptic NMDA receptors could activate a general but dominant pathway to oppose the effect of synaptic NMDA receptors, resulting in neuronal death [62, 68]. Of considerable relevance, this hypothesis has been further implicated in the pathology of ischemic stroke, consistent with studies showing that glutamate accumulation during ischemic phases could cause neuronal death by triggering extrasynaptic NMDA receptor activation [69, 70]. However, this synaptic versus extrasynaptic proposal has also been challenged by several recently conducted experiments revealing that synaptic NMDA receptors, alone, are capable of mediating excitotoxic neuronal injury [71, 72].

Postsynaptic Mechanisms of NMDA Receptor Signaling

In order to further develop the substantial understanding of the complex signaling processes in excitotoxicity, recent focus has been shifted to elucidating the compartmentalization of NMDA receptor signaling and the involvement of downstream neurotoxic signaling molecules that interact with NMDA receptors. Many of the sophisticated properties of NMDA receptor signaling have thus been substantiated.

The Postsynaptic Density

The postsynaptic terminal of a glutamatergic synapse is characterized in electron micrographs by an electron-dense thickening of the membrane known as the postsynaptic density (PSD)[73-75] (Fig. **1**). Electron microscopy (EM) observations have showed that the PSD forms a disk-like shape with approximately 200-800 nm in diameter and nearly 30-50 nm in thickness extending underneath the postsynaptic membrane [76]. Generally, the PSD component could be described as being composed of four major types of molecules [77-79]: i) Membrane-bound elements, including membrane receptors (mainly ionotropic glutamate receptors [80, 81]), and cell adhesion molecules. ii) Cytoskeletal elements. These mainly consist of cytoskeleton proteins and cytoskeletal regulators, such as actin, fodrin, tubulin and neurofilaments, which are important in localizing and clustering the PSD receptors and signal complexes [82]. iii) Scaffolding proteins. These are abundant components of the PSD, providing multiple functions including trafficking, anchoring and clustering of glutamate receptors; modulating the structure of the postsynaptic spine in an activity-dependent manner; as well as associating various components of the PSD and organizing them into large signaling complexes. Scaffolding proteins are the central organizers of the PSD architecture and the determinants of postsynaptic signaling compartmentalization. Of note, the membrane-associated guanylate kinase protein (MAGUK) superfamily, which comprises four homologous scaffold proteins (also referred to as the PSD-95 family proteins) : synapse-associated protein 90 (SAP90, also called PSD-95 or DLG4), SAP102 (also called DLG3), SAP97 (also called DLG1) and PSD-93 (also called chapsyn-110 or DLG2), act as key scaffolding proteins building up the NMDA receptor signaling machinery. Through these scaffolding proteins, NMDA receptors may interact with many of the prominent proteins in the PSD [83]. Specifically, the PSD-95 family proteins mainly organize the PSD through their common binding sites termed the PDZ (postsynaptic density-95 (PSD-95) / discs large (DLG) / zona occludens-1 (ZO1)) domain. These scaffold proteins are characterized by a tandem of three PDZ domains, where the interaction between the four NR2 subunits and the four members of the MAGUK family can be accommodated. In addition, the scaffold protein Homer, together with Shank (another scaffold protein forming the backbone of the PSD), may link metabotropic glutamate receptors (mGluRs) on the adjacent membrane to their downstream signaling molecules [84]. Interestingly, Shank may also link to the NMDA receptor complex via binding with guanylate kinase-associated protein (GKAP) which in turn binds to PSD-95, thus connecting the NMDA receptor complex to the mGluR complex [85]. iv) Signaling proteins. These PSD proteins may transmit glutamate receptor activity, as well as modulate the signaling properties of glutamate receptors, especially those of the NMDA receptors [86]. Regulation of downstream signaling initiated upon receptor activation requires phosphorylation events carried out by the abundant components in the PSD [87, 88]. Synergistically, these PSD proteins modulate signaling activity and mediate signal transduction into the cell, affecting transcription factors such as CREB, thus eventually regulating the properties of membrane receptors [89], the components of PSD [90, 91] and ultimately cell viability [92, 93].

NMDA Receptor Signaling Complexes

Given these substantial details in the organization of PSD, we draw back our focus to the aforementioned signaling divergence between the NR2A and NR2B subunits again. The NR2 subunits all possess a very large C-terminal domain, in which a type I PDZ interaction motif (T/SXV) is positioned. Consequently, the C-termini of NR2 subunits interact with the first two PDZ domains (PDZ1 and PDZ2) of the PSD-95 family proteins, linking them to downstream signaling molecules [94, 95]. In general, the divergence in signaling properties may result from the fact that the NR2A subunit and NR2B subunit do not share an identical C-terminal structure [96]. This difference leads not only to the varied phosphorylation properties [87, 97], but also the difference in intracellularly-coupled proteins due to their different affinities to various proteins at the PSD (including scaffold proteins such as PSD-95 and other signaling proteins)[28, 98]. The PDZ domains on PSD-95 family proteins provide advantages such as enabling interactions even with relatively weak affinities [99], but such benefit also contributes to promiscuous linking among the abundant components in the PSD [88], making discerning the difference in compartmentalization of NR2A and NR2B signaling difficult. For example, existing evidence has suggested that CaMKII directly binds to the C-terminal of the NR2B subunit but hardly binds to the NR2A subunit [100, 101]. Other studies have demonstrated that CaMKII can interact with both NR2A and NR2B subunits [102, 103]. Besides this, many reports postulate that NR2A subunits preferentially bind to PSD-95, whereas NR2B subunits preferentially bind to SAP-102 [104, 105]. With regard to ischemic stroke, recent studies have showed that NR2B could mediate neurotoxic signaling by interacting with neuronal nitric oxide synthase (nNOS) through binding to PSD-95 [106]. Disturbing the interaction between NR2B subunits and PSD-95 have significantly reduced nNOS activation and attenuated vulnerability to excitotoxicity [107, 108]. Furthermore, another difference in intracellular protein coupling between NR2A and NR2B subunits has been recently identified, which suggests that the synaptic Ras GTPase activating protein (SynGAP) is specifically associated with the NR2B subunit but not the NR2A subunit [66, 109] (Fig. **1**). Both NR2A- and NR2B-containing NMDA receptors could mediate NMDA receptor-dependent ERK activation [66]. However, SynGAP activation could act as a brake on ERK activation [110]. The ERK signaling cascade is believed to be a critical signaling pathway to activate transcription factors such as CREB and promote cell survival [111, 112]. Hence, the NR2A signaling complexes which lack SynGAP could promote ERK activation and lead to cell survival, while the NR2B signaling complexes to which SynGAP is preferentially coupled could inhibit ERK activation thus lead to pro-death signaling pathways. Such differences in NR2A and NR2B subunit signaling compartmentalization elegantly support the hypothesis that NR2A and NR2B have distinct signaling pathways.

NMDA Receptor Dynamics

The dynamics of NMDA receptors is an additional element that further complicates the properties of NMDA receptors. The signaling complexes of NMDA receptors are not fixed, as they change during development and can also redistribute through receptor diffusion. During ischemic stroke, the protein complex could be further altered by ischemic insult. Such NMDA receptor dynamics may directly or indirectly determine the distinct signaling of NMDA receptors in ischemic stroke.

Developmental Changes

During development, The protein complexes of NMDA receptors change dramatically [51, 113, 114]. In general, during the early stages of development after neonatal, synaptic and extrasynaptic NMDA receptors contain mainly NR2B subunits. On the other hand, in late stages of adulthood certain NMDA receptors in both locations change to contain NR2A subunits. At these stages, there is a relatively high proportion of NR2A subunits in the synaptic region and a high proportion of NR2B subunits in extrasynaptic areas, though the exact percentage varies according to the animal model, brain region and time phase [51, 90]. Such changes in distribution of NR2 subunits during development are mainly caused by differential expression of NR2 subunits. The expression of the NR2B subunit is extremely high between the later embryonic stages and neonatal stages, decreases until about 3 weeks after neonatal, and remains at a stable, relatively low expression level. NR2A subunits begin to express during the first week after birth, surpassing the level of NR2B subunits about one week later, and become dominant thereafter [115-117]. With respect to ischemic stroke, the increase in neuronal vulnerability to ischemic insults with advancing maturity may be attributable to this developmental switch [1, 113, 114].

Recent studies concerning the dynamics of NMDA receptors have confirmed such descriptions by revealing further details in the interactions between NMDA receptors and PSD-95 family proteins through development [118]. It has been proposed that SAP102 and SAP97 are highly expressed early in postnatal development, whereas PSD-95 and PSD-93 predominate at later stages [104, 119]. During the assembly of NR2B-containing NMDA receptors in the ER, they form a complex with SAP102, which promotes the membrane targeting of NR2B-containing NMDA receptors [120, 121]. In dendrites, NR2B-containing vesicles travel along microtubules to the cell surface and this transport is mediated by the interaction with a proteic complex including SAP97 and other kinesins [122-124]. In addition to NR2B subunit trafficking, NR2A subunit trafficking seems to be more complicated and highly activity dependent [125, 126]. It has been suggested that NR2A-containing NMDA receptors also interact with SAP97 during assembling in the ER. Contrastingly, the trafficking of the NR2A subunit and its binding to SAP97 is regulated by CaMKII-dependent phosphorylation. CaMKII phosphorylation would induce release of NR2A-containing NMDA receptors from the ER and subsequently disrupt the interaction between the NR2A subunit and SAP97, leading to membrane insertion of the NR2A-containing NMDA receptors [115]. As synaptic activity has a great tendency to cause CaMKII phosphorylation, such specific targeting of NR2A would promote an enriched distribution of NR2A subunits at excitatory locations, with the greatest probability at the center of PSD. Moreover, PSD-95 and PSD-93 are abundant in the PSD, especially due to their high palmitoylation degree [127], while SAP102 and SAP97 are widely distributed in dendrites and axons [83]. Also taking into consideration that PSD-95 is preferentially associated with NR2A, whereas SAP102 is more associated with NR2B, it could be concluded that the NR2B-SAP102 complex in immature synapses tends to be replaced by the NR2A-PSD-95 complex in mature synapses, or even be extruded by the insertion of the NR2A complex and pushed to extrasynaptic sites [128]. This is in agreement with the above suggestion that a high proportion of NR2A subunits are located in the synaptic region while extrasynaptic regions are enriched with NR2B subunits [129, 130]. Therefore, the aforementioned two hypotheses regarding the distinct signaling from NMDA receptors of different subunit compositions and localizations could converge, as NR2A and NR2B subunits recruit distinct signaling complexes, resulting in NR2A subunits preferentially located synaptically and NR2B subunits enriched at the extrasynaptic areas. This difference in signaling compartmentalization dominantly influences the role of NMDA receptors at both synaptic and extrasynaptic locations during ischemic stroke [65, 131, 132].

Receptor Diffusion

Receptor diffusion has recently emerged as a new element of NMDA receptor dynamics, which also has implicative roles in the pathology of ischemic stroke [133]. In principle, receptor redistribution would lead to a rearrangement of the synaptic and extrasynaptic subunit ratio and affect the balance between different signaling pathways [134, 135]. In ischemic stroke, NMDA receptor mislocalization has been reported to be linked with enhanced activation of extrasynaptic excitotoxic signaling cascades [136]. The nature of NMDA receptor diffusion is thermodynamic, as Brownian motion of NMDA receptors has been directly distinguished under microscopic observation [137]. While this thermodynamic motion is intrinsically spontaneous, it could be constrained by several biological processes [133]. For instance, at the PSD where a large number of NMDA receptors are condensed, the diffusion of NMDA receptors is highly restricted due to their interaction with the scaffolding meshwork. PSD-95 could stabilize NMDA receptors within the PSD through several mechanisms such as phosphorylation [138, 139], palmitoylation [140, 141], ubiquitination [142, 143] as well as protein-protein interactions [144-146]. NR2A subunits have more of a tendency to be retained at the PSD compared to NR2B subunits owing to their more concentrated localization at the center of PSD and their higher affinity for PSD-95, which might inversely account for the enrichment of NR2B subunits at extrasynaptic regions [115, 133]. This speculation is also supported by several studies showing NR2B subunits are more mobile than NR2A subunits [128, 147]. However, the retention mechanism for NMDA receptor stabilization could be modulated in an activity-dependent manner [90]. The calcium-dependent inactivation of NMDA receptors is principally mediated by Ca^{2+} influx that could induce α-actin displacement through binding to calmodulin, which subsequently uncouples NMDA receptors from the actin cytoskeleton, resulting in NMDA receptor redistribution to extrasynaptic sites [148-150]. Furthermore, calpain cleavage, a major event triggered by Ca^{2+} in ischemic stroke [151, 152], could regulate synaptic and extrasynaptic NMDA receptor localization via cleavage of the NMDA receptor C-terminus. In ischemic stroke, calpain cleavage has been proposed to be a key event in uncoupling NMDA receptors to their original downstream signaling pathways [153-155].

Lipid Raft: The Potential Extrasynaptic Signaling Platform?

A major contribution of NMDA receptor diffusion might be the recruitment of extrasynaptic signaling complexes [156]. Unfortunately, in contrast to recently formed knowledge about the signaling mechanisms in the PSD, the compartmentalization of extrasynaptic signaling still isn't well understood. The newly emerging concept of a "lipid raft" has been postulated to act as a extrasynaptic signaling platform which organizes different signaling complexes and mediate distinct signaling pathways [157-159]. Moreover, its roles in mediating excitotoxiciy have also been suggested [160-162]. Lipid rafts are dynamic membrane microdomains enriched in cholesterol and sphingolipids [163]. A recent study has evidenced that lipid rafts form highly organized regions on the extrasynaptic membrane, where diffusion of their associated molecules are limited [164-166], making it tempting to speculate that NMDA receptors that diffuse from the PSD might be recruited to lipid rafts. Likewise, several studies have showed that lipid rafts and the PSD share important signaling proteins including NMDA receptors, PSD-95 and other downstream kinases, but with differential organization [167-169]. It has also been reported that raft PSD-95 complexes contain less CaMKIIα and SynGap but enrichment in Src compared with PSD complexes [170]. The recruitment of a raft component has been speculated to be dependent on NMDA receptor activation, whereby signal transduction from raft NMDA receptors drives palmitoylation of PSD-95. This palmitoylation is sufficient to target itself to the raft and subsequently recruit other proteins such as CaMKII. Additionally, many studies have demonstrated that lipid rafts could reversibly diffuse into synaptic areas during ischemic insults, mediated by interactions with PSD-95 [171, 172]. However, the impact of this process on both PSD and raft signaling properties, as well as its role in ischemic stroke is still unknown. Several studies reported that NMDA receptors located in lipid rafts could mediate neurotoxicity [173, 174], whereas NMDA receptors outside of lipid rafts are responsible for glutamate-mediate growth cone guidance [168]. However, research exploring the role of lipid rafts in glutamate signaling events has just emerged in recent years [157, 158]. Although circumstantial evidence has supported the role of NMDA receptors in mediating excitotoxicity in ischemic conditions, many of the underlying mechanisms are yet to be identified.

AMPA/KA Receptor

Normally, AMPA receptors are not Ca^{2+}-permeable and are only thought to be the initiator of NMDA receptor activation, as prior glutamate activation of AMPA receptors would produce sufficient membrane depolarization to relieve the magnesium block of NMDA channels. Unexpectedly, recent studies have identified that AMPA receptors are also involved in mediating toxic levels of Ca^{2+} influx during ischemic stroke [175]. Similar to NMDA receptors, AMPA receptors are tetrameric assemblies comprised of four subunits: GluR1-4 (newly renamed GluA1-4). At mature hippocampal excitatory synapses, AMPA receptors consist primarily of GluA1/GluA2 or GluA3/GluA2 [176]. The GluA2 subunit is preferentially incorporated into the receptor and its presence profoundly determines the impermeability of AMPA receptors to Ca^{2+} [177, 178]. However, when subjected to ischemic stroke, it has been reported that AMPA receptor permeability to Ca^{2+} increases sharply [179]. Recent studies have suggested that ischemic insult could cause down regulation of the GluA2 gene, resulting in expression of AMPA receptors that are GluA2-lacking as well as those that contain unedited GluA2 subunits [180]. This would contribute to AMPA receptor permeability to Ca^{2+} . Studies to date provide strong evidence that GluA2-lacking or GluA2-unedited AMPA receptors have contributed to Ca^{2+}-mediated excitotoxic cell death [181, 182].

KA receptors are made up of five subunits: recently renamed GluK1-5 [183]. The properties of KA receptors are quite similar to AMPA receptors, in that they induce ion flux upon glutamate activation and are mostly impermeable to Ca^{2+}, and can be found both presynaptically and postsynaptically [184]. Functional studies have suggested that they mainly have a regulatory role in synaptic transmission rather than being the major ion transmitter receptor. Presynaptically, KA receptors are able to modulate presynaptic glutamate release, while postsynaptically, they may serve an analogous function to AMPA receptors in alleviating the Mg^{2+} blockade on NMDA receptors [185, 186]. Ca^{2+}-permeable KA receptors also exist [187], and together with Ca^{2+}-permeable AMPA receptors, they have been suggested to played a key role in mediating excitotoxicity among glial cells, causing destruction to myelin and white matter injuries in ischemic stroke [188, 189]. Studies have shown that Ca^{2+}-permeable AMPA/KA receptors expressed on oligodendrocytes have contributed to their high vulnerability to ischemic conditions [190, 191].

Metabotropic Glutamate Receptor

Metabotropic glutamate receptors are coupled to G proteins, and may mediate slow synaptic responses once activated. The mGluRs are built of eight subunits: mGluR1-8, and can be further classified into three groups: group I, II and III. Group I metabotropic glutamate receptors include mGluR1 and mGluR5. Activation of group I mGluRs are linked via G-proteins to the activation of phospholipase C, whose downstream effects include inositol triphosphate production and subsequent intracellular calcium release [192, 193]. They can also modulate excitatory postsynaptic potentials in a G-protein-independent fashion via tyrosine kinases [194]. Group II mGluRs include mGluR2 and mGluR3, while the remaining mGluRs all belong to the third group of mGluRs. The group II and III mGluRs are similar in their negative association with adenylyl cyclase signaling, which could modulate calcium channel influx [195]. With regard to ischemic stroke, the group I family of mGluRs appears to potentiate postsynaptic NMDA receptors [196], while the others are primarily located presynaptically and their activation could protect neurons against excitotoxic insult by reducing Ca^{2+} influx through NMDA receptors [197].

SYNAPTIC GLUTAMATE SIGNALING IN ISCHEMIC STROKE

The nature of ischemic stroke is a deprived energy supply to the brain, primarily caused by cerebral artery occlusion. The human brain is extremely vulnerable to such energy deprivation particularly because of the constraints of the elaborate signaling of synapses. It has been estimated that the human brain, while representing only 2% of the body weight, consumes nearly 20% of the whole body energy expenditure, whereas approximately 75% of the brain’s energy has been spent on events related to synaptic transmission and signal processes [198]. Hence, upon the onset of ischemic stroke, the extravagant energy consumption of synaptic signaling would severely constrain neuronal viability. During an ischemic episode, a hallmark cellular response event is the malfunction of Na^+/K^+-ATPase caused by an insufficient energy supply [199]. The failure of Na^+/K^+-ATPase leads to a profound loss of ionic gradients, subsequently followed by uncontrolled membrane depolarization in neurons as well as astrocytes [200]. Such massive degradation of ionic concentrations across the plasma membrane results in activation of voltage-gated calcium channels (VDCCs). Consequently, this unchecked intracellular Ca^{2+} elevation then initiates excessive release of presynaptic neurotransmitters, particularly glutamate [6, 9, 201].

Synaptic Glutamate Receptor-Mediated Ionic Imbalance

The extensive and sustained release of presynaptic glutamate evokes pathophysiological excitatory effects through the activation of postsynaptic glutamate receptors, especially AMPA and NMDA receptors. Prolonged activation of these ionotropic glutamate receptors would induce floods of ions and lead to a drastic elevation in the intracellular ionic concentration, especially with regard to Na^+ and Ca^{2+} [5, 6]. Therefore, glutamatergic transmission in ischemic stroke would bring about a devastating disturbance in postsynaptic ionic gradients, not only resulting in depolarization of the postsynaptic neurons, causing further release of glutamate and initiating “self-propagation” of excitotoxicity among neighbouring cells, but also aggravating energy consumption within the postsynaptic unit and accelerating cell death through toxic ion influxes (Fig. **2**). The insult of toxic ion influx following ischemic stroke can be described by two distinguishable stages. The first stage is marked by a rapid necrosis-like cell swelling, which is mainly Na^+-dependent. Cell swelling can be damaging on its own, while in addition it can alter the properties of glutamate receptors, leading to a greater ion influx upon glutamate activation of these receptors. For example, it has been demonstrated that the voltage-dependent Mg^{2+} blockade of NMDA receptors is weakened in mechanically injured neurons [202]. Besides, it has also been reported that AMPA-mediated currents are enhanced by applying stretch to neurons [203]. Apart from mechanical damage, intracellular accumulation of Na^+ would also reverse the activity of the Na^+/Ca^{2+} exchanger (NCX), leading to increased Na^+ efflux in exchange with Ca^{2+} influx [204]. High doses of Na^+ could also increase the activity of the Na^+/H^+ exchanger, resulting in H^+ overload and cellular acidification, which could in turn activate acid-sensing ion channels and permit yet more Ca^{2+} influx [205]. All of these mentioned events in this stage contribute to the rise of intracellular Ca^{2+}. Together with the Ca^{2+} flow through synaptic ionotropic glutamate receptors, the second wave of the insult thus forms, which is a delayed, predominantly Ca^{2+}-mediated cell degeneration [206-208].

As the cytoplasmic calcium concentration reaches non-physiological levels, the impaired intracellular calcium homeostasis will lead to activation and overstimulation of proteases, lipases, phosphatases and endonucleases. Alterations in activity of these enzymes could destroy the cell structure, resulting in a more severe alteration of the properties of membrane channels, and allowing even more toxic Ca^{2+} access to the neuronal cytoplasm. A significant event as a direct consequence of such overloading Ca^{2+} is mitochondrial dysfunction [209, 210]. Mitochondria are very important for cell survival, oweing to their capacity to store Ca^{2+} until the plasma membrane Ca^{2+}-ATPase succeeds in reducing cytoplasmic Ca^{2+} down to physiological levels. Tragically, it has also been reported that Ca^{2+}-ATPase can be cleaved by calpain or caspase during ischemic stroke, contributing to Ca^{2+} overload [211, 212]. Ca^{2+} is sequestered into the mitochondria matrix via a proton electrochemical gradient generated by the electron transport chain [213]. However, abnormal Ca^{2+} accumulation by mitochondria could depolarize the mitochondrial potential and decrease the electrochemical gradient, resulting in reduced ATP synthesis in mitochondria [214]. Concurrent with the desperate ATP consumption during ischemic stroke, these consequences synergistically contribute to the depletion of cellular ATP, thus sealing the fate of these cells. Despite the breakdown of cellular energy production, aberrations caused by prolonged Ca^{2+} accumulation in mitochondrial electron chain functioning would lead to excessive production of reactive oxygen species (ROS), which is another major force of excitotoxic insult during ischemic stroke [215, 216]. Finally, the devastating Ca^{2+} overload will initiate apoptosis via mitochondrial release of cytochrome c. The release of cytochrome c is mediated through the sustained opening of mitochondrial permeability transition pore (mtPTP), and once it is released, couples with the apoptosis protease-activating factor-1 (Apaf-1), which subsequently recruits and leads to the autoactivation of caspase-9 and caspase-3, to execute apoptotic cell death [217-219].

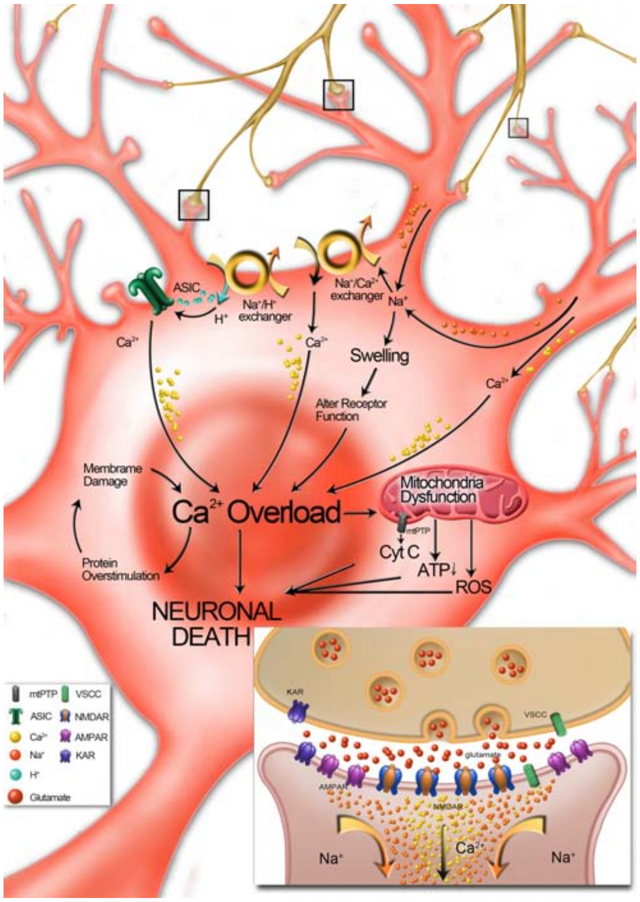

Fig. (2). Synaptic glutamate signaling-mediated ionic imbalance.

Upon the onset of ischemic stroke, energy depletion results in loss of normal membrane potential and excessive release of glutamate from presynaptic terminals (branches illustrated in isabelline). Excessive glutamate release causes overactivation of ionotropic glutamate receptors (N-methyl-D-aspartate receptor, NMDAR; α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor, AMPAR; and kainate receptor, KAR) on the postsynaptic membrane, inducing large amounts of Na^+ and Ca^{2+} influx into the postsynaptic neuron (illustrated in the part section view). Besides, Ca^{2+} also enters through voltage-sensitive calcium channels (VSCC). Drastic elevation of intracellular Na^+ causes rapid cell swelling, and also leads to further Ca^{2+} influx through swelling-induced receptor function alteration, Na^+/Ca^{2+} reversal, and Na^+/H^+ exchanger-mediated acid-sensing ion channel (ASIC) activation. Subsequently, Ca^{2+} overload overstimulates intracellular proteases, lipases, phosphatases and endonucleases, damaging the membrane and allowing more Ca^{2+} access. Furthermore, Ca^{2+} overloadcauses mitochondria dysfunction, resulting in neuronal death through initiating apoptosis by releasing cytochrome c (cyt c) through mitochondrial permeability transition pore (mtPTP), decreasing ATP synthesis and generating reactive oxygen species (ROS). As the cell gradually decreases its ATP levels and viability, it loses its normal membrane potential and repeats the excessive release of endogenous glutamate, thus propagating such synaptic glutamate signaling-mediated ionic imbalance and neuronal injury. This process is termed excitotoxicity [6, 7, 9].

The innocence of AMPA receptors in mediating excitotoxicity has been recently denied. AMPA receptors transduce fast excitatory postsynaptic responses to alleviate NMDA receptors' Mg^{2+} blockade, and they are highly kinetic, rapidly cycling in and out of the postsynaptic site [86, 178]. Their dynamic equilibrium is determined by a balance of endocytosis, exocytosis and lateral diffusion [220]. Physiologically, AMPA receptors are delivered to the surface at extrasynaptic sites. Upon NMDA receptor activation and Ca^{2+} influx, the lateral diffusion of AMPA receptors is enhanced, thus promoting the synaptic localization of AMPA receptors [178]. It has been suggested that the insertion of GluA1-containing AMPA receptors is regulated by NMDA receptor activation, while NR2A-containing NMDA receptors promote surface delivery of GluA1; NR2B-containing NMDA receptors inhibit this process [66, 221, 222]. Therefore, it could be said that overactivation of synaptic NMDA receptors would, at least transiently, lead to a rise in synaptic AMPA receptor numbers, inducing larger amounts of Na^+ influx. Furthermore, AMPA receptors are normally Ca^{2+}-impermeable, because of the pervasive incorporation of the GluA2 subunit. However, it has been demonstrated that 24-72 hours following ischemic insult, GluA2 protein is down-regulated [223] and deficiencies in mRNA editing of GluA2 occur [224]. This is presumably due to ADAR2 (the enzyme responsible for RNA editing of GluA2 subunit) cleavage and degradation caused by ischemia and NMDA receptor overactivation [180, 225]. Hence, the subsequent delivery of GluA2-lacking or GluA2-unedited AMPA receptors would result in the surface expression of Ca^{2+}-permeable AMPA receptors at post-ischemic stages, which significantly contribute to the glutamate receptor-mediated Ca^{2+} overload [180, 226]. Additionally, Ca^{2+}-permeable AMPA receptor-mediated excitotoxicity through the c-Jun N-terminal kinase (JNK) pathway has also been reported [227].

Neuroprotective Roles of Synaptic NMDA Receptor Activation and Ischemia-Induced Failure of Neuroprotection

As mentioned above, the clustering of the glutamatergic synaptic densities is, at least partially, dependent on excitatory stimulation during synapse development. Synaptic activity offers the tendency of promoting more components to be localized within the excitatory area, especially NR2A-containing NMDA receptors. Therefore, it seems reasonable to suspect that synaptic glutamatergic signaling would intrinsically be associated with pro-survival signaling pathways upon excitatory transmission. Indeed, recent studies have discovered several mechanisms that synaptic signaling could serve to preserve neuronal function and protect themselves against ischemic insults, most of which are mediated by synaptic NMDA receptors [62, 65]. The enhancement of cellular defense provided by NMDA receptor activity generally includes posttranslational modifications of proteins, *de nevo* gene expression, as well as protection against oxidative stress.

In principle, pro-survival signaling triggered by synaptic NMDA receptors are dependent on Ca^{2+} influx (Fig. **3**). Among all the signaling pathways, the activation of the transcription factor CREB is a predominant event that accounts for the protective effects of NMDA receptor activity [228-230]. CREB could be activated in two ways: i) Ca^{2+} influx through NMDA receptors that activate CaMKII and CaMK kinase (CaMKK), which in turn activate the nuclear Ca^{2+}/calmodulin dependent protein kinases IV (CaMKIV)[231, 232]. ii) Ca^{2+} entrance triggers the activation of the Ras/ERK pathway, which is mediated by the C-terminus interaction of NR2 subunits with downstream signaling complexes [233]. CaMKIV produces fast CREB phosphorylation, whereas the ERK1/2 pathway promotes CREB phosphorylation in a slow and sustained manner that could last beyond the episode of NMDA receptor activation [234]. In addition, calcineurin-dependent nuclear import of the transducer of regulated CREB activity (TORC), which is also supported by synaptic NMDA receptor-induced Ca^{2+} influx [235, 236], may assist the recruitment of CREB to its coactivator CREB binding protein (CBP) and initiate the activation of CREB [65, 237]. CREB activation then targets several activity-regulated inhibitors of death genes among the pool of nuclear Ca^{2+}-regulated genes [238]. This CREB-dependent gene expression could provide neurons with a long-lasting phase of protection against apoptotic insults during ischemic stroke, generally through rendering mitochondria a stronger resistance to cellular stress and toxic insults [221, 239, 240], as well as suppressing apoptotic cascades [240, 241]. Another potential candidate of synaptic Ca^{2+}-CREB signaling is Bdnf, the gene that encodes the brain-derived neurotrophic factor (BDNF)[242]. BDNF is known to have many neuroprotective properties [243] and could rescue neurons during ischemic phases [244, 245]. It has been reported that sustained CREB activation within different neuronal populations could protect them against ischemic death [246, 247]. Activation of CREB mediated by NMDA receptors in response to transient ischemic conditions has also been proposed to contribute to the establishment of ischemic tolerance [248, 249].

In addition to CREB activity, the PI3K (phosphoinositide-3-kinase) - Akt kinase cascade is another important neuroprotective signaling pathway triggered by synaptic NMDA receptors [250]. Ca^{2+}/calmodulin activated by NMDA receptor-mediated Ca^{2+} influx can further activate PI3K, which in turn catalyses the phosphorylation of the lipid PIP2 (phosphatidylinositol 4,5-biphosphate) to PIP3 (phosphatidylinositol 3,4,5-triphosphate) in the membrane, which then recruit PDK1 (phosphoinositide-dependent protein kinase) and its substrate Akt/PKB (protein kinase B) through their interactions with PIP3, subsequently triggering the phosphorylation and activation of Akt [62]. Synaptic NMDA receptor activity carries out sustained activation of the Akt pathway that could lead to neuronal survival and growth mainly via promoting the translocation of the nuclear FOXO (forkhead box O) subfamily [65]. Export of FOXOs could inactivate several death genes as well as pro-apoptotic signaling pathways, such as p53 [251], Apaf-1 [240], Bcl-2 family members [252], and the JNK/p38 pathway [253].

Moreover, as far as neuronal antioxidant defense is concerned, synaptic NMDA receptor activity could shield neurons from oxidative insults by triggering alterations of the thioredoxin-peroxiredoxin system [254]. It has been reported that synaptic activity enhances thioredoxin activity, which facilitates the reduction of overoxidized peroxiredoxins and could promote resistance to oxidative stress. This enhancement in oxidative defense has been attributed to a coordinate program of gene expression changes mediated by synaptic NMDA receptor activity [65].

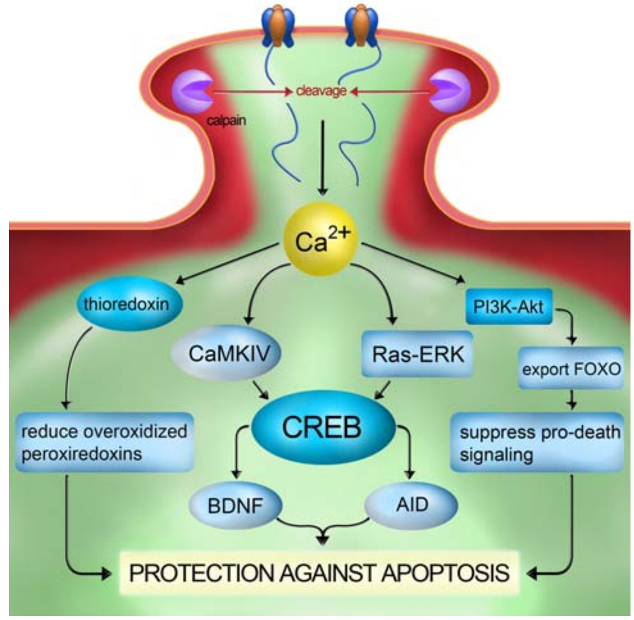

Fig. (3). Synaptic NMDA receptor-mediated neuroprotection.

Intrinsically, Ca^{2+} influx through synaptic NMDA receptors evokes several pro-survival signaling pathways to shield the neuron from dangers, which include: cyclic-AMP response element binding protein (CREB) activation that activates activity-regulated inhibitors of death genes (AID) and brain-derived neurotrophic factor (BDNF); the PI3K-Akt pathway that suppresses several pro-death signaling pathways by sustained export of nuclear forkhead box O (FOXO) subfamily proteins; and enhanced thioredoxin activity that facilitates reduction of overoxidized peroxiredoxins [62, 65]. However, overstimulation of synaptic NMDA receptors could lead to activation of calpain (illustrated in purple), which cleaves the C-terminus of NMDA receptors and uncouples its pro-survival signaling pathways [255, 256].

However, recent findings have revealed that upon ischemic stroke, calpain activation could result in a rapid and significant uncoupling of synaptic NMDA receptors from downstream survival pathways [255, 256]. Cleaved NR2 subunits remain associated with NR1 at the postsynaptic membrane, but no longer possess the ability to defend neurons against the lethal ischemic insults, which has been a newly proposed mechanism for mediating excitotoxicity. In spite of this discouraging fact, when novel treatment strategies for ischemic stroke are being designed the effect on NMDA receptor pro-survival signaling is worthwhile to keep in mind and should be carefully assessed if the strategy involves intervention in NMDA receptor function.

Synaptic Glutamate Signaling-Produced Neurotoxins

Conversely, excessive activation of synaptic NMDA receptors leads to production of toxic levels of ROS, which could exacerbate ischemic damage by triggering several devastating downstream effects. In fact, it has been well established that during ischemic stroke, a pronounced elevation of nitric oxide (NO) occurred following NMDA receptor activation [106, 257]. As described above, nNOS is concentrated near the NMDA receptor complex at the postsynaptic sites, through tethering to PSD-95. Under physiological conditions, low concentrations of NO synthesized by nNOS act as signal transmission molecules, which also play a role in neuroprotective effects [258, 259]. However, during ischemic stroke, the activity of nNOS is dramatically upshifted due to overactivation of NMDA receptors, producing high levels of NO [260]. Furthermore, overproduction of NO, together with the intracellular accumulation of ROS resulting from the aforementioned Ca^{2+} overload-mediated mitochondria dysfunction, such as superoxide anions (O_2^-) may combine, transforming superoxide into peroxynitrite ($ONOO^-$) - a powerful oxidative molecule [261-263]. These highly reactive molecules could cause membrane damage by lipid peroxidation [264], DNA strand breakdown by base deamination [265, 266], and DNA replication impairment by inhibiting ribonucleotide reductase [267]. In addition, NO/ONOO- could also suppress several mitochondrial respiratory chain enzymes, thus accelerating energy depletion in neurons [268].

The devastating role of ROS in ischemic stroke goes even beyond this primary damage. It has been recently discovered that ROS generation in excitotoxicity can promote the activation of transient receptor potential (TRP) channel superfamily members TRPM2 and TRPM7 [269]. These TRP channels are Ca^{2+} permeable and could induce further Ca^{2+} flow into the cell, aggravating the crisis of Ca^{2+} overload and oxidative stress during ischemia [270]. In addition to calcium, the TRPM7 channel has recently been reported to allow the entry of another divalent ion, zinc [271]. Zinc could also gain entry through ion channels or membrane transporters [272], or could be released endogenously via oxidative mechanisms [273]. Elevation of intracellular zinc levels have been associated with a number of deleterious effects, such as inhibiting energy metabolism [274, 275], increasing nNOS activity [276] and causing alterations in intracellular signaling [277]. Besides, there is evidence suggesting that AMPA/KA receptor activation could also lead to the formation of ROS [278] and induce Zn^{2+} influx [279, 280]. Taken together, these above mentioned mechanisms all provide positive feedback for lethal excitotoxic assaults and ROS-dependent cellular injuries (Fig. **4**).

More recently, it has been clarified that the NR2B subunit is mainly responsible for the NMDA receptor signaling to NO production in excitotoxicity. As discussed above, the C-terminal domain of NR2 subunits are divergent. Recent studies have revealed that during NMDA receptor-mediated excitotoxicity, NR2B subunit C-terminal domains could enhance toxic signaling, compared to NR2A subunit C-terminal domains, by exhibiting stronger coupling to the PSD-95-nNOS pathway [281]. Although we previously emphasized the enrichment of NR2A subunits at the PSD, the existence of NR2B subunits is not excluded [282, 283]. Additionally, nNOS is known to be intricately localized around postsynaptic sites [284], and promiscuously linked to NMDA receptor signaling complexes [277]. Interestingly, it has also been reported that the subcellular localization of nNOS is changed when subjected to glutamate overstimulation, indicating the possibility that the increased nNOS activity is due to the differential nNOS subcellular localization elicited by ischemia [284]. Despite all these unclear details, treatment of stroke by perturbing NR2B-containing NMDA receptor interaction with PSD-95 (which potentially blocks nNOS activation) has proved to be a promising therapeutic strategy [107, 108, 285]. Concurrently, this has been further supported by a finding that such neuroprotective effect is achieved exclusively by disturbing the interaction of the NR2B subunit C-terminal with PSD-95 and nNOS, rather than any other PDZ-domain containing proteins [286], indicating a surprising specificity of the PSD-nNOS pathway in excitotoxicity [88].

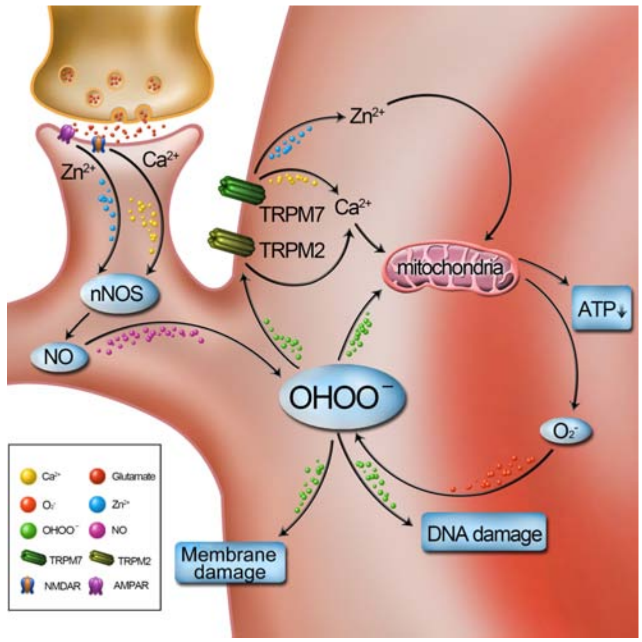

Fig. (4). Synaptic glutamate signaling-produced neurotoxins.

Overactivation of synaptic NMDA receptors (NMDAR) leads to excessive production of nitric oxide (NO) from the neuronal nitric oxide synthase (nNOS). This together with superoxide anions (O_2^-) generated from mitochondria dysfunction, forms the powerful peroxynitrite ($OHOO^-$). These reactive oxygen species (ROS) damage the membrane, DNA, and suppress several mitochondrial respiratory chain enzymes. To make things worse, these ROS assaults result in a positive feedback loop, by activating transient receptor potential (TRP) channel family members TRPM2 and TRPM7, which permit additional Ca^{2+} and Zn^{2+} influx that could further boost nNOS activity and aggravate mitochondria dysfunction. Zn^{2+} can also gain entry through synaptic AMPA receptors (AMPAR).

EXTRASYNAPTIC GLUTAMATE SIGNALING IN ISCHEMIC STROKE

Meanwhile, besides invading through the synaptic gate, toxic levels of glutamate simultaneously accumulate in extrasynaptic spaces. Normally, the CNS possesses a powerful glutamate uptake mechanism to control the extracellular concentration and prevent accumulation, which is primarily carried out by plasma-membrane glutamate transporters [287]. However, this transport process is mainly Na^+-dependent, which couples the co-transport of three sodium ions and a proton, as well as counter-transport of a potassium ion down their respective electrochemical gradients. During ischemic stroke, due to the aforementioned ionic imbalance and membrane depolarization caused by energy failure and synaptic transmission, the ionic gradients and membrane potential that power glutamate uptake against its electrochemical gradients are disrupted. In principle, the shift in ion gradients and membrane potential could result in reverse operation of the transporter and release glutamate into extracellular spaces [288]. Although excessive synaptically released glutamate also would lead to glutamate spillover into extrasynaptic areas through diffusion [289], it has been suggested that reverse uptake is the predominant process responsible for the accumulation of extrasynaptic glutamate, especially in severe brain ischemia [290-292]. Such accumulation of extrasynaptic glutamate is disastrous, for it could directly lead to the death of neurons, especially through the vicious activation of extrasynaptic NMDA receptors (Fig **5**).

Extrasynaptic NMDA Receptor-Mediated Pro-Death Signaling

To date, it has been the consensus that under many pathological conditions, overactivation of extrasynaptic NMDA receptors explicitly sets off several pro-death signaling pathways, many of which could even override the effect of synaptic NMDA receptor pro-survival signaling [20, 33, 65].

Above all, extrasynaptic NMDA receptor activity is coupled to a dominant CREB shut-off pathway [68], as CREB could undergo sustained activation by synaptic NMDA receptors [293]. So far, two sets of mechanisms discovered recently could provide an insight into this dichotomy. The first one is centered on juxtasynaptic attractor of caldendrin on dendritic boutons protein (Jacob)[294], a binding partner of the neuronal Ca^{2+} binding protein caldendrin (which itself is a PSD component). Jacob could cause CREB dephosphorylation when inside the nucleus, yet caldendrin controls this synapse-to-nuclear signaling by competing with Jacob's binding of importin-α, which is necessary for nuclear localization of Jacob. Such competition requires high levels of Ca^{2+} and hence is confined to the postsynaptic Ca^{2+} microdomain. Adversely, the activation of extrasynaptic NMDA receptors, which are predominantly NR2B-containing, fails to support this competition and consequently promote the nuclear accumulation of Jacob. The second mechanism concerns the ERK1/2 pathway, which can sustain the activation of CREB. It has been well documented in recent years that extrasynaptic NMDA receptor signaling can cause an overriding inactivation of ERK that was previously activated by synaptic NMDA receptors [131, 293]. This divergence could also be attributed to the fact that NR2B-containing NMDA receptors are abundant in the extrasynaptic region, and intrinsically, NR2B subunits are preferentially associated with the protein SynGAP, which acts as a brake on Ras-ERK signaling, as discussed previously [66]. Therefore, activation of extrasynaptic NMDA receptors can lead to the inhibition of the Ras-ERK signaling pathway and the subsequent inactivation of CREB.

In addition to CREB shut-off, other deadly effects evoked by extrasynaptic NMDA receptor signaling may include: i) nuclear import of FOXO [295, 296], because extrasynaptic NMDA receptor activity fails to provide sustained Akt activation, but instead promotes the translocation of nuclear FOXO and the associated death signaling (see above). ii) Cleavage of striatal enriched tyrosine phosphatase (STEP)[297]. STEP has been known to regulate two opposing signaling proteins, ERK1/2 and p38 (also see above), and it has been reported recently that synaptic stimulation is concomitant with ERK activation, whereas extrasynaptic stimulation invokes calpain activity and causes p38 activation because of the truncated cleavage product of STEP. iii) Overexpression of the pro-death gene Clca1 [298], which encodes a putative calcium-activated chloride channel, has been proposed recently to be involved in excitotoxicity mediated by extrasynaptic NMDA receptors.

Several recent studies have also shown additional NR2B subunit specific signaling pathways in ischemic neuronal injuries, which involve the dysfunction of PTEN-induced kinase-1 (PINK1)[299], a protein upstream of Akt, and the activation of the sterol regulatory element binding protein-1 (SREBP-1) transcription factor [300], which may regulate cellular cholesterol and lipid biogenesis [301]. These elements could presumably be involved in extrasynaptic glutamate signaling during ischemic stroke, but only on the assumption that NR2B-containing NMDA receptors are enriched at extrasynaptic areas. However, this mechanism still needs to be further elucidated.

Making it even worse, a more recent study has reported that extrasynaptic NMDA receptor activity is boosted during cerebral ischemia [302]. The authors postulated that despite an elevation of extracellular glutamate concentration, the channel conductance of NR2B-containing NMDA receptors at extrasynaptic locations could be enhanced via intracellular signaling cascades, which are constitutively mediated by the recruitment of death-associated protein kinase 1 (DAPK1) into the NR2B protein complex. DAPK1 selectively binds to the NR2B subunit C-terminal and phosphorylates the receptor, leading to enhanced channel conductance of Ca^{2+}. Genetic depletion of DAPK1 or disrupting DAPK1-NR2B interaction blocked the toxic Ca^{2+} influx through extrasynaptic NMDA receptors. Taken together, these results consistently suggest the explicit role of extrasynaptic NMDA receptor signaling in ischemic neuronal injuries.

Astrocytic Regulation of Glutamate Signaling in Ischemic Stroke

Astrocytes are the major type of glia cell distributed in the CNS, and also the most pervasive neighbour of neurons [303]. They play an important role in controlling the ambient glutamate concentration, as well as providing nutrients for neurons under both physiological and pathological conditions [304-306]. In fact, astrocytes are less susceptible to ischemic damage than neurons [200], generally because astrocytes have a relatively low density of ionotropic glutamate receptors, so they don't have to draw extra energy for signaling processes. Besides this, astrocytes also contain a larger energy substrate storage than neurons (i.e. glycogen storage) [307]. Therefore, during an ischemic episode, astrocytes can maintain their cellular ATP levels and membrane potentials longer than neurons. Consequently, their retained metabolism could protect neurons from glutamate excitotoxitcy during ischemia for a prolonged period through their powerful

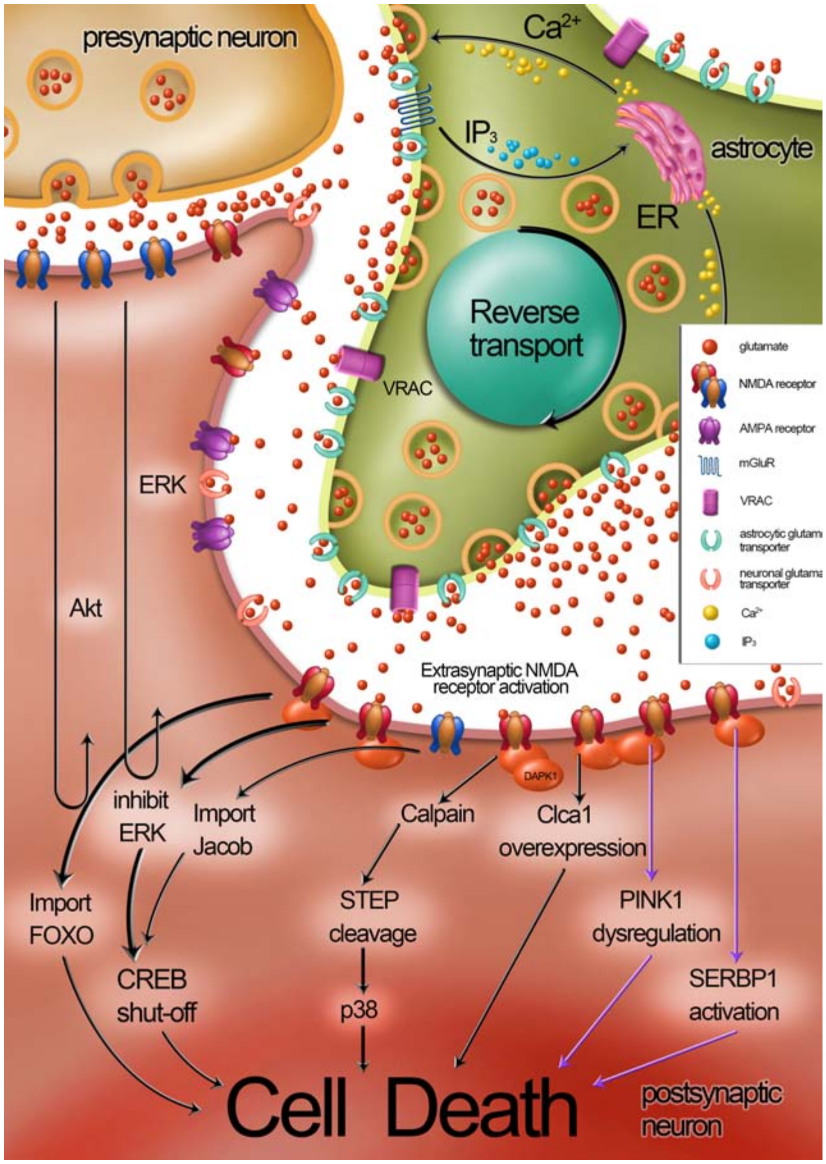

Fig. (5). Extrasynaptic glutamate signaling-mediated pro-death signaling.

Glutamate transporter reversal is the main cause of extracellular glutamate accumulation, whereas synaptic glutamate spillover also contributes partly. Astrocytes persist in glutamate uptake for a longer period than neurons during an ischemic episode, until they eventually fail to maintain their ATP levels and cellular ionic gradients. Consequently, astrocytes reverse transport the glutamate previously taken up, throwing this glutamate back into extracellular spaces inversely. Astrocytes also release glutamate through swelling-induced opening of volume-regulated anion channels (VRACs), as well as through a mechanism similar to synaptic vesicle exocytosis, which is mediated by intracellular Ca^{2+} elevation due to Ca^{2+} release from the endoplasmic reticulum (ER). Extracellular glutamate accumulation preferentially triggers the catastrophic activation of extrasynaptic NMDA receptors. Additionally, extrasynaptic NMDA receptor activity is enhanced during ischemic insults due to the recruitment of death-associated protein kinase 1 (DAPK1) to the C-terminal of the NR2B subunit [302]. Overactivation of extrasynaptic NMDA receptors explicitly sets off several pro-death signaling pathways (illustrated in black arrows), many of which could override synaptic pro-survival signaling (Akt and ERK activation) [20, 62, 65] (illustrated in two thick arrows originating from the extrasynaptic site which overrides two thin arrows originating from the synaptic site). Besides this, as NR2B-containing NMDA receptors are enriched at extrasynaptic regions, some additional NR2B subunit specific pro-death signaling might also be involved in extrasynaptic glutamate signaling-mediated ischemic neuronal injuries (NR2B subunit specific signaling pathways are illustrated in purple arrows).

glutamate uptake mechanism, and also by supplying neuronal energy requirements [308]. However, it has been recently characterized that under severe ischemic conditions, astrocytes can no longer protect their ailing neighbors, but instead release more glutamate into the extrasynaptic space through a variety of mechanisms, contributing to the eventual death of neurons.

Recent studies have revealed that at the early stages, reversed glutamate uptake mainly occurs at neuronal glutamate transporters [309], while astrocytes persist in taking up glutamate [310, 311]. However, as severe ischemia continues, astrocytes cannot afford such uptake any more due to the energy consumption required to sustain an ionic gradient, as well as to convert glutamate to glutamine [312]. Eventually, as the accumulation of intracellular Na^+ and glutamate increases [313], glutamate transporters on astrocytes succumb and operate backward. Thus the flood gates of astrocytic glutamate open, expelling the unconverted glutamate they have previously uptaken into extracellular space. Another mechanism evoked by ischemic stroke to cause glutamate release from astrocytes is mediated by the volume-regulated anion channels (VRACs)[314]. Because astrocytes also suffer greatly from swelling, presumably due to the aforementioned ionic imbalance, their endowed VRACs will open in response to swelling, releasing the previously absorbed anions, and also glutamate. As a corresponding consequence, astrocytic VRACs significantly contribute to the accumulation of extracellular glutamate [315]. Moreover, it has been suggested that the function of astrocytic glutamate transporters could be impaired by oxidative stress during ischemic stroke [316], and that the expression of glutamate transporters is also down-regulated due to ischemia-induced excessive activation of astrocytes [317, 318]. It is worth noting that a significant portion of extrasynaptic NMDA receptors are located adjacent to glial processes [319]. Therefore, it could be indicated that astrocytic reversed uptake would release glutamate that preferentially activates extrasynaptic NMDA receptors, triggering the death of their neighbours [292].

In addition to the ischemia-induced specific sources of glutamate release, an intrinsic mechanism of astrocytic glutamate release has been recently elucidated, which found out that astrocytes could release endogenous glutamate in response to the synaptically released glutamate [320]. Interestingly, this recent breakthrough in understanding glia-neuron interactions could revolutionize the classical pre- and post-synaptic model into a tripartite synapse system, although such a topic is currently still under heated debate [321-323]. As one of the dogmas of classical synaptic signaling theory, it has been believed that astrocytes are not involved in synaptic transmission because they neither fire action potentials nor have significant responses to synaptic neurotransmitter release, despite the fact that their processes could intimately contact both pre- and post-synaptic terminals [324]. However, recent studies suggested that the group I metabotropic glutamate receptors distributed on astrocytes could be activated by synaptically released glutamate [323, 325]. Based on an exhaustive amount of experimental data [322, 323, 326], it has been proposed that upon activation of astrocytic mGluRs, phospholipase C hydrolyzes the membrane lipid phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol and IP_3 (inositol 1,4,5-trisphosphate), leading to IP_3 receptor activation and Ca^{2+} release from the endoplasmic reticulum. Astrocytic intracellular Ca^{2+} elevation would then presumably cause astrocytic glutamate release in a mechanism similar to synaptic exocytosis. Thus, astrocytes could respond to synaptically-released glutamate by intracellular Ca^{2+} elevation that in turn would set off the release of further glutamate from astrocytes. Glutamate released under this mechanism could not only activate extrasynaptic NMDA receptors, but also directly participate in the regulation of synaptic signaling [327-329], as the released glutamate could also diffuse and bind to presynaptic mGluRs or NMDA receptors on neighboring presynaptic terminals. Glutamate activation of these presynaptic receptors could modulate Ca^{2+} influx into the presynaptic terminal, affecting the residual Ca^{2+} level, which could potentially be a key factor regulating synaptic transmission [330]. Although recent evidence has put into doubt the ability of astrocytes to release glutamate under physiological conditions [331, 332], it has been speculated that the severe rise in astrocytic Ca^{2+} during ischemic stroke would likely favor the induction of astrocytic glutamate release through this mechanism, as well as its consequential activation of extrasynaptic NMDA receptors [308]. Yet its role in regulating synaptic transmission is still under investigation and the exact effect of this astrocytic glutamate release during ischemic stroke remains to be investigated.

CONCLUDING REMARKS

This review has surveyed a series of synaptic and extrasynaptic glutamate signaling cascades that lead to neuronal death and cerebral injuries during ischemic stroke. Generally, after the onset of ischemic stroke, the initial charge upon brain cells is a Na^+-mediated ionic imbalance, caused by energy failure and a massive amount of uncontrolled synaptic transmission. Followed by this is the second wave, which is a Ca^{2+}-dependent neurodegeneration, while the subsequent generated ROS and neurotoxins mediate the third assault. Finally, as glutamate accumulates extracellularly mainly due to reversed glutamate transport and astrocytic glutamate release, the overactivated extrasynaptic NMDA receptors will doom neurons to their demise.

Many of the discussed signaling pathways might also occur under physiological conditions, but are simply overstimulated during ischemic stroke. Perhaps this has exactly reflected the very nature of ischemic stroke, whereby the devastating functional aberration is not caused by the CNS itself, but rather is a direct result of the deprived energy supply which pushes the metabolism of the CNS to its limitations, and eventually triggers the catastrophic cascade leading to its own destruction. This might also account for the reason why reinstating the blood supply seems to be the only effective treatment so far, as blocking glutamate signaling or blocking certain components of glutamate signaling (i.e. blocking NMDA receptors) bring about unpredictable side effects due to their pivotal roles in many physiological events. These straightforward blocking strategies would disturb the intrinsic systematic balance and increase the burden on the CNS, rather than protecting it.

Nevertheless, there is no doubt that many injurious effects in ischemic stroke are directly caused by glutamate signaling. But the question is how to deftly suppress this injurious signaling during stroke onset while maintaining a low influence on normal neurophysiological events? With recent advances into the sophisticated glutamate signaling mechanisms as have been discussed above, potential future strategies might include blocking glutamate signaling indirectly and specifically. Such signaling pathways might act as secondary signaling pathways under physiological conditions, but are greatly amplified during the ischemic period and contribute significantly to excitotoxic damage. For example, nNOS activity, as perturbing nNOS-PSD-95 interaction has already shown its promising potential in treatment of stroke [108, 285]; Suppressing NR2B-containing NMDA receptor activity and suppressing extrasynaptic NMDA receptor activity, as NR2B-containing NMDA receptors seem to be enriched at extrasynaptic sites and their overactivation during ischemic stroke is extremely lethal, but is naturally prevented by the CNS itself under physiological conditions. Furthermore, targeting astrocytes by supporting their function also seems to be a reasonable treatment strategy [333]. Yet, specific antagonists for the NR2B subunit or extrasynaptic NMDA receptors still need to be refined in order to minimize harm to patients, while strategies targeting astrocytes and other signaling pathways still need to be carefully assessed with regard to their practical influence on regular glutamate signaling before clinical treatment.

CONFLICTS OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

AID	=	Activity-regulated Inhibitors of Death
AMPA	=	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
Apaf-1	=	Apoptosis protease-activating factor-1
ASIC	=	Acid-Sensing Ion Channel
BDNF	=	Brain-Derived Neurotrophic Factor
CaMKII	=	Ca^{2+}/Calmodulin-dependent protein Kinase II
CaMKIV	=	Ca^{2+}/Calmodulin-dependent protein Kinase IV
CBP	=	CREB Binding Protein
CNS	=	Central Nervous System
CREB	=	Cyclic-AMP Response Element Binding protein
cyt c	=	cytochrome c
DAPK1	=	Death-Associated Protein Kinase 1
EM	=	Electron Microscopy
FOXO	=	Forkhead box O
GKAP	=	Guanylate Kinase-Associated Protein
IP_3	=	Inositol 1,4,5 -trisphosphate
Jacob	=	Juxtasynaptic attractor of caldendrin on dendritic boutons protein
JNK	=	c-Jun N-terminal Kinase
KA	=	Kainate
MAGUK	=	Membrane-Associated Guanylate Kinase proteins
MAPK	=	Mitogen activated protein kinase
mGluR	=	Metabotropic glutamate receptor
mtPTP	=	Mitochondrial Permeability Transition Pore
NCX	=	Na^{+}/Ca^{2+} Exchanger
NMDA	=	N-methyl-D-aspartate
nNOS	=	Neuronal Nitric Oxide Synthase
PDZ	=	Postsynaptic density-95/Discs large /Zona occludens-1
PINK1	=	PTEN-Induced Kinase-1
PKA	=	Protein Kinase A
PKB	=	Protein Kinase B
PKC	=	Protein Kinase C
PSD	=	Postsynaptic Density
ROS	=	Reactive Oxygen Species
SAP	=	Synapse-Associated Protein
SREBP-1	=	Sterol Regulatory Element Binding Protein-1
STEP	=	Striatal Enriched tyrosine Phosphatase
SynGAP	=	Synaptic Ras GTPase Activating Protein
TORC	=	Transducer Of Regulated CREB activity
TRP	=	Transient Receptor Potential
TRPM2	=	Transient Receptor Potential channel superfamily Member 2
TRPM7	=	Transient Receptor Potential channel superfamily Member 7
T/SXV	=	Threonine/Serine-X-valine-COOH
VDCC	=	Voltage-Gated Calcium Channel
VRAC	=	Volume-Regulated Anion Channel

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