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Review

Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury

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Abstract. Stroke and neurotrauma mediate neuronal death through a series of events that involve multiple interdependent molecular pathways. It has been suggested that these pathways are triggered following elevations in extracellular excitatory amino acids, primarily glutamate [1]. This report outlines mechanisms involving glutamate-mediated excitotoxicity with specific focus on (i) the role of Ca^{2+} in neurotoxicity, (ii) The concept of 'source specificity' of neurotoxicity, (iii) the role of the ionotropic N-methyl-D-aspartate (NMDA)-subtype glutamate receptor and its associated submembrane molecules that may give rise to signaling specificity in excitotoxicity and (iv) the role of glutamate-mediated free-radical generation and associated cell death pathways. We also highlight a novel, peptide-based approach for uncoupling NMDA receptors from excitotoxicity in the rat central nervous system subjected to focal ischemia, thereby reducing stroke infarct volume and improving neurological functioning.

Key words. Glutamate; NMDA receptor; calcium; nitric oxide; ROS; ischemia; traumatic brain injury; postsynaptic density protein-95; superoxide.

Glutamate-mediated neurotoxicity

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS). Following glutamate release, postsynaptic responses occur through both metabotropic and ionotropic receptors. Metabotropic receptors mediate their actions through GTP-binding-protein-dependent mechanisms that cause mobilization of Ca^{2+} from internal stores. Activation of ionotropic receptors leads to permeability to sodium, potassium and/or calcium in associated ion channels. There are three types of ionotropic glutamate receptors: N-methyl-D-aspartate receptor (NMDAR), 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) proprionate receptor (AMPAR) and kainate receptor subtypes [2, 3]. Although current research suggests the involvement of multiple members of the glutamatergic receptor superfamily in mediating excitotoxicity [4–6], the ionotropic receptors are recognized as being key players [7, 8].

In 1957, Lucas and Newhouse [9] found that L-glutamate injections could destroy the inner layers of the mouse retina, implicating glutamate as a putative neurotoxin. Approximately 2 decades later, Olney [10] indicated that kainate, a compound structurally related to glutamate, produced brain lesions in immature animals lacking a mature blood-brain barrier. Olney also found that the glutamate-induced retinotoxicity was accompanied by rapid cellular swelling. These morphological findings were most pronounced near dendrosomal components now known to express excitatory amino acid (EAA) receptors. In 1969, he coined the term 'excitotoxicity' to represent EAA-mediated degeneration of neurons [11].

In the early 80s, Kass and Lipton [12] and Rothman [13] investigated the role of excitotoxicity during hypoxia.

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They found that attenuating synaptic transmission by magnesium also leads to a concomitant reduction in hypoxic/anoxic neuronal death. Based on these observations, investigations incorporating glutamate receptor antagonists were conducted to further assess whether glutamate receptors were genuinely involved in propagating neuronal death following hypoxia. Experiments using ɣ-D-glutamylglycine, 2-amino-7-phosphnohepatonic acid and MK-801 to block glutamate receptors demonstrated that blocking excitotoxicity was neuroprotective in vitro [14] and in vivo.

Role of calcium in glutamate-mediated excitotoxicity

Glutamate receptor activation induces alterations in the concentration of intracellular ions, especially Ca^{2+} and Na^{+}. Investigators have shown that elevations of intracellular Na^{+} can be damaging to neuronal survival. Hippocampal cultures [15] and retina [16] exposed to glutamate exhibit irreversible, Na-mediated toxic swelling even in the absence of extracellular Ca^{2+}. However, based on the initial proposal by Berdichevski et al. [17], Choi and colleagues [5, 18] suggested that glutamate toxicity is primarily dependent on Ca influx. Using ion substitution experiments, they found that the removal of extracellular Na^{+} eliminates the acute neuronal swelling in cortical cell cultures exposed to glutamate. However, the absence of extracellular Na failed to prevent long-term (delayed) neuronal degeneration within this model. Furthermore, neuronal degeneration could only be fully attenuated following the concomitant removal of extracellular Ca^{2+}. These observations suggested that excitotoxicity is made up of two components. The first is an acute, Na^{+} and Cl^{-} dependent component which is marked by immediate cell swelling. The second component is Ca-dependent delayed cell degeneration, reproducible by the Ca^{2+} ionophore A23187.

It is now well established that a strong relationship exists between excessive Ca^{2+} influx and glutamate-triggered neuronal injury (reviewed in [7, 8, 19]. Glutamate-mediated pathological increases in intracellular Ca are thought to be mediated by either (i) prolonged activation of glutamate receptors, or by (ii) injury-induced alterations in receptor functioning leading to increased Ca^{2+} influx. Ca^{2+} overload can trigger many downstream neurotoxic cascades, including the uncoupling mitochondrial electron transfer from ATP synthesis, and the activation and overstimulation of enzymes such as calpains and other proteases, protein kinases, nitric oxide synthase (NOS), calcineurins and endonucleases. Alterations in activity of these enzymes can lead to (i) increased production of toxic reactive oxygen species (ROS) such as nitric oxide (NO), superoxide (O_2^{-}) and hydrogen peroxide, (ii) alterations in the organization of the cytoskeleton, (iii) activation of genetic signals leading to cell death (apoptosis) and (iv) mitochondrial dysfunction.

Although elevations in extracellular glutamate plays a central role in mediating both ischemic brain damage and traumatic brain injury [20, 21], altered glutamate receptor functioning has also been implicated as a potential mechanism mediating toxicity to neurons. For example, in vitro studies have shown that mechanical deformation of cells can alter the properties of glutamate receptors [22], leading to pathological increases in glutamate-induced intracellular Ca^{2+} concentrations. Ellis and colleagues (1995) used an in vitro cell injury model to demonstrate a reduction in voltage-dependent Mg^{2+} blockade of NMDA channels in mechanically injured neurons, leading to elevated intracellular Ca^{2+} levels in response to glutamate exposure. They also reported that stretch applied to neurons caused enhanced AMPA-mediated currents that appeared to be induced by a reduction in AMPA receptor desensitization [23].

As illustrated in the aforementioned examples, it is not surprising that many cytoprotective approaches have thus far been focused on antagonizing glutamate receptors in an attempt to block EAA-mediated pathological elevations of Ca^{2+}. Although it is intuitive to block Ca^{2+} influx at the level of the receptor, it is now apparent that blocking glutamate receptors is not the best approach. Specifically, glutamate receptor blockade has recently been documented to impair normal brain function and produce adverse side effects [24] (for details on clinical trials with glutamate antagonists, see www.stroketrials.org). Accordingly, current research has switched gears, and is now focused on preventing the intracellular signaling and regulatory pathways triggered by glutamate receptor overactivation in addition to reducing Ca^{2+} overload.

The ‘source-specificity’ hypothesis

It is now established that activation of distinct biochemical signaling pathways mediating independent physiological responses [25] is dependent on both the route of Ca^{2+} entry and the intracellular localization of Ca^{2+} microdomains [26]. Similarly, Ca-triggered neurotoxicity depends on the activation of distinct signaling pathways. These pathways are triggered more efficiently when neuronal Ca^{2+} influx occurs at specialized entry points-particularly at Ca-permeable glutamate receptors [27, 28]. This concept has been termed the ‘source-specificity’ hypothesis of Ca^{2+} neurotoxicity.

The source-specificity theory was developed following experiments indicating that Ca^{2+} loading through L-type voltage-sensitive channels were nontoxic to cells, whereas similar Ca^{2+} loads produced via NMDA receptors were highly neurotoxic [27]. These results suggested that al-

though cells underwent similar increases in intracellular Ca^{2+} concentrations, toxicity is contingent upon the location of Ca^{2+} entry. Based on these observations, the source-specificity hypothesis further proposed that rate-limiting enzymes or substrates responsible for excitotoxicity must also be directly associated with NMDA receptors. In this regard, this report will next outline the structure and function of NMDA receptors, and the implications of NMDA receptor activation to neurotoxicity. Because free-radical-mediated toxicity is also triggered by NMDA receptors, free-radical toxicity and cell death mechanisms will be examined.

NMDA glutamate receptors and neurotoxicity

The molecular structure of the NMDA receptor

NMDA receptors exist as either heterotetramer or -pentamer structures [29, 30]. Studies employing molecular cloning techniques have identified five NMDA receptor subunits, NR1and NR2A-D [29]. Each subunit is made up of four membrane domains, an extracellular amino terminal domain, and an intracellular C-terminal tail [29]. The NR1subunit contains eight functional splice variants (NR1a-h) and one nonfunctional truncated splice variant [31]. Among NR2 subunits, only NR2D has been shown to exhibit splice variants [32]. It has been shown that the receptors, Ca permeability is governed by an asparagine residue (N598) in the NR1subunit within the channel pore loop structure of the second membrane domain. This domain also determines the voltage-dependent Mg^{2+} block of NMDA receptors [33], control gating properties, potentiation and block by polyamines, inhibition by protons and Zn^{2+}, and affinity to glutamate and glycine [34, 35].

Functional heteromeric NMDA receptors require the presence of the NR1 subunit. When NR1 and NR2 subunits are coexpressed in nonneuronal cells, they form ion channels with functional and pharmacological properties similar to neuronal NMDA receptors [36, 37]. Studies using knockout mice have elucidated that although knocking out NR1 had minimal changes in the structure and function of neurons [38–40], the mice failed to survive. In contrast, NR2 A knockout mice both survived and showed normal development [41].

The NR1 subunit has many splice variants, and accordingly, a variety of NR1/NR2 combinations can be formed. The result of such multiplicity is the availability of many receptor complexes, with each exhibiting 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 [31]). In addition to the functional divergence offered by NR1, each NR2 subunit confers a unique set of characteristics upon the resultant NMDAR formation; these include sensitivity to Mg^{2+} block, glycine and glutamate affinity, and single-channel conductance [36, 37, 42].

The NR2 subunit may also produce agonist-like effects on its own receptor function. For example, Gallagher and colleagues (1997) showed using HEK 293 cultures transfected with NR1 A/NR2B chimera that specific mutations in the NR2B subunit sequence can both relieve proton inhibition and enhance polyamine stimulation of NMDA receptor complexes [42a]. Moreover, recombinant NMDAR1-NR2 A receptors have been shown to exhibit ~10-fold lower affinity for glycine relative to NMDAR1-NR2B receptors [42b, 42c]. This has also been observed in developing neurons, as the presence of the NR2B subunit during early development correlates with higher glycine affinity of NMDARs [42d].

NMDA receptor subunits exhibit variations in their intracellular C-terminal sequence. The C-terminal domains have been ascribed the role of mediating NMDA receptor interactions with multiple intracellular synaptic and cytoskeletal proteins. These form large receptor-linked multiprotein complexes in the postsynaptic density (PSD; reviewed in [43]). Interestingly, NMDA receptor interactions with PSD proteins exhibit high specificity, so that non-NMDA glutamate receptors interact with different PSD proteins altogether. This suggests that these interactions serve to link NMDA receptors to distinct downstream signaling molecules. Recent evidence suggests that specific protein-protein interactions may link glutamate-mediated Ca^{2+} fluxes through NMDA receptor channels to specific downstream signaling pathways involved with neurotoxicity (see below).

Involvement of NR1 and NR2 subunits in excitotoxicity

To date, researchers have investigated the role of the various NR1 splice variants (reviewed in [31]) in NMDA receptor function. This has been extensively observed in the context of synaptic organization and neurotransmission (e.g. [44–48]). Fewer, however, have addressed their role in excitotoxicity and survival. Kreutz and colleagues [49] studied the expression of alternatively spliced NR1 variants in the retinal ganglion cell layer following axonal trauma of the optic nerve. They found that neuronal survival following partial axonal trauma is highly dependent on the increased expression of NR1–4b. Reducing NR1–4b expression levels using antisense oligonucleotides resulted in significantly lower retinal ganglion cell survival. Based on these observations, the authors suggested that altered splicing can lead to both a different composition of the native NMDA receptor and different responses to glutamate activation. These variations in NMDA receptor compositions may be fundamental for the 'fine tuning' of neuronal responses to extracellular glutamate.

NMDA receptors are found both within synapses [50–53] as well as at extrasynaptic locations [54]. Interestingly, the final localization of NMDA receptors is in part determined by the CO domain of the NR1 subunit. The CO domain is a membrane-proximal segment of the NR1 cytoplasmic tail, which has been identified to interact with α-actinin, an actin-binding protein [55]. The anchoring of NMDA receptors to cytoskeletal elements in the synapse has been suggested to involve NR1 – α-actinin interactions. Specifically, F-actin depolymerization results in redistribution of NMDA receptor clusters to extrasynaptic sites [56–58]. Interestingly, Ca^{2+}/calmodulin has been identified to directly antagonize the binding of α-actinin to the NMDA receptor [47, 59]. Ca^{2+}/calmodulin has also been shown to bind to both the C0 and C1 segment of NR1. Its has been shown that binding of Ca^{2+}/calmodulin to NMDA receptor inhibits channel opening and reduces mean channel open time [47, 48]. In principle, the Ca^{2+} ions entering through synaptic NMDA receptors can bind to calmodulin, subsequently mediating displacement of α-actinin. When α-actinin is uncoupled from the NMDA receptor, it effectively uncouples NMDA receptors from the actin cytoskeleton. Accordingly, this event may not only result in their redistribution to extrasynaptic sites, but also play a role in optimizing synaptic function.

Actin-mediated anchoring of NMDA receptors at synaptic sites may also have bearing on the excitotoxic potential of synaptic NMDA receptors. Recent studies using cultured cortical neurons have shown that F-actin cytoskeleton depolymerization resulted in a redistribution of NMDA receptors away from dendritic spines. Depolymerizing F-actin also reduced the activity of synaptically activated NMDA receptors [60]. Although neurons treated with actin depolymerizing agents remained vulnerable to exogenously applied NMDA and glutamate, they were less vulnerable to excitotoxicity evoked by synaptic glutamate release [60]. Collectively, these results imply that the relative contributions of synaptic and extrasynaptic NMDA receptors to excitotoxicity may vary in accordance with the location of extracellular excitotoxin accumulation. Of note, the C1 cassette of NR1 subunits has also have also been identified to interact with two other proteins, Yotiao [61] and neurofilament-L [62]. However, the role of these proteins in NMDA receptor function is still unclear.

Increased resistance of immature cultured neurons to NMDA neurotoxicity is extensively documented in the current literature [18, 63–65]. It is believed that subsequent neuronal vulnerability to NMDA toxicity parallels the temporal expression pattern of different NR2 subunits (NR2B and NR2D expression may begin as early as E14, whereas NR2A and NR2C are first detected perinatally) [66–68]. Accordingly, increases in neuronal excitotoxic susceptibility with advancing maturity may be attributable to a developmental switch in NR2 subtype expression over time and ultimately dependent on neuronal activity [69, 70]. Bessho and colleagues [71] showed that K^+-induced depolarization in cerebellar granule initially promoted neuronal survival and cells upregulated the NR2A subunit of messenger RNA (mRNA) via Ca^{2+} influx through VSCCs [72, 73]. Paradoxically, these cells became increasingly vulnerable to NMDA-mediated toxicity after sustained K^+ depolarization, attributed by the authors to increased levels of NR2A and enhanced NMDA receptor-mediated Ca^{2+} influx.

Mizuta and colleagues [74] demonstrated that cortical neurons exhibited increased glutamate sensitivity on day 11 in culture, but were previously unaffected by glutamate exposure on culture days 7 through 9. Based on the findings that (i) levels of NR2B and NR1 were detected on both days 8 and 11, and (ii) NR2A protein levels were negligible on day 8 and 11, the authors concluded that glutamate toxicity in these neurons was mediated by a heteromeric NR1/NR2B receptor. Furthermore, Cheng and colleagues [75] found a temporal increase in the levels of neuronal NR1 and NR2A mRNA expression, whereas NR2B mRNA expression remained stable (following an initial increase during the first 10 days). Increases in NR2B mRNA correlated most closely with periods of glutamate-stimulated intracellular Ca^{2+} elevation and neuronal injury, suggesting a putative role for NR2B expression as a mediator of glutamate neurotoxicity. However, these findings do not preclude alternative explanations for a correlation between NR2B mRNA or protein levels and toxicity, as the aforementioned association may instead reflect simply an increase in the number of functional channels. Furthermore, there is no evidence to suggest that newly expressed NR2B subunits are necessarily incorporated into functional receptors at the plasma membrane. Experiments using heteromeric NR1-NR2 expression systems in non neuronal cell lines have shown that co transfection of NR1-NR2A resulted in more cell death than observed with NR1-NR2B co transfection. Furthermore, co transfection of NR1-NR2C receptors induced no toxicity [76, 77]. However, as nonneuronal cell lines and neurons may vary in their expression of downstream signaling pathways, the applicability of the aforementioned findings to neuronal models remains inconclusive.

The development of murine models has provided further insight into the role of distinct NR2 subunits in mediating neurotoxicity. Morikawa and colleagues [78] investigated the role of NR2A and NR2B subunits in brain ischemia in both NR2A [41, 79] and NR2A/2B knockout mice. NR2A and NR2A/2B deficiency both resulted in a pronounced reduction in infarct volume. However, no differences in infarct volume were observed between the two groups of knockouts. Although the authors interpreted these findings as suggestive of a role for NR2A in mediating glutamate neurotoxicity, the observed neuroprotection could alternatively result from a reduced number of

functional NMDA receptors (as NR2A knockout mice show decreased NMDA receptor channel activity) [41, 79]. Similar models of cerebral ischemia in NR2C knockouts were also shown to exhibit reduced injury [80]. In other studies, the expression of truncated NR2 subunits in mutant mice resulted in the production of synaptically activated gateable receptors with impaired intracellular signaling and synaptic localization [81]. These findings [82, 83] suggest that NR2 subunits link NMDA receptors to downstream mediators – thus facilitating a cellular response to the influx of ions through the NMDA receptor channel.

Many cytoplasmic PSD proteins bind with high specificity to distinct NMDA receptor subunits (reviewed in [43, 84, 85]) and 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 may be suggested that the coupling of receptor channels to downstream Ca^{2+} signaling machinery may serve to enhance the efficiency of signaling while permitting the compartmentalization of different Ca-dependent signaling pathways. This hypothesis is supported by various studies demonstrating that individual 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 [25, 26, 86, 87].

Studies demonstrate that Ca^{2+}-dependent neurotoxicity results in greater cell death when Ca^{2+} influx occurs through NMDA receptors as opposed to non-NMDA receptors or voltage-gated calcium channels (VSCCs), suggesting that discrete Ca^{2+} signaling pathways may also exist for NMDA receptor-mediated neurotoxicity [27, 28]. Furthermore, it has been hypothesized that lethal Ca^{2+} signaling by NMDA receptors is determined by the molecules with which they interact [27]. However, few 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/SAP90 subfamily of the membrane-associated guanylate kinase (MAGUK) superfamily (reviewed in [88, 89]).

MAGUKs

The MAGUK superfamily encompasses a collection of submembrane proteins involved in receptor clustering on the plasma membrane. There are several mammalian homologous families of MAGUKs, including PSD-95/synapse-associated protein 90 (SAP90) [90, 91], chapsyn-110/PSD-93 [92, 93], SAP102 [94] and SAP97/hdlg [95] (all of which are concentrated in brain synapses). MAGUKs are similar in domain organization; multiple N-terminal PDZ domains, a *src* homology (SH) domain 3 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 *Drosophila* protein found at septate junctions) and zona occludentes-1 (ZO-1, a vertebrate protein found at epithelial cell tight junctions [96] (see also [89, 97]. SH3 domains are also frequent sites of protein-protein interactions (for review see [98]). The specific function of the yeast GK homology domain is presently unclear.

Yeast two-hybrid systems have demonstrated that NMDA receptor subunits interact directly with members of the PSD-95/SAP90 family [99–101]. Specifically, the C-terminal cytoplasmic tail of NR2 subunits and certain splice variants of NR1 [102] can interact with the PDZ domains of PSD-95/SAP 90. The distal 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) and are required for interactions with the PDZ2 domain of PSD-95 [102–105]. Such interactions are paramount for both NMDA receptor clustering and synaptic targeting (reviewed in [106]).

PSD-95 family proteins are salient for synaptic organization, as they mediate the linkage of NMDA receptors to downstream signal-transduction enzymes. These interactions are achieved by either (i) PDZ domain interactions and/or by (ii) protein-protein interactions at other conserved domains of the MAGUKs. The binding of PSD-95 family members to intracellular signaling proteins is largely mediated via PDZ-PDZ domain interactions. For example, the second PDZ domain of both PSD-95 and PSD-93 [107, 108] interacts with the N-terminal PDZ domain of neuronal nitric oxide synthase (nNOS). Similarly, the PDZ2 domain of PSD-95 interacts with both the NMDA receptor C-terminus and nNOS. Although both NMDA receptors and nNOS appear to compete in vitro for binding at PDZ2 of PSD95, research has also shown that NR2 can independently bind the PDZ1 domain of PSD-95 [106]. Accordingly, PSD95 effectively mediates the linkage between NMDA receptors and nNOS. This molecular scaffold, which brings NMDARs 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 [109–112].

Involvement of PSD-95 in NMDA receptor-linked excitotoxicity

Current knowledge of PSD molecular organization suggests that NMDA receptor-mediated Ca^{2+} signals are linked to downstream neurotoxic signaling pathways through the aforementioned protein-protein interactions. Studies have demonstrated the expression of a truncated

PSD-95 mRNA had no effect on the total amounts of NR1, NR2 A and NR2B proteins. Similarly, the quantities of the PSD proteins PSD-93/chapsyn 110, SAP102 and SAP97 were unaltered in response to the expression of the mutant PSD-95. Furthermore, NMDA currents (including synaptic currents) and current-voltage relationship were also unchanged within this murine model. The distribution of NR1 as studied by electron microscopy in CA1 stratum radiatum was also unaffected. Thus, PSD-95 mutations produce no apparent compensatory alterations in either cellular protein expression or NMDA receptor function. Within this context, our research focused on elucidating a putative role for PSD-95 as an intermediary in an NMDA-associated toxic signaling cascade. Using murine cortical neuronal cultures, the expression of PSD-95 was suppressed with antisense oligonucleotides. Consistent with the aforementioned PSD-95 mutant studies, no changes were observed in NMDA receptor function, expression, current or Ca^{2+} loading. However, suppression of PSD-95 selectively attenuated Ca^{2+}-activated NO production by NMDA receptors (without affecting nNOS expression), resulting in the amelioration of NMDA-triggered excitotoxicity [113]. These findings suggest that PSD-95 is required for efficient coupling of NMDA receptor activity to NO toxicity, and imparts specificity to NMDA receptor-mediated excitotoxic Ca^{2+} signaling.

A new approach to uncouple NMDA receptors from downstream neurotoxic cascades

More recently, we investigated the possibility that the NMDAR/PSD-95 interaction might constitute a therapeutic target for diseases that involve excitotoxicity. Although NMDA receptors are implicated in mediating ischemic and traumatic brain injuries, direct and long-lasting inhibition of glutamate receptors in an attempt to reduce the damage is problematic. This may be because NMDA receptors are also responsible for mediating neuronal excitation that is essential to normal physiological CNS functioning. In light of this, the search for agents that act on NMDA receptors is currently focused on developing means to briefly block NMDARs and/or develop methods to allosterically modulate receptor functioning.

Targeting PSD-95/NMDA receptor interactions may represent an alternative therapeutic approach that may circumvent the negative consequences of blocking NMDAR function. Because suppression of PSD-95 is impractical as a therapy for brain injury and cannot be applied post-injury, we questioned whether interfering with the NMDAR/PSD-95 interaction could suppress excitotoxicity in vitro and ischemic brain damage in vivo. To treat both neurotoxicity and stroke without blocking NMDARs, neurons in vitro and in vivo were transduced with peptides that bind to modular domains that mediate classical NMDAR-PSD95 interactions. We constructed a peptide that is anticipated to bind the PDZ2 domain of PSD-95. Specifically, the peptide is comprised of the nine C-terminal residues of NR2B (KLSSIESDV; NR2B9c). We found that the NR2B9c peptide failed to transduce cells on its own. Accordingly, 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). It has been previously shown that this strategy permits proteins to transduce cell membranes in a rapid, dose-dependent manner independent of receptors and transporters (see [114]). We found that 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. Interestingly, the treatment was effective when applied either before, or 1 h after, the onset of excitotoxicity in vitro and cerebral ischemia in vivo [115]. We believe that similar strategies, based on a molecular understanding of excitotoxic mechanisms, may be employed to study and modulate signaling pathways responsible for other components of traumatic and ischemic CNS injury.

Role of ROS in executing glutamate neurotoxicity

As previously mentioned, elevated extracellular glutamate plays a central role in mediating both ischemic brain damage [20] and traumatic brain injury [21]. The resultant effect of excess glutamate is the prolonged activation of glutamate receptors and, thus, increased intracellular Ca^{2+} levels. Mitochondria are unique among cellular organelles by virtue of their oxygen consumption [116], production of free radicals [116] and mobilization of intracellular Ca^{2+} [116]. Under conditions of excess intracellular Ca^{2+}, mitochondria are very important for cell survival, as they have the ability to sequester large amounts of Ca^{2+}. However, abnormal Ca^{2+} accumulation by mitochondria is also a cause for mitochondrial dysfunction [117]. Ca^{2+} is sequestered into the mitochondria matrix via a proton electrochemical gradient generated by the electron transport chain, thus depolarizing the mitochondrial potential [118–120]. This influx of Ca^{2+} decreases the electrochemical gradient, and subsequently reduces ATP synthesis. In response to elevated Ca^{2+}, cells redirect ATP expenditure to drive plasma membrane Ca^{2+} pumps to extrude Ca^{2+}. The concurrent accumulation of intramitochondrial Ca^{2+}, reduced ATP synthesis and increased ATP usage has been suggested to be a primary cause of cell death [116]. In addition to Ca^{2+}-mediated reduction in mitochondrial ATP production, aberrations in mitochondrial electron chain functioning can result in

excessive ROS production, leading to neurotoxicity [121–124]. Increased superoxide anion production (O_2^-) has been documented in both ischemic insults and traumatic brain injury [125].

ROS are also produced in the neuronal cytoplasm following elevations in intracellular Ca^{2+}. The Ca^{2+}-induced activation of proteases mediates the conversion of xanthine dehdrogenase (XDH) into xanthine oxidase (XO). XO uses molecular oxygen as an electron acceptor, thus resulting in elevations of O_2^- [126]. Rises in intracellular Ca also activate phospholipases, such as phospholipase A2, that release arachidonic acid [127]. Arachidonic acid metabolism by oxidases results in production of oxygen free radicals [128, 129]. Glutamatergic-induced ROS production can also be mediated by a pathway specifically associated with the NMDA receptor subtype. Neuronal NO production is linked with NMDA activation via the postsynaptic density scaffolding protein, postsynaptic density 95 and neuronal nitric oxide (NOS1) [130] (see above).

The accumulation of intracellular ROS by many of the aforementioned mechanisms can mediate cellular destruction and cause the cell to die. It has been argued that NMDA receptor-mediated neurotoxicity results from overproduction of both NO and O_2^-. The reaction product of these two species is peroxynitrite ($ONOO^-$), a chemically complex molecule that has the activity of both the hydroxyl radical and the nitrogen dioxide radical [131]. Peroxynitrite can nitrate and hydroxylate aromatic rings on amino acid residues [132], and oxidize lipids [133], protein and DNA [134, 135]. Both NO and $ONOO^-$ can damage DNA, leading to activation of the nuclear DNA repair enzyme, poly (ADP ribose) synthase (PARS). Although PARS activity is centered on repairing damaged DNA, this enzyme is both energy dependent and highly promiscuous. Thus, prolonged PARS activity can rapidly deplete cellular energy reserves. In addition to DNA damage, NO/$ONOO^-$ has been shown to inhibit mitochondrial respiratory chain enzymes.

Traumatic brain injury [136], ischemia [137] and NMDA receptor-mediated neurotoxicity have all been described to induce apoptosis [138] in certain cell populations. In many cases, apoptosis is initiated via mitochondrial release of cytochrome c, a component of the mitochondrial electron transport chain [139]. Cytochrome c release from the mitochondria is, in part, mediated through both free radical-dependent mechanisms and/or mitochondrial pores triggered by Bax oligomerization (for review see [140]). Cytoplasmic cytochrome c has been shown to couple with apoptosis protease-activating factor-1 (Apaf-1), a cytosolic protein containing a caspase recruitment domain (CARD) [141]. Subsequent to their interaction, the CARD domain becomes exposed, leading to both, recruitment and autoactivation of multiple procaspase 9 molecules. Active caspase 9 then cleaves procaspase 3 into caspase 3 (active form of the enzyme), a salient effector enzyme characteristic of neuronal apoptosis [139, 142].

Inactive caspase 3 exists as a 32-kDa proenzyme (CPP32), and activation generates large (17–24 kDa) [143, 144] and small (10–12 kDa) fragments [145]. In the resting state, the caspase-activated DNase, also known as DNA fragmentation factor-40/caspase-3-activated DNase (DFF40/CAD) [146], remains inactive via interactions with the inhibitory subunit DNA fragmentation factor-45/inhibitor of caspase-3-activated DNase (DFF45/ICAD) by forming an inactive complex. Upon interaction between activated caspase-3 and the inactive complex, the inhibitory DFF45/ICAD protein is cleaved into 24- and 12-kDa fragments [143], thus releasing DFF40/CAD. DFF40/CAD subsequently translocates to the nucleus and cleaves genomic DNA – a salient step in apoptosis [144]. The hallmarks of apoptosis include somal shrinkage and neuronal condensation, externalization of phosphatidylserine, nuclear membrane breakdown, internucleosomal DNA cleavage and the formation of apoptotic bodies ([147]; for review see [148]).

Apart from cytochrome c release, research indicates that the tumor suppressor gene p53 is an important upstream initiator of apoptosis following neuronal injury. Specifically, it has been shown that p53 is upregulated in response to excitotoxins, hypoxia and ischemia [149–151], and neurotrauma [152, 153]. Following oxidative DNA damage p53 levels increase and trigger apoptosis through the upregulation of several regulatory enzymes, including Bax, CD95 and DR5 (a receptor for the death ligand TRAIL), which are all classical members of the core apoptosis pathways (for review see [140]).

In addition to caspase-mediated cell death mechanisms, researchers have recently discovered caspase-independent forms of programmed cell death. These mechanisms involve the release of mitochondrial proteins such as endonuclease g (endo g) or apoptosis inducing factor (AIF) subsequent to mitochondrial depolarization. Mitochondrial release of endo g and AIF initiates an apoptotic program that occurs concurrent with those executed by caspase activity (for review see [140]). Endo g is a 30-kDa nuclease that has been proposed to function in mitochondrial DNA replication ([154], for review see [140]). However, once released from the mitochondria, endo g is capable of inducing nuclear DNA cleavage through caspase-independent mechanisms [155–157]. Nuclear translocation of AIF is associated with large-scale (50–700 kbp) DNA fragmentation, and does not produce classical apoptotic features such as internucleosomal DNA fragmentation, somal shrinkage or nuclear condensation [158]. Recent work by Zhang and colleagues (2002) suggests that neuronal death subsequent to experimental brain injury involves AIF translocation from the mitochondria to cell nuclei [159]. Interestingly, they also found that the direct application of $ONOO^-$ to cortical

neurons leads to mitochondrial release of AIF [159]. These findings suggest that NMDAR-mediated excitotoxicity may be associated with mitochondrial AIF release, as $ONOO^-$ is a potent free radical specifically associated with mitochondrial depolarization, superoxide generation and NMDA receptor-mediated NO production. As can be inferred from the aforementioned, ROSs are important contributors to glutamate-mediated excitotoxicity, as their activity can either directly or indirectly mediate toxicity. Although the downstream consequences associated with enhanced ROS production are varied, they all appear to impinge on neuronal integrity.

Concluding remarks

To date, consensus suggests that glutamate-mediated excitotoxicity is involved in many brain pathologies such as ischemia and traumatic brain injury. Recent advances have provided a more comprehensive understanding of the mechanisms governing glutamate-mediated neurotoxicity. Among the consequences of glutamate-mediated Ca^{2+} overload, the activation of potentially lethal second messengers and enzymes, mitochondrial dysfunction and free-radical formation are known key players in executing cell death. More recently, Ca^{2+}-dependent neurotoxicity has been identified to occur via distinct intracellular signaling pathways. This is likely mediated through physical interactions of cell membrane receptors with specialized submembrane molecules. Thus, the molecular characterization of neurotoxic signal-transduction pathways is a critical step for the identification of potential therapeutic targets for future pharmacological and genetic intervention strategies.

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1 Faden A. I., Demediuk P., Panter S. S. and Vink R. (1989) The role of excitatory amino acids and NMDA receptors in traumatic brain injury. Science **244:** 798–800
2 Simon R. P., Swan J. H. and Meldrum B. S. (1984) Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. Science **226:** 850–852
3 Ozyurt E., Graham D. I., Woodruff G. N. and McCulloch J. (1988) Protective effect of the glutamate antagonist, MK-801 in focal cerebral ischemia in the cat. J. Cereb. Blood Flow. Metab. **8:** 138–143
4 Choi D. W. (1985) Glutamate neurotoxicity in cortical cell culture is calcium dependent. Neurosci. Lett. **58:** 293–297
5 Choi D. W. (1987) Ionic dependence of glutamate neurotoxicity. J. Neurosci. **7:** 369–379
6 Choi D. W. (1995) Calcium: still center-stage in hypoxic-ischemic neuronal death. Trends Neurosci. **18:** 58–60
7 Choi D. W. (1988) Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. Trends Neurosci. **11:** 465–467
8 Tymianski M. (1996) Cytosolic calcium concentrations and cell death in vitro. In: Advances in Neurology: Cellular and Molecular Mechanisms of Ischemic Brain Damage, Siesjo B. K. and Wieloch T. (eds), pp. 85–105, Lippincott-Raven, Philadelphia
9 Lucas D. R. and Newhouse J. P. (1957) The toxic effect of sodium L-glutamate on the inner layers of the retina. Arch. Ophthalmol. **58:** 193–201
10 Olney J. W. (1978) Neurotoxicity of excitatory amino acids. In: Kainic Acid as a Tool in Neurobiology, McGeer E. G., Olney J. W. and McGeer P. L. (eds), Raven Press, pp. 95–121, New York
11 Olney J. W. (1969) Brain lesion, obesity and other disturbances in mice treated with monosodium glutamate. Science **164:** 719–721
12 Kass I. S. and Lipton P. (1982) Mechanisms involved in irreversible anoxic damage to the in vitro rat hippocampal slice. J. Physiol. **332:** 459–472
13 Rothman S. M. (1983) Synaptic activity mediates death of hypoxic neurons. Science **220:** 536–537
14 de Weille J. R., Schweitz H., Maes P., Tartar A. and Lazdunski M. (1991) Calciseptine, a peptide isolated from black mamba venom, is a specific blocker of the L-type calcium channel. Proc. Natl. Acad. Sci. USA **88:** 2437–2440
15 Rothman S. M. (1985) The neurotoxicity of excitatory amino acids is produced by passive chloride influx. J. Neurosci. **5:** 1483–1489
16 Olney J. W., Price M. T., Samson L. and Labruyere J. (1986) The role of specific ions in glutamate neurotoxicity. Neurosci. Lett. **65:** 65–71
17 Berdichevsky E., Riveros N., Sanches-Armass S. and Orrego F. (1983) Kainate, N-methylaspartate and other excitatory amino acids increase calcium influx onto rat brain cortex cells in vitro. Neurosci. Lett. **36:** 75–80
18 Choi D. W. (1987) Maulucci-Gedde M, Kriegstein AR. Glutamate neurotoxicity in cortical cell culture. J. Neurosci. **7:** 357–368
19 Tymianski M. and Tator C. H. (1996) Normal and abnormal calcium homeostasis in neurons: a basis for the pathophysiology of traumatic and ischemic central nervous system injury. Neurosurgery **38:** 1176–1195
20 Hossmann K. A. (1994) Glutamate-mediated injury in focal cerebral ischemia: the excitotoxin hypothesis revised. Brain Pathol. **4:** 23–36
21 Siesjo B. K., Zhao Q., Pahlmark K., Siesjo P., Katsura K., Folbergrova J. (1995) Glutamate, calcium and free radicals as mediators of ischemic brain damage. Ann. Thorac. Surg. **59:** 1316–1320
22 Zhang L., Rzigalinski B. A., Ellis E. F. and Satin L. S. (1996) Reduction of voltage-dependent Mg^{2+} blockade of NMDA current in mechanically injured neurons. Science **274:** 1921–1923
23 Goforth P. B., Ellis E. F. and Satin L. S. (1999) Enhancement of AMPA-mediated current after traumatic injury in cortical neurons. J. Neurosci. **19:** 7367–7374
24 Lees K. R. (1998) Does neuroprotection improve stroke outcome? [see comments]. Lancet **351:** 1447–1448
25 Ghosh A. and Greenberg M. E. (1995) Calcium signalling in neurons: Molecular mechanisms and cellular consequences. Science **268:** 239–247
26 Bading H., Ginty D. D. and Greenberg M. E. (1993) Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. Science **260:** 181–186
27 Tymianski M., Charlton M. P., Carlen P. L. and Tator C. H. (1993) Source specificity of early calcium neurotoxicity in-cultured embryonic spinal neurons. J. Neurosci. **13:** 2085–2104
28 Sattler R., Charlton M. P., Hafner M. and Tymianski M. (1998) Distinct influx pathways, not calcium load, determine neuronal vulnerability to calcium neurotoxicity. J. Neurochem. **71:** 2349–2364

29 Hollmann M. and Heinemann S. (1994) Cloned glutamate receptors. Ann. Rev. Neurosci. **17:** 31–108

30 Laube B., Kuhse J. and Betz H. (1998) Evidence for a tetrameric structure of recombinant NMDA receptors. J. Neurosci. **18:** 2954–2961

31 Zukin R. S. and Bennett M. V. (1995) Alternatively spliced isoforms of the NMDARI receptor subunit. Trends Neurosci. **18:** 306–313

32 Ishii T., Moriyoshi K., Sugihara H., Sakurada K., Kadotani H., Yokoi M. et al. (1993) Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. J. Biol. Chem. **268:** 2836–2843

33 Burnashev N., Schoepfer R., Monyer H., Ruppersberg J. P., Gunther N., Seeburg P. H. et al. (1992) Control by asparagine residues of calcium permeability and magnesium blockade in the NMDA receptor. Science **257:** 1415–1419

34 Schneggenburger R. and Ascher P. (1997) Coupling of permeation and gating in an NMDA-channel pore mutant. Neuron **18:** 167–177

35 Traynelis S. F, Burgess M. F., Zheng F., Lyuboslavsky P. and Powers J. L. (1998) Control of voltage-independent zinc inhibition of NMDA receptors by the NR1 subunit. J. Neurosci. **18:** 6163–6175

36 Monyer H., Sprengel R., Schoepfer R., Herb A., Higuchi M., Lomeli H. et al. (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science **256:** 1217–1221

37 Kutsuwada T., Kashiwabuchi N., Mori H., Sakimura K., Kushiya E., Araki K. et al. (1992) Molecular diversity of the NMDA receptor channel. Nature **358:** 36–41

38 Forrest D., Yuzaki M., Soares H. D., Ng L., Luk D. C., Sheng M. et al. (1994) Targeted disruption of NMDA receptor 1 gene abolishes NMDA response and results in neonatal death. Neuron **13:** 325–338

39 Yuzaki M., Forrest D., Verselis L. M., Sun S. C., Curran T. and Connor J. A. (1996) Functional NMDA receptors are transiently active and support the survival of Purkinje cells in culture. J. Neurosci. **16:** 4651–4661

40 Li Y., Erzurumlu R. S., Chen C., Jhaveri S. and Tonegawa S. (1994) Whisker-related neuronal patterns fail to develop in the trigeminal brainstem nuclei of NMDAR1 knockout mice. Cell **76:** 427–437

41 Sakimura K., Kutsuwada T., Ito I., Manabe T., Takayama C., Kushiya E. et al. (1995) Reduced hippocampal LTP and spatial learning in mice lacking NMDA receptor epsilon 1 subunit. Nature **373:** 151–155

42 Stern P., Behe P., Schoepfer R. and Colquhoun D. (1992) Single-channel conductances of NMDA receptors expressed from cloned cDNAs: comparison with native receptors. Proc. R. Soc. Lond. B Biol. Sci. **250:** 271–277

42a. Gallagher M. J., Huang H., Grant E. R. and Lynch D. R. (1997) The NR2B-specific interactions of polyamines and protons with the N-methyl-D-aspartate receptor. J. Biol. Chem. **272:** 24971–24979

42b. Kutsuwada T., Kashiwabuchi N., Mori H., Sakimura K., Kushiya E., Araki K. et al. (1992) Molecular diversity of the NMDA receptor channel. Nature **358:** 36–41

42c. Williams K., Russell S. L., Shen Y. M. and Molinoff P. B. (1993) Developmental switch in the expression of NMDA receptors occurs in vivo and in vitro. Neuron **10:** 267–278

42d. Kew J. N. C., Richards J. G., Mutel V. and Kemp J. A. (1998) Developmental changes in NMDA receptor glycine affinity and ifenprodil sensitivity reveal three distinct populations of NMDA receptors in individual rat cortical neurons. J. Neurosci. **18:** 1935–1943

43 Sheng M. and Pak D. T. (2000) Ligand-gated ion channel interactions with cytoskeletal and signaling proteins. Annu. Rev. Physiol. **62:** 755–778

44 Standley S., Roche K. W., McCallum J., Sans N. and Wenthold R. J. (2000) PDZ domain suppression of an ER retention signal in NMDA receptor NR1 splice variants. Neuron **28:** 887–898

45 Okabe S., Miwa A. and Okado H. (1999) Alternative splicing of the C-terminal domain regulates cell surface expression of the NMDA receptor NR1 subunit. J. Neurosci. **19:** 7781–7792

46 Logan S. M., Rivera F. E. and Leonard J. P. (1999) Protein kinase C modulation of recombinant NMDA receptor currents: roles for the C-terminal C1 exon and calcium ions. J. Neurosci. **19:** 974–986

47 Ehlers M. D., Zhang S., Bernhardt J. P. and Huganir R. L. (1996) Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. Cell **84:** 745–755

48 Zhang S., Ehlers M. D., Bernhardt J. P., Su C. T. and Huganir R. L. (1998) Calmodulin mediates calcium-dependent inactivation of N-methyl-D-aspartate receptors. Neuron **21:** 443–453

49 Kreutz M. R., Bockers T. M., Bockmann J., Seidenbecher C. I., Kracht B., Vorwerk C. K. et al. (1998) Axonal injury alters alternative splicing of the retinal NR1 receptor: the preferential expression of the NR1b isoforms is crucial for retinal ganglion cell survival. J. Neurosci. **18:** 8278–8291

50 Halpain S., Hipolito A. and Saffer L. (1998) Regulation of F-actin stability in dendritic spines by glutamate receptors and calcineurin. J. Neurosci. **18:** 9835–9844

51 Jiang M., Lee C. L., Smith K. L. and Swann J. W. (1998) Spine loss and other persistent alterations of hippocampal pyramidal cell dendrites in a model of early-onset epilepsy. J. Neurosci. **18:** 8356–8368

52 Jeffrey M., Goodsir C. M., Bruce M. E., McBride P. A. and Fraser J. R. (1997) In vivo toxicity of prion protein in murine scrapie: ultrastructural and immunogold studies. Neuropathol. Appl. Neurobiol. **23:** 93–101

53 Garey L. J., Ong W. Y., Patel T. S., Kanani M., Davis A., Mortimer A. M. et al. (1998) Reduced dendritic spine density on cerebral cortical pyramidal neurons in schizophrenia [see comments]. J. Neurol. Neurosurg. Psychiatr. **65:** 446–453

54 Rao A. and Craig A. M. (1997) Activity regulates the synaptic localization of the NMDA receptor in hippocampal neurons. Neuron **19:** 801–812

55 Wyszynski M., Lin J., Rao A., Nigh E., Beggs A. H., Craig A. M. et al. (1997) Competitive binding of alpha-actinin and calmodulin to the NMDA receptor. Nature **385:** 439–442

56 Matus A., Ackermann M., Pehling G., Byers H. R. and Fujiwara K. (1982) High actin concentrations in brain dendritic spines and postsynaptic densities. Proc. Natl. Acad. Sci. USA **79:** 7590–7594

57 Kaech S., Fischer M., Doll T. and Matus A. (1997) Isoform specificity in the relationship of actin to dendritic spines. J. Neurosci. **17:** 9565–9572.

58 Allison D. W., Gelfand V. I., Spector I. and Craig A. M. (1998) Role of actin in anchoring postsynaptic receptors in cultured hippocampal neurons: differential attachment of NMDA versus AMPA receptors. J. Neurosci. **18:** 2423–2436

59 Krupp J. J., Vissel B., Thomas C. G., Heinemann S. F. and Westbrook G. L. (1999) Interactions of calmodulin and alpha-actinin with the NR1 subunit modulate Ca^{2+}-dependent inactivation of NMDA receptors. J. Neurosci. **19:** 1165–1178

60 Sattler R., Xiong Z., Lu W. Y., MacDonald J. F. and Tymianski M. (2000) Distinct roles of synaptic and extrasynaptic NMDA receptors in excitotoxicity. J. Neurosci. **20:** 22–33

61 Lin J. W., Wyszynski M., Madhavan R., Sealock R., Kim J. U. and Sheng M. (1998) Yotiao, a novel protein of neuromuscular junction and brain that interacts with specific splice variants of NMDA receptor subunit NR1. J. Neurosci. **18:** 2017–2027

62 Ehlers M. D., Fung E. T., O'Brien R. and Huganir R. L. (1998) Splice variant-specific interaction of the NMDA receptor sub-

unit NR1 with neuronal intermediate filaments. J. Neurosci. **18:** 720–730
63 Barish M. E. and Mansdorf N. B. (1991) Development of intracellular calcium responses to depolarization and to kainate and N-methyl-D-aspartate in cultured mouse hippocampal neurons. Brain Res. Dev. Brain Res. **63:** 53–61
64 Marks J. D., Friedman J. E. and Haddad G. G. (1996) Vulnerability of CA1 neurons to glutamate is developmentally regulated. Brain Res. Dev. Brain Res. **97:** 194–206
65 Wahl P., Schousboe A., Honore T. and Drejer J. (1989) Glutamate-induced increase in intracellular Ca2+ in cerebral cortex neurons is transient in immature cells but permanent in mature cells. J. Neurochem. **53:** 1316–1319
66 Monyer H., Burnashev N., Laurie D. J., Sakmann B. and Seeburg P. H. (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron **12:** 529–540
67 Sheng M., Cummings J., Roldan L. A., Jan Y. N. and Jan L. Y. (1994) Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. Nature **368:** 144–147
68 Wenzel A., Fritschy J. M., Mohler H. and Benke D. (1997) NMDA receptor heterogeneity during postnatal development of the rat brain: differential expression of the NR2 A, NR2B and NR2C subunit proteins. J. Neurochem. **68:** 469–478
69 Audinat E., Lambolez B., Rossier J. and Crepel F. (1994) Activity-dependent regulation of N-methyl-D-aspartate receptor subunit expression in rat cerebellar granule cells. Eur. J. Neurosci. **6:** 1792–1800
70 Lindlbauer R., Mohrmann R., Hatt H. and Gottmann K. (1998) Regulation of kinetic and pharmacological properties of synaptic NMDA receptors depends on presynaptic exocytosis in rat hippocampal neurones. J. Physiol. **508 (Pt 2):** 495–502
71 Bessho Y., Nawa H. and Nakanishi S. (1994) Selective up-regulation of an NMDA receptor subunit mRNA in cultured cerebellar granule cells by K(+)-induced depolarization and NMDA treatment. Neuron **12:** 87–95
72 Gallo V., Suergiu R. and Levi G. (1987) Functional evaluation of glutamate receptor subtypes in cultured cerebellar neurones and astrocytes. Eur. J. Pharmacol. **138:** 293–297
73 Balazs R., Jorgensen O. S. and Hack N. (1988) N-methyl-D-aspartate promotes the survival of cerebellar granule cells in culture. Neuroscience **27:** 437–451
74 Mizuta I., Katayama M., Watanabe M., Mishina M. and Ishii K. (1998) Developmental expression of NMDA receptor subunits and the emergence of glutamate neurotoxicity in primary cultures of murine cerebral cortical neurons. Cell. Mol. Life Sci. **54:** 721–725
75 Cheng C., Fass D. M. and Reynolds I. J. (1999) Emergence of excitotoxicity in cultured forebrain neurons coincides with larger glutamate-stimulated [Ca(2+)](i) increases and NMDA receptor mRNA levels. Brain Res. **849:** 97–108
76 Anegawa N. J., Lynch D. R., Verdoorn T. A. and Pritchett D. B. (1995) Transfection of N-methyl-D-aspartate receptors in a nonneuronal cell line leads to cell death. J. Neurochem. **64:** 2004–2012
77 Chazot P. L., Coleman S. K., Cik M. and Stephenson F. A. (1994) Molecular characterization of N-methyl-D-aspartate receptors expressed in mammalian cells yields evidence for the coexistence of three subunit types within a discrete receptor molecule. J. Biol. Chem. **269:** 24403–24409
78 Morikawa E., Mori H., Kiyama Y., Mishina M., Asano T. and Kirino T. (1998) Attenuation of focal ischemic brain injury in mice deficient in the epsilon1 (NR2 A) subunit of NMDA receptor. J. Neurosci. **18:** 9727–9732
79 Kiyama Y., Manabe T., Sakimura K., Kawakami F., Mori H. and Mishina M. (1998) Increased thresholds for long-term potentiation and contextual learning in mice lacking the NMDA-type glutamate receptor epsilon1 subunit. J. Neurosci. **18:** 6704–6712
80 Kadotani H., Namura S., Katsuura G., Terashima T. and Kikuchi H. (1998) Attenuation of focal cerebral infarct in mice lacking NMDA receptor subunit NR2C. Neuroreport **9:** 471–475
81 Sprengel R., Suchanek B., Amico C., Brusa R., Burnashev N., Rogov A. et al. (1998) Importance of the intracellular domain of NR2 subunits for NMDA receptor function in vivo. Cell **92:** 279–289
82 Mori H., Manabe T., Watanabe M., Satoh Y., Suzuki N., Toki S. et al. (1998) Role of the carboxy-terminal region of the GluR epsilon2 subunit in synaptic localization of the NMDA receptor channel. Neuron **21:** 571–580
83 Steigerwald F., Schulz T. W., Schenker L. T., Kennedy M. B., Seeburg P. H., Kohr G. (2000) C-Terminal truncation of NR2 A subunits impairs synaptic but not extrasynaptic localization of NMDA receptors. J. Neurosci. **20:** 4573–4581
84 Garner C. C., Nash J. and Huganir R. L. (2000) PDZ domains in synapse assembly and signalling. Trends Cell Biol. **10:** 274–280
85 Scannevin R. H. and Huganir R. L. (2000) Postsynaptic organization and regulation of excitatory synapses. Nat. Rev. Neurosci. **1:** 133–141
86 Lerea L. S. and McNamara J. O. (1993) Ionotropic glutamate receptor subtypes activate c-fos transcription by distinct calcium-requiring intracellular signalling pathways. Neuron **10:** 31–41
87 Friedlander R. M., Gagliardini V., Hara H., Fink K. B., Li W., MacDonald G. et al. (1997) Expression of a dominant negative mutant of interleukin-1 beta converting enzyme in transgenic mice prevents neuronal cell death induced by trophic factor withdrawal and ischemic brain injury. J. Exp. Med. **185:** 933–940
88 Sheng M. (1997) Glutamate receptors put in their place. Nature **386:** 221–223
89 Ponting C. P., Phillips C., Davies K. E. and Blake D. J. (1997) PDZ domains: targeting signalling molecules to sub-membranous sites. Bioessays **19:** 469–479
90 Cho K. O., Hunt C. A. and Kennedy M. B. (1992) The rat brain postsynaptic density fraction contains a homolog of the Drosophila discs-large tumor suppressor protein. Neuron **9:** 929–942
91 Kistner U., Wenzel B. M., Veh R. W., Cases-Langhoff C., Garner A. M., Appeltauer U. et al. (1993) SAP90, a rat presynaptic protein related to the product of the Drosophila tumor suppressor gene dlg-A. J. Biol. Chem. **268:** 4580–4583
92 Brenman J. E., Christopherson K. S., Craven S. E., McGee A. W. and Bredt D. S. (1996) Cloning and characterization of postsynaptic density 93, a nitric oxide synthase interacting protein. J. Neurosci. **16:** 7407–7415
93 Kim E. and Sheng M. (1996) Differential K+ channel clustering activity of PSD-95 and SAP97, two related membrane-associated putative guanylate kinases. Neuropharmacology **35:** 993–1000
94 Muller B. M., Kistner U., Kindler S., Chung W. J., Kuhlendahl S., Fenster S. D. et al. (1996) SAP102, a novel postsynaptic protein that interacts with NMDA receptor complexes in vivo. Neuron **17:** 255–265
95 Muller B. M., Kistner U., Veh R. W., Cases-Langhoff C., Becker B., Gundelfinger E. D. et al. (1995) Molecular characterization and spatial distribution of SAP97, a novel presynaptic protein homologous to SAP90 and the *Drosophila* discs-large tumor suppressor protein. J. Neurosci. **15:** 2354–2366
96 Kennedy M. B. (1995) Origin of PDZ (DHR, GLGF) domains [letter; comment]. Trends Biochem. Sci. **20:** 350–350
97 Fanning A. S. and Anderson J. M. (1996) Protein-protein interactions: PDZ domain networks. Curr. Biol. **6:** 1385–1388

98 Pawson T. (1995) Protein modules and signalling networks. Nature **373:** 573–579

99 Ehlers M. D., Mammen A. L., Lau L. F. and Huganir R. L. (1996) Synaptic targeting of glutamate receptors. Curr. Opin. Cell Biol. **8:** 484–489

100 Gomperts S. N. (1996) Clustering membrane proteins: It's all coming together with the PSD-95/SAP90 protein family. Cell **84:** 659–662

101 Kornau H. C., Seeburg P. H. and Kennedy M. B. (1997) Interaction of ion channels and receptors with PDZ domain proteins. Curr. Opin. Neurobiol. **7:** 368–373

102 Kornau H. C., Schenker L. T., Kennedy M. B. and Seeburg P. H. (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. Science **269:** 1737–1740

103 Kim E., Niethammer M., Rothschild A., Jan Y. N. and Sheng M. (1995) Clustering of Shaker-type K+ channels by interaction with a family of membrane-associated guanylate kinases. Nature **378:** 85–88

104 Doyle D. A., Lee A., Lewis J., Kim E., Sheng M. and MacKinnon R. (1996) Crystal structures of a complexed and peptide-free membrane protein-binding domain: molecular basis of peptide recognition by PDZ. Cell **85:** 1067–1076

105 Morais Cabral J. H., Petosa C., Sutcliffe M. J., Raza S., Byron O., Poy F. et al. (1996) Crystal structure of a PDZ domain. Nature **382:** 649–652

106 Niethammer M., Kim E. and Sheng M. (1996) Interaction between the C terminus of NMDA receptor subunits and multiple members of the PSD-95 family of membrane-associated guanylate kinases. J. Neurosci. **16:** 2157–2163

107 Brenman J. E., Chao D. S., Gee S. H., McGee A. W., Craven S. E., Santillano D. R. et al. (1996) Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. Cell **84:** 757–767

108 Stricker N. L., Christopherson K. S., Yi B. A., Schatz P. J., Raab R. W., Dawes G. et al. (1997) PDZ domain of neuronal nitric oxide synthase recognizes novel C-terminal peptide sequences. Nat. Biotechnol. **15:** 336–342

109 Craven S. E. and Bredt D. S. (1998) PDZ proteins organize synaptic signaling pathways. Cell **93:** 495–498

110 Bredt D. S. and Snyder S. H. (1990) Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc. Natl. Acad. Sci. USA **87:** 682–685

111 Bredt D. S. and Snyder S. H. (1989) Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc. Natl. Acad. Sci. USA **86:** 9030–9033

112 Garthwaite J., Charles S. L. and Chess-Williams R. (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as inter-cellular messenger in the brain. Nature **336:** 385–388

113 Sattler R., Xiong Z., Lu W. Y., Hafner M., MacDonald J. F., Tymianski M. (1999) Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. Science **284:** 1845–1848

114 Schwarze S. R. and Dowdy S. F. (2000) In vivo protein transduction: intracellular delivery of biologically active proteins, compounds and DNA. Trends Pharmacol. Sci. **21:** 45–48

115 Aarts M., Liu Y., Liu L., Besshoh S., Arundine M., Gurd J. W. et al. (2002) Treatment of ischemic brain damage by perturbing NMDA rece. Science **298:** 846–850

116 Schinder A. F., Olson E. C., Spitzer N. C. and Montal M. (1996) Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. J. Neurosci. **16:** 6125–6133

117 Atlante A, Calissano P, Bobba A, Giannattasio S, Marra E, Passarella S. (2001) Glutamate neurotoxicity, oxidative stress and mitochondria. FEBS Lett. **497:** 1–5

118 Akerman K. E. (1978) Changes in membrane potential during calcium ion influx and efflux across the mitochondrial membrane. Biochim. Biophys. Acta **502:** 359–366

119 Gunter T. E. and Pfeiffer D. R. (1990) Mechanisms by which mitochondria transport calcium. Am. J. Physiol. **258:** C755–C786

120 Loew L. M., Carrington W., Tuft R. A. and Fay F. S. (1994) Physiological cytosolic Ca2+ transients evoke concurrent mitochondrial depolarizations. Proc. Natl. Acad. Sci. USA **91:** 12579–12583

121 Bauer M. K., Vogt M., Los M., Siegel J., Wesselborg S. and Schulze-Osthoff K. (1998) Role of reactive oxygen intermediates in activation-induced CD95 (APO-1/Fas) ligand expression. J. Biol. Chem. **273:** 8048–8055

122 Green D. R. and Reed J. C. (1998) Mitochondria and apoptosis. Science **281:** 1309–1312

123 Antonsson B., Conti F., Ciavatta A., Montessuit S., Lewis S., Martinou I. et al. (1997) Inhibition of Bax channel-forming activity by Bcl-2. Science **277:** 370–372

124 Zamzami N., Marchetti P., Castedo M., Decaudin D., Macho A., Hirsch T. et al. (1995) Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J. Exp. Med. **182:** 367–377

125 Kumar C., Okuda M., Ikai I. and Chance B. (1990) Luminol enhanced chemiluminescence of the perfused rat heart during ischemia and reperfusion. FEBS Lett. **272:** 121–124

126 Sussman M. S. and Bulkley G. B. (1990) Oxygen-derived free radicals in reperfusion injury. Methods Enzymol. **186:** 711–723

127 Juurlink B. H. (1997) Response of glial cells to ischemia: roles of reactive oxygen species and glutathione. Neurosci. Biobehav. Rev. **21:** 151–166

128 Chanock S. J., el Benna J., Smith R. M. and Babior B. M. (1994) The respiratory burst oxidase. J. Biol. Chem. **269:** 24519–24522

129 Shami P. J., Sauls D. L. and Weinberg J. B. (1998) Schedule and concentration-dependent induction of apoptosis in leukemia cells by nitric oxide. Leukemia **12:** 1461–1466

130 Sattler R., Xiong Z., Lu W. Y., Hafner M., MacDonald J. F. and Tymianski M. (1999) Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. Science **284:** 1845–1848

131 Koppenol W. H., Moreno J. J., Pryor W. A., Ischiropoulos H. and Beckman J. S. (1992) Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. Chem. Res. Toxicol. **5:** 834–842

132 Beckman J. S., Ischiropoulos H., Zhu L., van der Woerd M., Smith C., Chen J. et al. (1992) Kinetics of superoxide dismu. Arch. Biochem. Biophys. **298:** 438–445

133 Radi R., Beckman J. S., Bush K. M. and Freeman B. A. (1991) Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. Arch. Biochem. Biophys. **288:** 481–487

134 Darley-Usmar V. M., Hogg N., O'Leary V. J., Wilson M. T. and Moncada S. (1992) The simultaneous generation of superoxide and nitric oxide can initiate lipid peroxidation in human low density lipoprotein. Free Radic. Res. Commun. **17:** 9–20

135 Moreno J. J. and Pryor W. A. (1992) Inactivation of alpha 1-proteinase inhibitor by peroxynitrite. Chem. Res. Toxicol. **5:** 425–431

136 Buki A., Okonkwo D. O., Wang K. K. and Povlishock J. T. (2000) Cytochrome c release and caspase activation in traumatic axonal injury. J. Neurosci. **20:** 2825–2834

137 Graham S. H. and Chen J. (2001) Programmed cell death in cerebral ischemia. J. Cereb. Blood Flow Metab. **21:** 99–109

138 Sakhi S., Bruce A., Sun N., Tocco G., Baudry M. and Schreiber S. S. (1997) Induction of tumor suppressor p53 and DNA fragmentation in organotypic hippocampal cultures following excitotoxin treatment. Exp. Neurol. **145:** 81–88

139 Liu X., Kim C. N., Yang J., Jemmerson R. and Wang X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell **86:** 147–157

140 Wang X. (2001) The expanding role of mitochondria in apoptosis. Genes Dev. **15:** 2922–2933
141 Zou H., Li Y., Liu X. and Wang X. (1999) An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J. Biol. Chem. **274:** 11549–11556
142 Rodriguez J. and Lazebnik Y. (1999) Caspase-9 and APAF-1 form an active holoenzyme. Genes Dev. **13:** 3179–3184
143 Sabol S. L., Li R., Lee T. Y. and Abdul-Khalek R. (1998) Inhibition of apoptosis-associated DNA fragmentation activity in nonapoptotic cells: the role of DNA fragmentation factor-45 (DFF45/ICAD). Biochem. Biophys. Res. Commun. **253:** 151–158
144 Sakahira H., Enari M. and Nagata S. (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature **391:** 96–99
145 Woo M., Hakem R., Soengas M. S., Duncan G. S., Shahinian A., Kagi D. et al. (1998) Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev. **12:** 806–819
146 Enari M., Sakahira H., Yokoyama H., Okawa K., Iwamatsu A. and Nagata S. (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature **391:** 43–50
147 Raghupathi R., Graham D. I. and McIntosh T. K. (2000) Apoptosis after traumatic brain injury. J. Neurotrauma **17:** 927–938
148 Hengartner M. O. (2000) The biochemistry of apoptosis. Nature **407:** 770–776
149 Xiang H., Kinoshita Y., Knudson C. M., Korsmeyer S. J., Schwartzkroin P. A., Morrison R. S. (1998) Bax involvement in p53-mediated neuronal cell death. J. Neurosci. **18:** 1363–1373
150 Banasiak K. J. and Haddad G. G. (1998) Hypoxia-induced apoptosis: effect of hypoxic severity and role of p53 in neuronal cell death. Brain Res. **797:** 295–304
151 McGahan L., Hakim A. M. and Robertson G. S. (1998) Hippocampal Myc and p53 expression following transient global ischemia. Brain Res. Mol. Brain Res. **56:** 133–145
152 Lu J., Moochhala S., Kaur C. and Ling E. (2000) Changes in apoptosis-related protein (p53, Bax, Bcl-2 and Fos) expression with DNA fragmentation in the central nervous system in rats after closed head injury. Neurosci. Lett. **290:** 89–92
153 Napieralski J. A., Raghupathi R. and McIntosh T. K. (1999) The tumor-suppressor gene, p53, is induced in injured brain regions following experimental traumatic brain injury. Brain Res. Mol. Brain Res. **71:** 78–86
154 Cote J. and Ruiz-Carrillo A. (1993) Primers for mitochondrial DNA replication generated by endonuclease G. Science **261:** 765–769
155 Liu X., Zou H., Slaughter C. and Wang X. (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell **89:** 175–184
156 Liu X., Li P., Widlak P., Zou H., Luo X., Garrard W. T. et al. (1998) The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis. Proc. Natl. Acad. Sci. USA **95:** 8461–8466
157 Li L. Y., Luo X. and Wang X. (2001) Endonuclease G is an apoptotic DNase when released from mitochondria. Nature **412:** 95–99
158 Joza N., Susin S. A., Daugas E., Stanford W. L., Cho S. K., Li C. Y. et al. (2001) Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature **410:** 549–554
159 Zhang X., Chen J., Graham S. H., Du L., Kochanek P. M., Draviam R. et al. (2002) Intranuclear localization of apoptosis-inducing factor (AIF) and large scale DNA fragmentation after traumatic brain injury in rats and in neuronal cultures exposed to peroxynitrite. J. Neurochem. **82:** 181–191