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Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity

Mark Arundine [a,b], Michael Tymianski [a,b,*]

[a] *Toronto Western Hospital Research Institute, Lab 11-416, Suite 4W-435, 399 Bathurst Street, Toronto, Ont., Canada M5T 2S8*
[b] *Department of Physiology, University of Toronto, Toronto, Ont., Canada M5G 1X8*

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Abstract

Excitotoxicity contributes to neuronal degeneration in many acute CNS diseases, including ischemia, trauma, and epilepsy, and may also play a role in chronic diseases, such as amyotrophic lateral sclerosis (ALS). Key mediators of excitotoxic damage are Ca ions (Ca^{2+}), which under physiological conditions govern a multitude of cellular processes, including cell growth, differentiation, and synaptic activity. Consequently, homeostatic mechanisms exist to maintain a low intracellular Ca^{2+} ion concentration so that Ca^{2+} signals remain spatially and temporally localized. This permits multiple independent Ca-mediated signaling pathways to occur in the same cell.

In excitotoxicity, excessive synaptic release of glutamate can lead to the disregulation of Ca^{2+} homeostasis. Glutamate activates post-synaptic receptors, including the ionotropic *N*-methyl-D-aspartate (NMDA), 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) proprionate (AMPA), and kainate receptors. Upon their activation, these open their associated ion channel to allow the influx of Ca^{2+} and Na^{+} ions. Although physiological elevations in intracellular Ca^{2+} are salient to normal cell functioning, the excessive influx of Ca^{2+} together with any Ca^{2+} release from intracellular compartments can overwhelm Ca^{2+}-regulatory mechanisms and lead to cell death.

Although Ca^{2+} disregulation is paramount to neurodegeneration, the exact mechanism by which Ca^{2+} ions actually mediate excitotoxicity is less clear. One hypothesis outlined in this review suggests that Ca^{2+}-dependent neurotoxicity occurs following the activation of distinct signaling cascades downstream from key points of Ca^{2+} entry at synapses, and that triggers of these cascades are physically co-localized with specific glutamate receptors. Thus, we summarize the importance of Ca^{2+} regulation in mammalian neurons and the excitotoxicity hypothesis, and focus on the molecular determinants of glutamate receptor-mediated excitotoxic mechanisms.

Keywords: Neuronal degeneration; Ca^{2+} regulation; Excitotoxicity; Glutamate receptors; Post-synaptic density; PSD-95

1. Introduction

1.1. Calcium ion homeostasis

Calcium ions are important intracellular messengers governing cellular functions, such as differentiation and growth, membrane excitability, exocytosis, and synaptic activity. Neurons possess specialized homeostatic mechanisms to ensure a tight command of cytosolic Ca^{2+} levels. In their resting state, free calcium levels are maintained at low levels (100 nM). Thus, localized Ca^{2+} elevations that occur in the vicinity of an ion channel pore or at intracellular Ca^{2+} release sites can efficiently activate enzymes or neighboring ion channels. Neurons control both intracellular Ca^{2+} levels and the location of Ca^{2+} ions through a complex interplay between Ca^{2+} influx, Ca^{2+} efflux, Ca^{2+} buffering, and internal Ca^{2+} storage. Under physiological conditions, these processes enable multiple Ca^{2+}-regulated signaling cascades to occur independently within the same cell. However, excessive Ca^{2+} influx or release from intracellular stores can elevate Ca^{2+} loads to levels that exceed the capacity of Ca^{2+}-regulatory mechanisms. This leads to the inappropriate activation of Ca^{2+}-dependent processes that are normally dormant or operate at low levels, causing metabolic derangements and eventual cell death [1–3]. For example, excessive elevations in intracellular Ca^{2+} may overactivate proteases, lipases, phosphatases, and endonucleases that either directly damage cell structure or induce the formation of oxidative free radicals that mediate cell death.

Although cellular Ca^{2+} overload is unlikely to be the sole mechanism mediating neuronal death, several lines of evidence support a close relationship between excessive Ca^{2+}

* Corresponding author. Tel.: +1-416-603-5896; fax: +1-416-603-5298.
E-mail address: mike_t@uhnres.utoronto.ca (M. Tymianski).

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influx and neuronal injury [2,4,5]. Beginning with the work by Schlaepfer and Bunge [6], indicating that degeneration of amputated axons involved extracellular Ca^{2+} ions, much research has focused on the implications of Ca^{2+} overload and the "calcium hypothesis" whereby "neuronal Ca^{2+} overload leads to subsequent neurodegeneration".

1.2. The role of glutamate in neurotoxicity

Glutamate is the major excitatory neurotransmitter in the mammalian CNS and is present in millimolar concentrations in central gray matter. Once released, postsynaptic responses occur via pharmacologically and functionally distinct metabotropic and ionotropic receptors. Metabotropic receptors mediate their actions through GTP-binding protein-dependent mechanisms that cause mobilization of Ca^{2+} from internal stores. Ionotropic receptors are associated with an ion channel, and include the *N*-methyl-D-aspartate (NMDA) receptor, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) proprionate (AMPA) receptor, and kainate receptor subtypes. Ionotropic receptor activation leads to permeability to sodium, potassium, and/or calcium ions.

Experiments by Lucas and Newhouse [7], which showed that L-glutamate injections could destroy the inner layers of the mouse retina, were the first to suggest that glutamate could be a neurotoxin. These observations were replicated and expanded by Olney [8], who confirmed the retinotoxicity of glutamate and further indicated that the structurally related compound kainate produces brain lesions in immature animals that do not possess a fully developed blood–brain barrier. Olney also reported that the glutamate-induced retinotoxicity is accompanied by rapid cellular swelling which is most pronounced near dendrosomal components that are currently known to express excitatory amino acid (EAA) receptors. In 1969, he coined the term "excitotoxicity", to indicate neurodegeneration mediated by EAAs [9]. The role of excitotoxicity in damaging neurons during hypoxia was established when Kass and Lipton [10] and Rothman [11] reported that attenuating synaptic transmission by magnesium leads to a reduction in hypoxic/anoxic neuronal death. Soon thereafter, experiments with glutamate receptor antagonists, such as γ-D-glutamylglycine, 2-amino-7-phosphnohepatonic acid, and MK-801, showed that blocking excitotoxicity could be neuroprotective in vitro [12] and in vivo [13,14]. To date, virtually every glutamate receptor subtype has been implicated in mediating neurotoxicity, largely by a Ca-dependent process [15–17]. Of these, however, the ionotropic glutamate receptors remain recognized as playing key roles [4,5].

1.3. The role of calcium in glutamate-mediated excitotoxicity

Glutamate receptor activation causes changes in intracellular ions, especially Ca^{2+} and Na^{+}. Na^{+} influx may be damaging in isolation, as hippocampal cultures [18] and retina [19] exhibit irreversible, Na-mediated toxic swelling even in the absence of extracellular Ca^{2+}. However, as initially proposed by Berdichevsky et al. [20], Choi et al. [16,21] emphasized the role of Ca^{2+} influx in glutamate neurotoxicity. They indicated in ion substitution experiments that although the removal of extracellular Na^{+} eliminates the acute neuronal swelling in cortical cell cultures exposed to glutamate, neurons still undergo delayed degeneration unless extracellular Ca^{2+} is removed. Their observations suggested two components of excitotoxicity; first, an acute, Na^{+}- and Cl^{-}-dependent component marked by immediate cell swelling, and a second delayed cell degeneration that could be mimicked by the Ca^{2+} ionophore A23187. Choi et al. concluded that at lower glutamate exposures the Ca^{2+} component is the more significant cause of neuronal death.

A strong relationship exists between excessive Ca^{2+} influx and glutamate-triggered neuronal injury (reviewed in refs. [2,4,5]). Thus, the focus of initial cytoprotective approaches was on blocking the excessive Ca^{2+} rise produced by glutamate through antagonizing glutamate receptors. However, it is now apparent that blocking glutamate receptors is not the best approach, as this also impinges on normal brain function and produces adverse side effects [22] (see www.stroketrials.org for details on clinical trials with glutamate antagonists). Thus, current research is not only focused on preventing Ca^{2+} overload, but on the intracellular signaling and regulatory pathways triggered by glutamate receptor overactivation. It is now established that the route of Ca^{2+} entry and the intracellular localization of Ca^{2+} microdomains give rise to the activation of distinct biochemical signaling pathways that mediate independent physiological responses [23,24]. Similarly, Ca-mediated neurotoxicity requires distinct signaling pathways to be triggered in cells, and such pathways are more efficiently triggered when Ca^{2+} ions enter neurons at specific entry points—particularly at Ca-permeable glutamate receptors [25,26]. This has been coined the "source-specificity" hypothesis of Ca^{2+} neurotoxicity.

The source-specificity hypothesis was originally based on experiments performed with free Ca^{2+} indicators [25], and later by measuring total cellular Ca^{2+} accumulation with radiolabeled $^{45}Ca^{2+}$ [26]. The aim was to determine whether equivalent increases in free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) or total $^{45}Ca^{2+}$ loads produced through different Ca^{2+} influx pathways would be equally toxic. The results revealed that Ca^{2+} loading through L-type voltage-sensitive Ca^{2+} channels (VSCCs) were not harmful, whereas similar Ca^{2+} loads produced via NMDA receptors were highly neurotoxic. The source-specificity hypothesis proposed that rate-limiting enzymes or substrates responsible for excitotoxicity must be co-localized with NMDA receptors. Analogous mechanisms may also participate in toxicity produced by other glutamate receptor subtypes, especially AMPA receptors. Therefore, we focus below on

the structure and function of glutamate receptors and their associated submembrane molecules that may give rise to signaling specificity in excitotoxicity.

2. AMPA receptors

2.1. Molecular structure of the AMPA receptor

AMPA receptors are heteromeric structures composed of subunits encoded by four genes GluR1–4 (GluR-A–D). These receptors exhibit higher affinity to glutamate and AMPA as compared to kainic acid [27]. GluR1–3 mRNAs are expressed throughout the CNS whereas GluR4 mRNA exhibits a more restricted spatial and temporal distribution [28]. Each AMPA receptor subunit has four hydrophobic membrane spanning domains with an extracellular N-terminal domain and a cytoplasmically disposed C-terminal tail [27]. Neural tissues contain a wide variety of functionally distinct receptor isoforms due to alternatively spliced exonic sequences in the respective mRNA as well as to RNA editing of the different subunits [27]. The GluR2 subunit is unique in that it can undergo RNA editing to encode a positively charged arginine (R) residue in the membrane-associated segment 2 of the subunit, while unedited subunits contain a neutral glutamine (Q) residue at this position [29,30]. This RNA editing is fundamental for determining the Ca^{2+} permeability of the GluR2-containing AMPA receptor complexes, and makes GluR2(R)-containing AMPA receptors impermeable to Ca^{2+} or other divalent cations [31]. Heteromeric AMPA receptors that lack GluR2 exhibit a high permeability to Ca [31].

AMPA receptor subunits also show divergence from each other in the sequence and length of their C-terminal tail, a region that is currently understood to mediate interactions with a variety of intracellular proteins. These interactions are considered to be paramount in regulating AMPA receptor functioning, targeting, and trafficking [32–34]. For example, the common C-terminal sequence (t-SVKI) shared by both GluR2 and GluR3 interacts with glutamate receptor-interacting proteins 1 and 2 (GRIP1, GRIP2), as well as AMPA receptor-binding protein (ABP). These proteins all contain multiple protein–protein binding domains, also referred to as PDZ domains, which enables them to assemble and regulate protein signaling complexes around the AMPA receptor [33,35]. For example, GRIP1 and GRIP2 have been shown to bind to signaling proteins, such as the Eph receptor and its ligand ephrin-B1, as well as GRASP-1, a novel neuron-specific Ras guanine nucleotide exchange factor [36,37]. The interaction of these proteins with AMPA receptors has only recently been proposed to have a role in governing the AMPA receptors' pharmacological properties [38], activity-dependent and -independent receptor targeting and trafficking [7,39], as well as synaptic transmission and plasticity [40,41].

2.2. Calcium-dependent neurotoxicity mediated by AMPA receptors

Prior to the cloning of AMPA receptor subunits, AMPA receptors were regarded as Ca^{2+}-impermeable channels. Accordingly, in glutamate-triggered neurotoxicity, they were thought to induce membrane depolarization via Na^{+} influx. The AMPA-mediated depolarization, in turn, opened both VSCCs and removed the Mg^{2+} block from NMDA receptors, thus allowing Ca^{2+} influx through these pathways.

It is now understood that Ca^{2+}-permeable AMPA receptors exist in the CNS [42], and experiments suggesting that AMPA receptor antagonists impart neuroprotection from excitotoxicity (reviewed in ref. [43]) indicate that AMPA receptors are important in neurological disorders. Recent research has focused on the GluR2 subunit as it governs Ca^{2+} permeability.

GluR2 mRNA is widely expressed in the CNS. Though most AMPA receptor-containing neurons express GluR2, the expression levels vary. In many brain regions, about 8–15% of neurons express Ca^{2+}-permeable (GluR2-lacking) AMPA receptors [26,44,45]. These neuronal subpopulations are selectively destroyed in a Ca^{2+}-dependent manner following exposures to either AMPA or kainate [45]. However, even AMPA receptors expressing the GluR2 subunit exhibit a small permeability to Ca^{2+} and other divalent cations. Also, GluR2 expression levels are heterogeneous between cells in a given brain region, with some neurons expressing lower (but non-zero) levels relative to other AMPA receptor subunits [46–48]. The low but ever-present permeability to Ca^{2+} in heterogeneous cell populations may explain why mammalian neurons that are highly vulnerable to AMPA-mediated damage, such as hippocampal granule and pyramidal neurons [27] and cortical neurons [49], still express GluR2.

Whether a low Ca^{2+} permeability is solely responsible for AMPA receptor-mediated excitotoxic cell death is uncertain, but unlikely. Experiments in mice lacking a functional GluR2 gene revealed that despite increased Ca^{2+} influx in CA1 neurons, these mice did not exhibit neuropathological lesions suggestive of excitotoxicity [50]. Also, experiments in cortical cultures obtained from GluR2-deficient mice revealed that the elevated Ca^{2+} permeability in these neurons did not correlate with increased vulnerability to excitotoxicity [51]. The vulnerability to a given concentration of kainate was higher in GluR2-deficient neurons. However, these neurons also exhibited increased kainate potency and larger ionic currents, explaining their selective vulnerability on the basis of overall ionic current size, and not Ca^{2+} influx. The same study revealed that in vivo vulnerability of CA1 hippocampal neurons to stereotactic kainate injections, and CA3 neurons to intraperitoneal kainate administration, was independent of the levels of GluR2 [51]. Although such data do not exclude the role for Ca^{2+} ions in AMPA receptor-mediated excitotoxicity, it strongly suggests that Ca^{2+} permeability is not the sole determinant of neurotoxic

vulnerability. Rather, the GluR2 subunit may contribute to other aspects of AMPA receptor function, and its absence may impact synaptic organization of channel pharmacological properties, such as agonist potency and channel conductance, that impact on ionic currents.

One possibility is that AMPA receptor-mediated ion fluxes could be coupled to downstream neurotoxic second messengers via interactions with submembrane proteins. To date, none of the identified submembrane AMPA-interacting proteins have been implicated in AMPA-mediated excitotoxicity. However, many have been ascribed a role in the dynamic regulation of synaptic structure by regulating receptor trafficking; a concept thought to underlie cellular models of synaptic plasticity (i.e. long-term potentiation and depression) [34,39]. When considering that interactions between AMPA receptors and submembrane proteins are salient to AMPA receptor-mediated physiological signaling, the hypothesis that AMPA-interacting proteins could have an important role in mediating excitotoxicity is highly plausible.

For example, the interaction of GRIP1, a GluR2-interacting protein, with GRASP-1 may couple AMPA receptors to Ras signaling [37]. Interestingly, Ye et al. showed that GRASP-1 is a neuronal substrate for caspase-3 [37], and is cleaved in apoptotic neurons in a time-dependent manner during development and ischemia [52]. Although the consequence of the caspase cleavage of GRASP-1 remains less clear, it seems to uncouple the C-terminal GRIP-binding domain of GRASP-1 from the rasGEF domain and may thereby disrupt the regulation or targeting of the GEF. They also showed that overexpression of GRASP-1 downregulates synaptic targeting of AMPA receptors, and that when it is cleaved into two fragments via caspase-3, it loses such activity. Ye et al. suggested that cleavage of GRASP-1 by caspase-3 under pathological conditions may incapacitate the regulation of AMPA receptor synaptic targeting and result in increased synaptic AMPA receptors. The increased synaptic expression resulting from GRASP-1 may render neurons more susceptible to excitotoxicity. It has also been suggested that GRASP-1 may serve as a scaffolding protein for the c-jun N-terminal kinase (JNK) signaling pathway. The JNK pathway is integral to stress responses and cell death [53,54], which may indicate a role of GRASP-1 in AMPA receptor-mediated cell death. All these possibilities, as yet, remain to be determined.

An alternative role of GluR2-interacting proteins in excitotoxicity may be that the presence of GluR2 is required to maintain synaptic structure and organization. Accordingly, the toxicity observed in GluR2-deficient neurons may result from the effects on synaptic organization and function rather than due to AMPA receptor Ca^{2+} permeability. An interesting candidate protein is the *N*-ethylmaleimide-sensitive fusion protein (NSF), as it has been shown both to interact with GluR2 and to mediate membrane-fusion events [55–57]. It has been suggested that GluR2 surface expression involves the interaction between NSF and GluR2, and that disrupting this interaction can lead to the elimination of functional AMPA receptors at synapses [55,58]. Interestingly, NSF expression increases following an ischemic insult [59]. It is not yet clear whether an increase in NSF leads to an increase of surface expression of existing GluR2-containing AMPA receptors following ischemia. If so, one can speculate that increased GluR2 surface expression may decrease Ca^{2+} permeability through AMPA receptors, and restore synaptic organization. Taken together, these activities may serve as a feedback mechanism to protect neurons from further degeneration.

3. NMDA receptors

3.1. Molecular structure of NMDA receptors

Molecular cloning studies have identified five NMDA receptor subunits, NR1 and NR2A–D [27]. The structure of each subunit is very similar to AMPA subunits. Specifically, each subunit is composed of four membrane domains, an extracellular amino-terminal domain, and an intracellular C-terminal tail [27]. The NR1 subunit is known to have eight functional splice variants (NR1a–h) and one non-functional truncated splice variant [60], while among NR2 subunits only NR2D has been shown to exhibit splice variants [61]. NMDA receptors exist as either hetero-tetramer or -pentamer structures [27,62]. An asparagine residue (N598) in the NR1 subunit within the channel pore loop structure of the second membrane domain controls the receptor's Ca^{2+} permeability. Interestingly, the position of the asparagine is homologous to the Q/R sites of GluR1–4 [63,64]. This residue not only determines the voltage-dependent Mg^{2+} block of NMDA receptors [65], but also controls gating properties, potentiation and block by polyamines, inhibition by protons and Zn^{2+}, and affinity to glutamate and glycine [66,67].

The NR1 subunit is essential for the assembly of functional NMDA receptors. Co-expression of NR1 with NR2 subunits in non-neuronal cells results in ion channels with functional and pharmacological properties similar to NMDA receptors in neurons [68,69]. NR1 knockouts are lethal in the first few postnatal days, though there are only minimal changes in the structure or function of neurons of the brain [70–72], while NR2A knockout mice survive and develop normally [73]. In light of the large array of NR1 subunit splice variants, different combinations of NR1 and NR2 subunits can result in a variety of receptor complexes, with each having different ligand affinities. For example, various NR1 splice variants exhibit different sensitivity to agonists, antagonists, Ca^{2+}, Zn^{2+}, polyamines, and phosphorylation by protein kinase C (reviewed in ref. [60]). Furthermore, each NR2 subunit confers a unique set of characteristics upon the resultant NMDA receptor, such as sensitivity to Mg^{2+} block, glycine and glutamate affinity, and single-channel conductance [68,69,74].

Like AMPA receptors, NMDA receptor subunits exhibit divergence in their C-terminal sequence. The C-terminal

domains enable NMDA receptors to interact with a variety of intracellular synaptic and cytoskeletal proteins. These form large receptor-linked multiprotein complexes in the postsynaptic density (PSD; reviewed in ref. [75]) whose functional significance is only beginning to be understood. However, the interactions of NMDA receptors with proteins in the PSD exhibit high specificity, thereby linking NMDA receptors to distinct downstream signaling molecules. Evidence is emerging that these specific protein–protein interactions may link Ca^{2+} fluxes through NMDA receptor channels to neurotoxic downstream signaling pathways. This is reviewed below.

3.2. Role of NR1 subunits in excitotoxicity

Functional NMDA receptors require the NR1 subunit [68]. Studies in cultured neurons obtained from NR1 knockout mice revealed them to be resistant to NMDA- and glutamate-induced excitotoxicity [76]. Similar results can be obtained in cultures by suppressing NR1 expression with antisense oligonucleotides [77], a procedure that also reduced focal ischemic infarction in vivo [77]. Other investigations using mice expressing NR1 mutations in the site critical for Ca^{2+} permeation (N598Q and N598R) showed that NMDA receptor-mediated signaling was perturbed and that the mice failed to develop autonomic functions, such as feeding and breathing [78]. All these observations can only suggest that functional NMDA receptors are necessary for triggering receptor-dependent excitotoxicity, but cannot indicate whether the NR1 subunit plays a distinct role in mediating neuronal death or survival.

Some studies have addressed the role of the various NR1 splice variants (reviewed in ref. [60]) in NMDA receptor function, especially in the context of synaptic organization and neurotransmission (e.g. refs. [79–83]). Fewer have addressed their role in excitotoxicity and survival. Kreutz et al. [84] studied the effects of axonal trauma of the optic nerve on expression of alternatively spliced NR1 variants in the retinal ganglion cell layer. They showed that the increased expression of NR1–4b is crucial for neuronal survival after partial axonal trauma, as decreasing NR1–4b expression levels using antisense oligonucleotides significantly decreased cell survival of retinal ganglion cells. The authors propose that altered splicing leads to a different composition of the native NMDA receptor and different responses to glutamate activation.

Some NR1 splice variants have been implicated in NMDA receptor-mediated toxicity in heterologous cells and primary neuronal cultures. NR1 pore mutations that block Ca^{2+} entry and NR1 C-terminal tail deletions decreased NMDA-induced neurotoxicity significantly when transfected into CHO cells. Expression of these constructs in primary cortical neurons was shown to protect cells from NMDA receptor-dependent toxicity [85]. The studies suggested that while the N1 cassette of the amino-terminus exhibits very low toxicity on its own, C1 and C2 enhance NMDA receptor toxicity.

3.3. Interactions of NR1 subunits with submembrane proteins and excitotoxicity

Excitotoxicity in cultured neurons and neurological diseases, such as epilepsy, schizophrenia, and aging, results in a profound loss of dendritic spines [86–89], the sites of most NMDA receptors. This suggests that dendritic spines constitute the subcellular compartments where NMDA-mediated excitotoxicity occurs. However, NMDA receptors are also found extrasynaptically [90–92], raising the possibility that both synaptic and extrasynaptic NMDA receptors have physiological and pathological roles. The ultimate localization of NMDA receptors is partly governed by the NR1 subunit CO domain, a membrane-proximal segment of the NR1 cytoplasmic tail, which interacts with α-actinin, an actin-binding protein [93]. NR1–α-actinin interactions may serve to anchor NMDA receptors to cytoskeletal elements in the synapse as depolymerizing F-actin results in a redistribution of NMDA receptor clusters to extrasynaptic sites [94–96]. Interestingly, Ca^{2+}/calmodulin has been identified to directly antagonize the binding of α-actinin to the NMDA receptor [82,97]. Ca^{2+}/calmodulin has been shown to bind to both the C0 and C1 segment of NR1 and its binding inhibits NMDA receptor opening and reduces mean channel open time [82,83]. Therefore, Ca^{2+} ions that enter through synaptic NMDA receptors and bind to calmodulin may lead to the displacement of α-actinin. This may release NMDA receptors from the actin cytoskeleton, leading to their redistribution to extrasynaptic sites. Thus, though actin-mediated anchoring of NMDA receptors at synaptic sites may have evolved to optimize synaptic function [32,98], it may also have bearing on the excitotoxic potential of synaptic NMDA receptors. In recent studies in cultured cortical neurons, depolymerizing the F-actin cytoskeleton caused a redistribution of NMDA receptors away from dendritic spines and reduced the activity of synaptically activated NMDA receptors [99]. Neurons treated with actin depolymerizing agents remained vulnerable to exogenously applied NMDA and glutamate, indicating that NMDA receptors are equally capable of triggering excitotoxicity within and outside of synapses. However, neurons treated with actin depolymerizing agents were less vulnerable to excitotoxicity evoked by synaptic glutamate release [99]. Thus, the relative contributions of synaptic and extrasynaptic NMDA receptors to excitotoxicity may vary in accordance with the location of extracellular excitotoxin accumulation.

NR1 subunits containing the C1 cassette have also been shown to interact with two other proteins, Yotiao [100] and neurofilament-L [101]. This finding may explain previous observations indicating C1-dependent clustering of NR1 in heterologous cells [102], and may provide further evidence supporting that the NR1 subunit of NMDA receptors is

salient for synaptic localization of the receptor. The role of these proteins in NMDA receptor function is still unclear. Yotiao has been identified to bind to both the protein phosphatase 1 and protein kinase A, but these proteins' role in excitotoxicity is unclear.

3.4. Role of the NR2 subunit in excitotoxicity

It is well documented that immature cultured neurons are less vulnerable to NMDA neurotoxicity [21,103–105]. This vulnerability parallels the temporal expression pattern of different NR2 subunits, as the expression of NR2B and NR2D begins at least as early as E14, whereas NR2A and NR2C are first detected perinatally [106–108]. Thus, a developmental switch in NR2 subtype expression may underlie the increase in susceptibility to excitotoxicity over time. This developmental switch in NR2 subtype expression is dependent on neuronal activity [109,110]. For example, Bessho et al. [111] showed that a K^+-induced depolarization in cerebellar granule cells, which initially promotes neuronal survival [112,113], upregulated NR2A subunit mRNA via Ca^{2+} influx through VSCCs. Interestingly, these cells become more vulnerable to NMDA-mediated toxicity after prolonged K^+ depolarization, which the authors suggested to be partially due to the increased levels of NR2A and an enhanced NMDA receptor-mediated Ca^{2+} influx.

Mizuta et al. [114] demonstrated that cortical neurons exhibited increased glutamate sensitivity on day 11 in culture, whereas they were not affected by glutamate on culture days 7–9. The authors concluded that glutamate neurotoxicity in these neurons was mainly mediated by a heteromeric NR1–NR2B receptor, as they detected levels of NR2B and NR1 on both days (8 and 11), while NR2A protein levels were hardly detectable on either day 8 or 11. Furthermore, Cheng et al. [115] found that NR1 and NR2A mRNA levels increased continuously over time in neuronal cultures, whereas NR2B mRNA increased dramatically during the first 10 days and subsequently remained stable. The time-course of NR2B mRNA increase correlated most closely with increases in glutamate-stimulated intracellular Ca^{2+} elevations and neuronal injury. These authors also concluded that NR2B expression might be a critical determinant of glutamate neurotoxicity. However, these data still do not exclude other explanations for the correlations between NR2B mRNA or protein levels and toxicity, as this could also reflect an increase in the number of functional channels, or the newly expressed subunit may not be incorporated into functional receptors at the plasma membrane. Experiments using heteromeric NR1–NR2 expression systems in non-neuronal cell lines have shown that the co-transfection of NR1 and NR2A resulted in more cell death than transfections of NR1 with NR2B. Furthermore, co-transfection of NR1–NR2C receptors failed to induce toxicity [116,117]. How these results relate to the data in neurons is less certain, as non-neuronal cell lines do not necessarily express the same downstream signaling pathways as neurons. More detailed studies are necessary to address these issues.

The development of mutant mice provides further insights into the role of distinct NR2 subunits in neurotoxicity. Morikawa et al. [118] investigated the role of NR2A and NR2B subunits in brain ischemia using mutant mice deficient in NR2A [73,119] and double mutants deficient in NR2A and NR2B. NR2A deficiency resulted in a pronounced reduction in infarct volume. Infarct volume in mice lacking both NR2A and NR2B was no different than in NR2A deficiency alone. The authors suggested that NR2A plays an important role in glutamate neurotoxicity. However, an alternative suggestion is that the observed protective effect could also result from a reduced number of functional NMDA receptors, as NR2A knockout mice show decreased NMDA receptor channel activity [73,119]. Mutant mice deficient in NR2C have also been subjected to an animal model of cerebral ischemia and were shown to exhibit reduced injury [120]. Alternatives to knockout mice are mutant mice expressing truncated NR2 subunits. Most express gateable receptors that are synaptically activated, but are defective in intracellular signaling and synaptic localization [121–123]. This suggests that NR2 subunits link NMDA receptors to downstream molecules that mediate cellular responses to ions entering through the NMDA receptor channel.

Many cytoplasmic PSD proteins bind with high specificity to distinct NMDA receptor subunits (reviewed in refs. [33,34,75]). Some have been shown to physically couple NMDA receptors to downstream signaling enzymes. Given that incoming Ca^{2+} ions diffuse rapidly away from the ion channel pore, it seems logical that coupling the receptor channel to the downstream Ca^{2+} signaling machinery would enhance the efficiency of signaling while permitting the compartmentalization of different Ca-dependent signaling pathways. This idea is supported by studies showing that different Ca^{2+}-dependent processes, including synaptic plasticity and gene expression, are separately regulated through distinct signaling pathways linked to specific routes of Ca^{2+} influx [23,24,124,125].

Studies suggest that such distinct Ca^{2+} signaling pathways would also exist for NMDA receptor-mediated neurotoxicity. Ca^{2+}-dependent neurotoxicity is triggered most efficiently when Ca^{2+} influx occurs through NMDA receptors and cannot be reproduced by loading neurons with equivalent quantities of Ca^{2+} through non-NMDA receptors or VSCCs [25,26]. This led to the hypothesis that lethal Ca^{2+} signaling by NMDA receptors is determined by the molecules with which they interact [25]. However, very few of the identified NMDA receptor-interacting proteins have been ascribed a functional role in channeling Ca^{2+} signals to intracellular second messengers. Below, we describe the contribution of one major family of NR2-interacting PSD proteins: the PSD-95/synapse-associated protein 90 (SAP90) subfamily of the membrane-associated guanylate kinase (MAGUK) superfamily (reviewed in refs. [126,127]).

3.5. Membrane-associated guanylate kinases

The MAGUK superfamily represents submembrane proteins involved with receptor clustering on the plasma membrane. Several mammalian homologous families of MAGUKs exist, including PSD-95/SAP90 [128,129], PSD-93/chapsyn-110 [130,131], SAP102 [132], and SAP97/hdlg [133], all of which are concentrated in brain synapses. MAGUKs share a common domain organization; multiple N-terminal PDZ domains, a *src* homology (SH) domain 3 (SH3), and a carboxy-terminal yeast guanylate kinase (GK) homology domain. Each domain can function as a site for protein–protein interactions. The PDZ domains are named after three of the homologous proteins that contain them: PSD-95/SAP90, discs large (Dlg-A, a *Drosophilia* protein found at septate junctions), and zona occludentes-1 (ZO-1, a vertebrate protein found at epithelial cell tight junctions) [134] (see also refs. [127,135]). SH3 domains are also frequent sites of protein–protein interactions (for review, see ref. [136]). The specific function of the yeast GK homology domain is presently unclear.

Direct interactions between NMDA receptor subunits and members of the PSD-95/SAP90 family have been demonstrated using the yeast two-hybrid system [137–139]. These interactions occur through the C-terminal cytoplasmic tail of NR2 subunits and certain splice variants of NR1 [140], with the PDZ domains of PSD-95/SAP90. In brief, the last three amino acids of the NR2 subunit C-terminus define a consensus motif threonine/serine X valine (T/SXV, where "X" is any amino acid) essential for binding with the PDZ2 domain of PSD-95 [140–143]. These interactions are important for the synaptic targeting and clustering of NMDA receptors (reviewed in ref. [144]). In addition, PSD-95 family proteins may participate in synaptic organization by linking NMDA receptors to downstream signal-transduction enzymes. These linkages may be mediated both by PDZ domain interactions and by protein–protein interactions at other conserved domains of the MAGUKs. SH3 domain motifs are found in a variety of signaling molecules (for review, see ref. [145]). Though a binding partner for the MAGUK SH3 domain has yet to be found, a direct protein–protein interaction between the SH3 domain and the GK region in PSD-95 forms an intramolecular SH3–GK interaction that may modulate the clustering activity of PSD-95. GK domains typically mediate the catalysis of GMP to GDP using adenosine 5′-triphosphate (ATP), suggesting a signaling function. Although GKs in MAGUKs do not exhibit kinase activity, a family of proteins has been identified that binds to the guanylate kinase homology domain of PSD-95: GK associated protein [85,146] or PSD-95/SAP90 associated proteins [147]. Despite the fact that their function is unknown, it is speculated that they are involved in intracellular signaling pathways by acting as an adapter molecule between PSD-95 and as yet unidentified intracellular signaling molecules.

PDZ–PDZ domain interactions govern the binding of PSD-95 family members to cytoplasmic signaling proteins. For example, the PDZ domain of neuronal nitric oxide synthase (nNOS) binds to the second PDZ domains of both PSD-95 and PSD-93 [148,149]. Although NR2 and nNOS compete in vitro for binding at the PDZ2 domain of PSD-95, NR2 can independently bind the PDZ1 domain of PSD-95 [144]. Thus, PSD-95 allows for the formation of a ternary complex between NMDA receptors, PSD-95, and nNOS. This molecular scaffold, which brings NMDA receptors into close proximity with nNOS, may explain the preferential activation of nNOS by Ca^{2+} coming through the NMDA receptors over Ca^{2+} entry through other channels [150–153].

3.6. Involvement of PSD-95 in NMDA receptor-mediated neurotoxicity

Current knowledge of the molecular organization of the PSD provides a blueprint for explaining the toxicity of Ca^{2+} ions that enter neurons through NMDA receptors. It suggests that NMDA receptor-mediated Ca^{2+} signals are linked to downstream neurotoxic signaling pathways through the types of protein–protein interactions described above. Recently, studies in mutant mice expressing a truncated form of PSD-95 revealed that the PSD-95 mutation did not produce compensatory alterations in the total amount of NR1, NR2A, and NR2B proteins nor in the other PSD proteins, PSD-93/chapsyn-110, SAP102, and SAP97. NMDA currents, including synaptic currents, and current–voltage relationship were also unchanged. Moreover, the distribution of NR1 as studied by electron microscopy in CA1 stratum radiatum was unaltered. Thus, PSD-95 mutations do not produce apparent compensatory changes in other proteins, or in NMDA receptor function. Therefore, we examined in our laboratory whether PSD-95 might act as a link between NMDA receptor activity and downstream signaling. To this end, we suppressed the expression of PSD-95 in cultured cortical neurons using antisense oligonucleotides. Consistent with results from PSD-95 mutant mice, the function of NMDA receptors was not altered, as receptor expression, NMDA currents, and Ca^{2+} loading via NMDA receptors were unchanged. However, suppressing PSD-95 selectively attenuated Ca^{2+}-activated NO production by NMDA receptors without affecting nNOS expression. This attenuated excitotoxicity triggered via NMDA receptors, but not through other glutamate or Ca^{2+} channels [154]. PSD-95 is thus required for the efficient coupling of NMDA receptor activity to NO toxicity, and imparts specificity to NMDA receptor-mediated excitotoxic Ca^{2+} signaling.

3.7. From molecular interactions to clinical treatments?

We have recently investigated the idea that the NMDA receptor/PSD-95 interaction might constitute a therapeutic target for diseases that involve excitotoxicity. NMDA receptors mediate ischemic brain damage, but also neuronal excitation essential to CNS function. Thus, blocking them to

treat stroke is problematic. Targeting PSD-95 protein therefore represents an alternative therapeutic approach that may circumvent the negative consequences of blocking NMDA receptor function. However, mutation or suppression of PSD-95 is impractical as a therapy for brain injury and cannot be applied after an injury has occurred. Therefore, rather than alter PSD-95 expression, we questioned whether interfering with the NMDA receptor/PSD-95 interaction could suppress excitotoxicity in vitro and ischemic brain damage in vivo. To treat stroke without blocking NMDA receptors, neurons in vitro and in vivo were transduced with peptides that bind to modular domains governing interactions of NMDA receptors with PSD-95. We used a peptide comprised of the nine C-terminal residues of NR2B (KLSSIESDV; NR2B9c), which is anticipated to bind the PDZ2 domain of PSD-95. NR2B9c did not enter cells and therefore, we fused it to a peptide corresponding to the cell membrane-transduction domain of the HIV-1-Tat protein (YGRKKRRQRRR; Tat) to obtain a 20-amino acid peptide (Tat-NR2B9c). This strategy permits proteins to transduce cell membranes in a rapid, dose-dependent manner independent of receptors and transporters (see ref. [155]). The Tat-NR2B9c peptide protected cultured neurons from excitotoxicity and, in rats subjected to transient focal cerebral ischemia, dramatically reduced cerebral infarction while improving neurological function. The treatment was effective when applied either before, or one hour after, the onset of excitotoxicity in vitro and cerebral ischemia in vivo [156]. This and similar strategies based on a molecular understanding of excitotoxic mechanisms may amount to practical future treatments for human neurological disorders.

4. Calcium-dependent non-excitotoxic neuronal death

Although glutamate-mediated excitotoxicity has been ascribed a prominent role in mediating neuronal death, several human clinical trials employing glutamate receptor antagonists have failed to protect patients against stroke or head injuries. This prompted criticism of the excitotoxicity hypothesis, and a re-evaluation of the validity of in vitro and in vivo animal models used to derive the initial promising results of anti-excitotoxicity therapy [22,157]. There is controversy about whether excitotoxicity is a truly significant player in the stroke process, and some have suggested that mechanisms additional to excitotoxicity may be important [158]. Accordingly, we will briefly explore alternative mechanisms of Ca^{2+} influx that may contribute to anoxic/excitotoxic neuronal death.

The degree of neurotoxic Ca^{2+} influx through glutamate channels may mask alternative mechanisms of toxic Ca^{2+}entry. Of these, the best characterized process is Ca^{2+} influx via the reverse operation of the plasma membrane Na^+/Ca^{2+} exchanger. Membrane depolarization causes intracellular Na^+ accumulation—which drives the Na^+/Ca^{2+} exchanger in reverse. This causes neurotoxic Ca^{2+} accumulation in both CNS gray [159,160] and white matter (reviewed in refs. [161,162]).

Additional pathways of Ca^{2+} entry into neurons also exist, though their relationship to neuronal death remains less well understood. For example, neuronal nicotinic acetylcholine receptors (nAChRs) containing the α-7 subunit are highly permeable to Ca^{2+} and have an even greater Ca^{2+} permeability than NMDA glutamate receptors [163]. They are concentrated in dendrites where they may serve in regulating plasticity and other physiologic processes (for reviews, see refs. [164,165]). High levels of α-7 transcripts are found by in situ hybridization in the hippocampus and cerebral cortex [163], which are susceptible to ischemia. However, despite evidence that nAChRs play a role in physiological calcium-dependent processes in central neurons, their role in inducing neurotoxicity is unclear. To date, most publications suggest that activating nAChRs promotes, rather than detracts from neuronal survival [166–170].

In addition to glutamate, extracellular ATP has been shown to mediate excitatory transmission in both peripheral [171,172] and central [173,174] nervous systems. Neurons also express ATP-gated ion channels (P2X receptors) that are structurally distinct from the nicotinic and glutamate superfamilies [175]. They are widely distributed in the peripheral and central nervous systems and mediate fast excitatory transmission at nerve–muscle and nerve–nerve synapses [176–178] They have also been implicated in presynaptic modulation of transmitter release [179]. Seven P2X cDNAs have been cloned [175]. The main properties of neuronal P2X receptors are reproduced fairly well by heterologously expressed $P2X_1$–$P2X_4$ and native receptor channels in neurons and smooth muscle are permeable to sodium and potassium, but have very limited permeability to large cations, such as *N*-methyl-D-glucamine ($NMDG^+$) [175]. The receptor channels are also permeable to Ca^{2+} ions [180–182], and resemble their counterparts in the nicotinic and glutamate superfamilies in kinetics of activation and permeability [183]. P2X receptor activation has been shown to directly mediate a rapid increase in intracellular Ca^{2+} concentrations [174,184]. Sustained ATP application causes P2X receptors to develop an increased permeability to large cations and Ca^{2+}, and is capable of causing death of HEK293 cells [185]. Although present in central neurons, the role of P2X receptors has not been addressed. Though extracellular ATP levels are anticipated to drop in anoxia/ischemia [10,186], ADP, the product of ATP hydrolysis, may rise under metabolic impairment [187,188]. As ADP also gates P2X receptors [176], this may be a dominant mechanism of their activation in anoxia.

Undoubtedly, there exists a large diversity of pathways for Ca^{2+} entry into neurons, and some may play key roles in neuronal death pathways. Such pathways will likely be the focus of future research.

5. Concluding remarks

Recent advances have provided a better understanding of the mechanisms of Ca^{2+}-dependent neurotoxicity. A wide range of negative consequences of Ca^{2+} overload, such as free radical formation and the activation of second messengers and enzymes, have been identified. There now exists a consensus that Ca^{2+}-dependent neurotoxicity, as with many physiological events, occurs through distinct intracellular signaling pathways, likely through physical interactions of cell membrane receptors with specialized submembrane molecules. The molecular characterization of neurotoxic signal-transduction pathways may provide therapeutic targets for future pharmacological or genetic therapies for human neurological diseases.

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