Novel concepts in excitotoxic neurodegeneration after stroke

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Brain injury following cerebral ischaemia (stroke) involves a complex combination of pathological processes, including excitotoxicity and inflammation leading to necrotic and apoptotic forms of cell death. At the cellular level, excitotoxicity is mediated by glutamate and its cognate receptors, resulting in increased intracellular calcium and free radical production, and eventual cell death. Recent evidence suggests that scaffolding molecules that associate with glutamate receptors at the postsynaptic density allow coupling of receptor activity to specific second messengers capable of mediating excitotoxicity. These findings have important implications in the search for effective neuroprotective therapies in treating stroke.

Ischaemic stroke represents a major cause of morbidity and mortality in westernised society (reviewed in Refs 1, 2). The syndrome is initiated by a transient or permanent reduction in cerebral blood flow that results in a low oxygen state, leading to hypoxia in the tissue. The ischaemic insult can occur as (1) focal brain ischaemia, caused when a major cerebral artery is blocked by embolism or thrombosis, or (2) global ischaemia, caused by a generalised reduction in blood flow to the brain, for example after cardiac arrest or following severe traumatic brain or lung injuries. The extent of tissue death through lack of oxygen (known as the area of infarct) depends heavily on the degree and duration of blood flow reduction in the ischaemic

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Institute URL: http://www.uhn.ca/

Accession information: DOI: 10.1017/S1462399403007087; Vol. 5; 16 December 2003
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https://doi.org/10.1017/S1462399403007087 Published online by Cambridge University Press

area of the brain. At the stroke focus, where focal ischaemia is the most severe, cell death occurs within minutes. Cells are killed rapidly by lipolysis, proteolysis and the membrane breakdown that follows profound bioenergetic failure, with consequent loss of ion homeostasis. By contrast, neurons within the region that surrounds the ischaemic focus, known as the penumbra, experience moderate blood flow reduction and retain structural integrity but lose function. In animal models of ischaemic stroke, the penumbra can progress to infarction as a result of ongoing excitotoxicity or to secondary deleterious events, such as spreading depolarisation, postischaemic inflammation and apoptosis. Furthermore, in the event of rescue by restoring blood flow to an organ or tissue (reperfusion), for instance by dissolving a thrombus, neurons within this region might still be at risk of selective and delayed neuronal injury (reperfusion injury). Thus, the prime goal of neuroprotection has been to salvage the ischaemic penumbra and thereby limit the extent of damage.

The molecular mechanisms that underlie neurotoxicity are becoming clearer. A prime role for neurotransmitter receptors at the postsynaptic density (PSD), a specialised structure located beneath the postsynaptic membrane of neurons, and the scaffolding proteins that couple receptor activity to specific second messengers has been identified in the neurotoxicity that follows ischaemic damage. These findings, discussed here, have important clinical implications for treating stroke.

Stroke pathophysiology: an introduction

Our understanding of the mechanisms of cellular and molecular brain injury and the approach to stroke treatment have changed dramatically in the past two decades. Stroke pathophysiology involves a complex combination of processes including excitotoxicity, inflammation, necrosis and apoptosis (reviewed in Ref. 3). Focal impairment of cerebral blood flow restricts the delivery of oxygen and glucose, and impairs the maintenance of ionic gradients. There is an ensuing loss of membrane potential, and neurons depolarise. Consequently, dendritic as well as presynaptic voltage-dependent calcium (Ca^{2+}) channels become activated, and the rise in Ca^{2+} triggers the release of excitatory amino acids (EAAs), such as glutamate, into the extracellular space. EAA receptors subsequently mediate the crucial excitotoxic events that cause ischaemic neuronal death (Fig. 1).

Extensive research has shown that Ca^{2+} influx via EAA receptors is critical in mediating ischaemic neurodegeneration. More recently, increasing knowledge of the synaptic organisation of EAA receptors and their associated proteins has allowed us to propose specific, neurotoxic signalling pathways that transduce Ca^{2+}-dependent neurotoxic events during ischaemia. For instance, nitric oxide (NO) synthesised by the Ca^{2+}-dependent enzyme neuronal nitric oxide synthase (nNOS) reacts with a superoxide anion to form the highly reactive species peroxynitrite, which promotes tissue damage. The important role played by reactive oxygen species (ROS) in cell damage during stroke is emphasised by the fact that treatment with free radical scavengers can be effective in salvaging brain tissue in experimental models of focal cerebral ischaemia. Oxygen free radicals also serve as important signalling molecules that trigger inflammation and apoptosis.

Excitotoxicity

Excitotoxicity, the process whereby EAAs cause neurodegeneration (Ref. 4), is now considered to be a key mechanism of cell death in diseases such as stroke, central nervous system (CNS) trauma, epilepsy and chronic neurodegenerative disorders. Excitotoxicity results from an excessive release and inadequate reuptake of synaptic glutamate, the major excitatory neurotransmitter in the mammalian CNS. The role of glutamate in hypoxic neurodegeneration was established in the 1980s by studies that showed reduced neuronal sensitivity to hypoxia when postsynaptic glutamate receptors were blocked (Refs 5, 6). The majority of glutamate receptor subtypes have since been implicated in mediating neurotoxicity and there is general agreement that the mechanism is largely dependent on Ca^{2+} (Refs 7, 8). Glutamate receptor overactivation, and the resultant failure of Ca^{2+} channels to maintain Ca^{2+} balance, activates intracellular signalling events leading to free radical production and cell death.

Ca^{2+} ions are important intracellular messengers governing cellular functions such as differentiation and growth, membrane excitability, exocytosis and synaptic activity. Neurons tightly regulate Ca^{2+} homeostasis, controlling both intracellular Ca^{2+} levels and the location of Ca^{2+}

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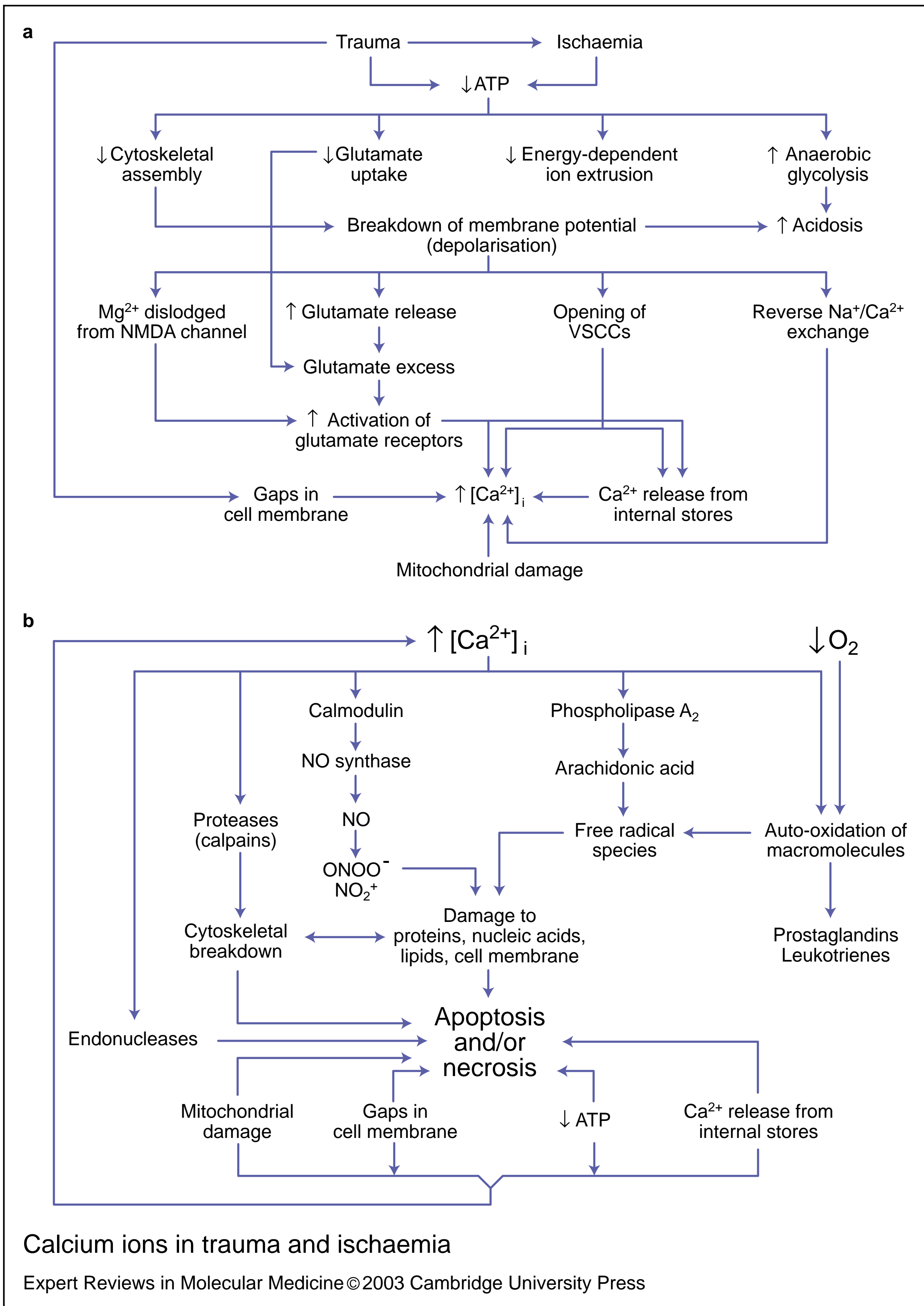

Figure 1. Calcium ions in trauma and ischaemia. *(See next page for legend.)*

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Figure 1. Calcium ions in trauma and ischaemia. *(Legend; see previous page for figure.)* (a) Pathways leading to elevations in intracellular calcium ions (Ca^{2+}) as a result of trauma and ischaemia. Following traumatic and ischaemic injuries, neuronal energy failure results in inadequate ATP synthesis, resulting in a loss of Ca^{2+} homeostasis triggered by mechanisms including: increased Ca^{2+} influx, decreased Ca^{2+} efflux, and altered Ca^{2+} buffering and sequestration. Energy failure results in reduced activity of energy-dependent ionic pumps (e.g. Na^+/K^+-ATPases) and reduced activity of processes associated with cytoskeletal assembly, which might then contribute to both membrane depolarisation and the subsequent activation of mechanisms associated with elevating intracellular Ca^{2+}. Such mechanisms include reverse operation of the Na^+/Ca^{2+} exchanger, opening of voltage-sensitive Ca^{2+} channels (VSCCs), removal of the Mg^{2+} block from NMDA channel pores (facilitates activation of NMDA currents and Ca^{2+} influx by excitatory amino acids), and increased glutamate release from synaptic terminals. Rises in extracellular glutamate, resulting from both increased synaptic release and reduced neuronal and glial uptake, can lead to elevations in intracellular Ca^{2+} by the increased activation of NMDA and non-NMDA glutamatergic receptors. Other salient processes contributing to pathological rises in intracellular Ca^{2+} might involve Ca^{2+} release from intracellular stores, mitochondrial damage, and injury-associated physical aberrations of the plasma membrane. (b) Pathways leading to cell injury as a result of elevations in intracellular Ca^{2+}. Elevations in intracellular Ca^{2+} arising from sources including those associated with mitochondrial damage, cell membrane disruption, reduced energy (ATP) production, and increased release from intracellular stores can trigger neurotoxicity by a variety of mechanisms. Sustained elevations in intracellular Ca^{2+} during ischaemia and traumatic brain injury can induce calpains, a family of Ca^{2+}-dependent cysteine proteases. Uncontrolled calpain activity can result in pronounced cytoskeletal breakdown, leading to cell injury. Increased intracellular Ca^{2+} can also mediate cell injury by activating processes associated with increased production of free radical species (e.g. activation of phospholipases) and increased reactive nitrogen species production (e.g. activation of Ca^{2+}/calmodulin-dependent enzymes including neuronal nitric oxide synthase). The collective rise in reactive oxygen and nitrogen species can aberrantly affect intracellular proteins, nucleic acids, lipids and cell membranes. Elevated Ca^{2+} can also activate endonucleases, which can subsequently fragment DNA. The collective contributions of all the aforementioned Ca^{2+}-dependent mechanisms of cell injury might be pivotal in mediating two well-recognised forms of cell death: necrosis and apoptosis. Abbreviations: ATP, adenosine triphosphate; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; $ONOO^-$, peroxynitrite; NO_2^+, nitronium ion.

ions through a complex interplay between Ca^{2+} influx and efflux, Ca^{2+} buffering and internal Ca^{2+} storage (for review see Ref. 3). Thus, localised 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 neighbouring ion channels. Under physiological conditions, these processes enable multiple Ca^{2+}-regulated signalling cascades to occur independently within the same cell (for review see Ref. 3). 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 (for review see Ref. 3). 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 (Refs 3, 9, 10). For example, excessive elevations in intracellular Ca^{2+} can activate proteases, lipases, phosphatases and endonucleases that either directly damage cell structure or induce the formation of oxidative free radicals that mediate cell death.

Although loss of Ca^{2+} homeostasis is an important factor in excitotoxic neurodegeneration, there is a compelling body of evidence that shows a dissociation between Ca^{2+} accumulation and neuronal death. Several studies have shown that Ca^{2+}-channel blockers can prevent Ca^{2+} accumulation but not neurotoxicity during anoxia (Refs 11, 12). Thus, a general elevation in Ca^{2+} does not necessarily predict neuronal death, and additional factors might influence the outcome of Ca^{2+} influx. We know that the various Ca^{2+}-dependent processes are regulated via distinct signal pathways linked to specific routes of Ca^{2+} influx (Refs 13, 14). The 'source specificity hypothesis' (Ref. 15) reasons that Ca^{2+} toxicity occurs not simply as a function of increased Ca^{2+} concentration, but is also linked to the route of Ca^{2+} entry and the distinct second messenger pathways activated as a result. Source specificity was originally based on experiments performed with free Ca^{2+} indicators, showing that Ca^{2+} loads produced by voltage-sensitive Ca^{2+} channels were not harmful, whereas similar intracellular Ca^{2+} increases via *N*-methyl-D-aspartate receptors (NMDARs; see below) were toxic (Ref. 15). Thus, distinct influx pathways, rather than Ca^{2+} load, determine neuronal vulnerability to glutamate and Ca^{2+} (Ref. 16). The source specificity hypothesis proposes that molecular targets, such

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as rate-limiting enzymes, are physically linked or colocalised with glutamate receptors and can be manipulated to block Ca^{2+}-dependent neurotoxicity, and might therefore be utilised in therapeutic neuroprotective strategies.

Glutamate receptors and excitotoxicity

Inhibition of oxygen supply to the brain for as little as 4–5 min is sufficient to induce irreparable damage to brain tissue. Paradoxically, in vitro studies have identified that, in certain culture conditions, neurons are quite resilient to oxygen deprivation (Refs 17, 18). Specifically, neurons have been shown to survive many hours under hypoxia in the presence of glutamate receptor antagonists (Ref. 17). The same is observed when neurons lacking functional glutamate are exposed to hypoxic conditions (Ref. 17). However, in conditions where glutamate receptors are not blocked, neurons lose their resilience to hypoxia (Ref. 19). Collectively, these examples indicate that glutamate receptors are key players mediating ischaemic damage (Ref. 20).

Postsynaptic responses to the excitatory neurotransmitter glutamate are mediated via pharmacologically and functionally distinct metabotropic (mGluR) or ionotropic (iGluR) glutamate receptor families. iGluR family members can be divided into pharmacologically distinct subfamilies based on their affinity for the agonists AMPA/KA (α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid/kainite) and NMDA. An additional subfamily has been identified – namely, the orphan glutamate receptor delta2 (GluRdelta2) (reviewed in Ref. 21). Mutations in the GluRdelta2 gene result in a constitutively active glutamate receptor ion channel, and have been shown to trigger neurodegeneration in the lurcher mouse (Ref. 22). During ischaemia, glutamate receptors are believed to mediate an imbalance in neuronal ion homeostasis: mGluRs through the inositol trisphosphate-stimulated release of intracellular Ca^{2+}; and iGluRs through increased permeability to Na^{+}, K^{+} and Ca^{2+}. These changes in ion status are responsible for activating the membrane depolarisations and signal cascades that initiate both immediate and delayed neuronal death.

mGluRs

mGluRs are G-protein-coupled receptors that belong to the same family as the Ca^{2+}-sensing and gamma-aminobutyric acid B (GABA-B) receptors. There are eight subtypes (mGlu1–8) of the metabotropic family, each discernable by its pharmacological profile and its transduction pathways (Ref. 23) (Table 1). Metabotropic receptors mediate their actions through GTP-binding-protein-dependent mechanisms linked to phospholipase C (PLC) and phosphoinositide turnover that mobilise internal Ca^{2+} stores (Refs 23, 24). Although mGluR subtypes are not implicated directly in excitotoxicity, during stroke they play an important modulatory role in the neuronal response to glutamate signalling. Group I mGluRs have been demonstrated to downregulate K^{+} channels and upregulate nonselective cation channels, inhibit GABA receptor activity and potentiate iGluR function, resulting in enhanced neuronal excitability (Refs 25, 26, 27, 28). Group II and III mGluRs located in the presynaptic terminals modulate the release of glutamate and the inhibitory neurotransmitter GABA (Ref. 23). Thus, the mGluRs play an important role in mediating neuronal plasticity, nociception (pain), and, in some instances, neurodegeneration. Agonists of group I mGluRs (mGlu1 and mGlu5) amplify NMDA-mediated excitotoxicity in vitro (Ref. 29), and both competitive and noncompetitive group I antagonists reduce neuronal toxicity to NMDA and are neuroprotective in experimental stroke (Refs 29, 30).

iGluRs

iGluRs are heteromeric structures assembled from a combination of subunits (Table 1) that form selective ligand-gated ion channels that lead to permeability to extracellular Na^{+}, K^{+} or Ca^{2+} ions upon activation. Each subunit has four membrane domains with an extracellular N-terminus and an intracellular C-terminal tail (Ref. 31). iGluRs play an important role in mediating the synaptic plasticity that is implicated in our ability to learn and form memories. In addition, much of the toxicity associated with glutamate overactivity has been attributed to stimulation of the iGluRs (Ref. 32). The pharmacologically distinct iGluR subfamilies are the AMPA/KA receptors (AMPARs/KARs) and the NMDA receptors (NMDARs).

AMPARs/KARs

AMPARs/KARs mediate the fast excitatory component of glutamate neurotransmission and are responsible for propagating membrane

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Table 1. Glutamate receptor family subunits

Subfamily	Receptor subunits (Rat)	Agonists
mGluR	mGluR1, mGluR2, mGluR3, mGluR4, mGluR5, mGluR6, mGluR7, mGluR8	Glutamate
GluR	GluR1, GluR2[a], GluR3, GluR4	AMPA-selective
GluR	GluR5[a], GluR6[a], GluR7	Kainate-selective
KAR	KA1, KA2	Kainate-selective
NR2	NR2A, NR2B, NR2C, NR2D	NMDA
NR1	NR1	NMDA
NR3	NR3A, NR3B	NMDA

[a] Transcripts encoding these subunits undergo RNA editing to yield Gln to Arg substituted Ca^{2+}-impermeable isoforms.
Abbreviations: AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid; GluR, glutamate receptor; KAR, kainate receptor; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate. NR2, NMDA receptor subunit 2; NR1, NMDA receptor subunit 1; NR3, NMDA receptor subunit 3.

depolarisation by the opening of voltage-stimulated Ca^{2+} channels (Ref. 33). AMPARs are tetraheteromeric structures assembled from combinations of GluR1–4 (GluR-A–D) subunits (Ref. 34). KARs, although similar in structure and function, can be pharmacologically distinguished from the AMPAR subtype: they display a higher affinity for kainic acid and glutamate as opposed to AMPA. KARs comprise subunits (high-affinity KA1 and KA2 and lower-affinity GluR5–7) that are homologous to AMPAR subunits. AMPARs are permeable to K^+ and Na^+ but are normally impermeable to Ca^{2+} in principal neurons. The majority of AMPARs arise from GluR2 transcripts that are edited within the region encoding the second transmembrane domain such that a glutamine residue is replaced with a positively charged arginine; this makes these AMPARs impermeable to extracellular Ca^{2+} (Ref. 35). The loss of the GluR2 subunit has been implicated in certain disease states, such as delayed neuronal death in ischaemic brain damage (Refs 36, 37). AMPARs are also considered to be key mediators of axonal damage during ischaemia (Ref. 38). AMPARs undergo trafficking from intracellular pools to synaptic sites, a property that is implicated in the activity-dependent modulation of synaptic strength (Ref. 39). AMPAR activity can also be modulated by subunit phosphorylation: receptor activation is potentiated by Ser/Thr phosphorylation by protein kinase A (PKA), PKC and Ca^{2+}/calmodulin-dependent kinase type II (CaMKII) (Refs 40, 41).

A variety of intracellular proteins bind to GluR subunits (Fig. 2a) and function in the targeting or clustering of receptors at the synapse as well as modulating receptor activity and the activation of second messenger pathways. AMPAR subunits diverge from one another in the sequence and length of their C-terminal tails, a region that mediates interaction with intracellular proteins. The intracellular C-terminus of GluR1 binds synapse-associated protein (SAP)-97 (a member of the PSD-95/SAP-90 family) (Ref. 42), as well as GluR1-interacting protein (GRIP) (Ref. 43). The GluR2 subunit associates with the Src-related kinase Lyn and the ATPase NSF (*N*-ethylmaleimide-sensitive fusion protein) (Ref. 44). NSF and its associated SNAPs (synaptic NSF attachment proteins) act as a chaperone complex for insertion of AMPARs into the synaptic membrane. Several studies indicate that NSF is crucial for the rapid turnover and regulation of AMPAR function (Refs 45, 46). GluR2 and 3 subunits also contain a C-terminal motif (SVKI) that allows them to bind to the scaffolding proteins PICK-1 (protein interacting with C kinase), GRIP-1 and ABP (AMPAR-binding

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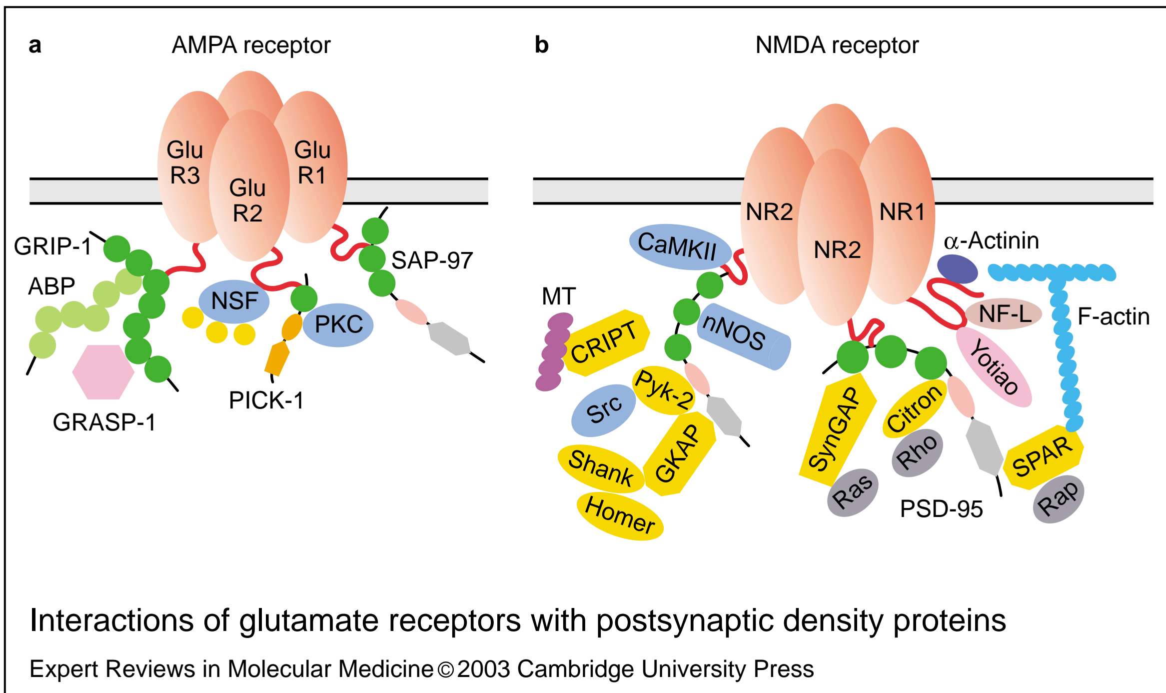

Figure 2. Interactions of glutamate receptors with postsynaptic density proteins. The wide array of proteins found within the postsynaptic density (PSD) interact to form the glutamatergic signal transduction machinery. Such interactions govern the activity-dependent and -independent receptor targeting and trafficking that is important for synaptic plasticity and excitotoxic signalling. (a) AMPA receptors (AMPARs) interact with PDZ-containing proteins including PSD-95 family members PICK-1, ABP and GRIP1, which might be important for membrane targeting and link the receptor to intracellular enzymes. In addition enzymes such as NSF regulate AMPAR trafficking to the plasma membrane. (b) NMDA receptors (NMDARs) are clustered within the PSD by binding to scaffolding proteins (PSD-95 members, α-actinin), which in turn bind effector molecules (CRIPT, SPAR, citron), cytoskeletal proteins (F-actin, MT, NF-L) and regulatory enzymes (nNOS, Src, CaMKII). The synaptic organisation of glutamate receptors brings them into close association with intracellular pathways capable of mediating excitotoxicity, such as the coupling of Ca^{2+}-dependent activation of nNOS to NMDAR signalling via PSD-95. It is important to note that protein interactions within the PSD are dynamic and might be altered by glutamatergic signalling. Abbreviations: ABP, AMPAR-binding protein; CaMKII, Ca^{2+}/calmodulin-dependent kinase II; CRIPT, cysteine-rich interactor of PDZ3; GKAP, guanylate kinase-associated protein; GRASP-1, GRIP-associated protein; GRIP-1, glutamate-receptor-interacting protein 1; Homer, PSD protein family involved with crossinteraction between metabotropic glutamate receptors and intracellular signal transduction systems; MT, microtubule; NF-L, neurofilament L; nNOS, neuronal nitric oxide synthase; NSF, *N*-ethylmaleimide-sensitive fusion protein; PICK-1, protein interacting with C-kinase; PKC, protein kinase C; PSD, postsynaptic density; Pyk-2, proline-rich tyrosine kinase 2; Ras, Rho and Rap, GTPases of the Ras superfamily; SAP-97, synapse-associated protein 97; Shank, proline-rich synapse-associated protein; SPAR, a Rap-specific GTPase-activating protein; SynGAP, synaptic GTPase-activating protein; Yotiao, NR1-interacting adaptor protein.

protein) through the PDZ domains of these proteins (PDZ domains are named after three of the homologous proteins that contain them – PSD-95, discs large, and zona occludentes 1 – and are discussed further below). These scaffolding proteins have been implicated in the clustering and trafficking of AMPARs at the synapse (Refs 47, 48, 49), and might be salient mediators of AMPAR-triggered second-messenger cascades.

NMDARs

NMDARs are slow-gating channels that are highly permeable to Ca^{2+} and Na^{+} (Ref. 50). Permeability to Na^{+} contributes to further membrane depolarisation, whereas it is the influx of exogenous Ca^{2+} that generates the intracellular Ca^{2+} transients that are responsible for the physiological effects of NMDAR signalling (reviewed in Ref. 51). NMDAR channels are made

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up of various subunits with distinct temporal and spatial distributions, pharmacological properties and signal transduction. NMDAR subunits share amino acid sequence and structural homology with AMPARs, and similarly form tetrameric structures (Ref. 52). Three families of subunits have been identified for NMDARs: the ubiquitously expressed NR1 subunit, four distinct NR2 subunits (A–D) and two NR3 members (A and B). Although recombinant NR1 subunits can form homomeric channels responsive to glutamate, they are only weakly permeable to Ca^{2+} and generate little current response (Refs 53, 54). Highly active, functional NMDARs are produced only when they contain both NR1 and NR2 subunits and, in some cases, NR3 subunits (Refs 55, 56).

At least a portion of the signalling properties of NMDARs is governed by the interactions of its intracellular domains with cytoskeletal and signal transduction molecules (Fig. 2b). The intracellular tail of NR2 subunits is critical for the proper function of NMDARs, as noted by mouse genetic studies. Mouse mutants with targeted deletion of the NR2 cytoplasmic tails are phenotypically indistinguishable from the corresponding NR2 subunit knockouts (Ref. 57). For instance, both knockout of NR2B and deletion of its C-terminus are neonatal-lethal, and both knockout of NR2A and deletion of its tail result in mice with impaired synaptic plasticity and memory (Refs 57, 58, 59). The C-terminal tails possess multiple motifs for phosphorylation and protein–protein interactions. The regulatory protein CaMKII, activated upon glutamate stimulation, phosphorylates serine residues within the C-terminal tail of NR2A and NR2B, and might play a role in regulating synaptic plasticity (Refs 60, 61). NMDAR signalling can be upregulated by tyrosine phosphorylation by Src kinase family members and inhibited by the actions of the phosphatase calcineurin (Refs 62, 63, 64). NMDAR assembly can also be modulated by phosphorylation. For instance, PKC phosphorylation of NR1 plays a role in subunit clustering (Ref. 65). Interaction of NR1 and NR2B with α-actinin-2, an actin-binding protein enriched in the PSD, might be important for the clustering of NMDARs at the synapse (Refs 66, 67). Ca^{2+}/calmodulin binds multiple high- and low-affinity sites on the tail of NR1 where it inhibits channel opening and decreases open time in response to Ca^{2+} (Refs 68, 69). The intracellular tail of NR subunits also binds structural proteins such as spectrin, neurofilament subunit NF-L and yotiao, although their role is as yet uncertain (Refs 70, 71, 72). Each NR2 subunit ends in a consensus tSXV (terminal SXV) motif [ESDV (NR2A, 2B) or ESEV (NR2C, 2D)] that allows them to bind members of the PSD-95 family of scaffold proteins (Ref. 73). PSD-95 in turn links NMDARs to signalling partners, regulatory enzymes and adaptor molecules.

Postsynaptic organisation and glutamate receptors

The PSD is a specialised structure located beneath the postsynaptic membrane aligned with active zones of presynaptic terminals within the CNS (Ref. 74; for an electron micrograph image also see Ref. 74). It is an electron-dense region comprising multiple membrane-bound, scaffolding and cytoskeletal proteins; in addition, signal molecules with the potential to act as neurotoxic triggers downstream of glutamate receptors most probably exist within the PSD. The PSD is relatively insoluble and can be isolated from synaptosomal fractions by differential centrifugation (Ref. 75). Several functions including cell–cell adhesion, regulation of receptor clustering and modulation of receptor function have been attributed to the PSD. Different types of PSDs have been demonstrated: type I PSDs are asymmetric structures that exceed the nerve terminal thickening of the presynaptic active zone (Ref. 76) and are particularly enriched in excitatory glutamate receptors, whereas type II or symmetric PSDs are characteristic of inhibitory GABA synapses (Ref. 77). The PSD at glutamatergic synapses is not a static entity and its morphology can be influenced by synaptic activity. For instance, events triggered by glutamate receptor activation affect not only signal transmission but also structural remodelling of the PSD (Ref. 78).

PSD proteins

There are four major types of molecules that constitute the PSD: membrane-bound proteins, cytoskeletal proteins, scaffolding proteins and modulatory enzymes. iGluRs are the most abundant membrane proteins found in the PSD but cell–cell adhesion molecules such as neuroligins and β-neurexin (Ref. 79) are also present, as are some subtypes of the mGluR family (Ref. 80). The enzymes found associated with the PSD play important roles in modulating

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iGluR signalling. Members of the Src family of nonreceptor protein tyrosine kinases, as well as CaMKII, PKC, ERK2-type mitogen-activated kinase and calcineurin regulate phosphorylation of PSD components and second messengers (Ref. 81). Other enzymes found in the PSD are key effectors in the glutamate receptor signal pathway: nNOS is activated by NMDAR Ca^{2+} influx and can modulate NMDAR signalling; SPAR [a Rap-specific GTPase-activating protein (RapGAP) that forms a complex with PSD-95 and NMDARs in brain] (Ref. 82), synaptic GTPase-activating protein (SynGAP) (Ref. 83) and the more recently identified citron are effectors of the Rap and Ras signalling pathways (Ref. 84).

Cytoskeletal proteins are considered to be important to the localisation and clustering of PSD receptors and signal complexes, whereas scaffolding proteins are the 'glue' that function in bringing the various PSD components into association. The abundant cytoskeletal elements include actin, fodrin, tubulin and neurofilaments, and scaffolding proteins include spectrin, α-actinin-2, AKAP 79 (a kinase-anchoring protein 79) and PDZ-containing proteins. α-Actinin-2 serves as the intermediary that links actin to NMDAR subunits, thereby influencing the clustering of the receptors (Ref. 66). The PSD is particularly enriched in specialised scaffolding proteins that contain PDZ domains. The PDZ proteins of excitatory synapses fall into two main families related to either PSD-95 or GRIP-1.

There are four members of the PSD-95 family found in mammalian synapses: PSD-95/SAP-90, SAP-97, PSD-93/chapsyn-110 and SAP-102 (reviewed in Ref. 85). All these are membrane-associated guanylate kinases (MAGUKs) and each member is characterised as having three N-terminal PDZ domains followed by a Src-homology 3 (SH3) and guanylate kinase (GK)-like domain. Each domain is capable of mediating protein–protein interactions with multiple binding partners. The PDZ domains are roughly 100 amino acids long and bind to small consensus motifs (tSXV) at the C-terminus of associated proteins. In addition, PDZ domains can self-associate, furthering their ability to cluster PSD proteins into functional complexes.

The second family of PDZ proteins includes GRIP and ABP, with seven and six PDZ domains, respectively (Refs 48, 49). These proteins do not contain SH3 or GK domains and their PDZ interactions with AMPAR GluR2/3 subunits occur via a class II hydrophobic interaction, which is distinct from the class I PSD-95–NMDAR interaction. GRIP and ABP are also capable of forming homo- and heteromultimers through PDZ–PDZ interactions (Ref. 49).

Neurotoxic signalling of glutamate receptors within the PSD

The wide array of PSD proteins interact to constitute the glutamatergic signal transduction machinery. In addition, PSD components govern trafficking and function of the iGluR AMPARs and NMDARs.

AMPARs and neuronal death

AMPAR stability and movement at synapses are important factors controlling synaptic strength. Indeed, preventing the interaction of the AMPAR GluR2 subunit with GRIP-1/ABP proteins impairs long-term depression in both the hippocampus and cerebellum (Ref. 86). Synaptic localisation of Ca^{2+}-impermeable GluR2 subunits is thought to be important in modulating the neurotoxic effects of AMPAR signalling (Ref. 87). Several studies have suggested that CA1 hippocampal neurons, vulnerable to delayed death following global ischaemia, have downregulated synaptic expression of GluR2 (Refs 88, 89). Whereas increased Ca^{2+} permeability might play a role in the CA1 vulnerability, there are conflicting studies that indicate that other processes might affect AMPAR-mediated toxicity (Refs 88, 89). As yet, none of the AMPAR-associated proteins has been ascribed a role in excitotoxic signalling; however, these proteins have the potential for mediating excitotoxic insult via interactions with other synaptic molecules. For example, GRIP-1 binding to GRASP-1 might couple AMPAR activation to the Ras signalling pathway (Ref. 90). GRASP-1 is cleaved in apoptotic neurons during ischaemia, disrupting its regulation of Ras signalling. In addition, GRASP-1 downregulates synaptic targeting of AMPARs (Ref. 42). Thus, its cleavage during ischaemia might result in increased synaptic AMPAR activity and vulnerability to glutamate overactivity.

NMDARs and neuronal death

Close coupling of receptor channels to downstream signal machinery allows for efficient, local Ca^{2+}-dependent activation of signalling cascades. Unlike the case for the AMPARs, compelling evidence exists demonstrating that

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Ca^{2+}-dependent neuronal death is triggered most efficiently through NMDARs (Ref. 15). These data indicate that the synaptic organisation of NMDARs brings them into close association with intracellular pathways capable of causing neurotoxicity. NR2A and NR2B subunits bind to the first two PDZ domains of PSD-95. The third PDZ domain has been shown to bind neuroligins, which in turn bind the cell–cell adhesion molecule β-neurexin. This interaction indicates that PSD-95 binding is important for synaptic NMDAR clustering. However, studies of PSD-95-knockout mice and of cultured neurons in which PSD-95 expression is suppressed demonstrate normal synaptic localisation of NMDARs and normal synaptic NMDA currents, suggesting interaction with PSD-95 is not the sole factor governing clustering (Ref. 91). For example, synaptic localisation of NMDARs is partly governed by the interaction of the NR1 subunit with α-actinin-2 and the actin cytoskeleton. Depolymerising F-actin results in redistribution of synaptic NMDAR clusters to extrasynaptic sites (Refs 67, 92). Interestingly, calmodulin, a negative regulator of NMDAR signalling, has been shown to antagonise α-actinin-2 binding to NR1 directly (Ref. 93). Our laboratory has shown that depolymerising F-actin reduces signalling and excitotoxicity evoked by synaptic glutamate release but not by exogenous glutamate application (Ref. 92). These data indicate that synaptic and extrasynaptic NMDARs are equally capable of mediating neurotoxicity, although, depending on conditions, synaptic NMDAR activity might actually be neuroprotective by preventing apoptotic cell death (Ref. 21). Thus, although actinin and calmodulin interactions with NR1 are optimised for synaptic signalling, some other interaction might be critical in regulating excitotoxic signal cascades. It should be noted here that PSD-95-null mutant mice show a dramatic increase in long-term potentiation (LTP), indicating an important role for NR2–PSD-95 interactions in NMDAR signal transduction. Indeed, through its various protein-binding domains, PSD-95 can bring an array of signal molecules into close proximity to Ca^{2+} influx through the NMDAR channel.

The discovery of distinctive signal molecules as binding partners of PSD-95 led to the testing of the hypothesis that PSD-95 acts as the scaffolding link between incoming Ca^{2+} ions and intracellular signal molecules such as nNOS (Refs 94, 95). By suppressing the expression of PSD-95, we selectively attenuated excitotoxicity triggered by NMDARs but not other GluR or Ca^{2+} channels (Ref. 92). Further study of the NMDAR signalling mechanism revealed that suppressing PSD-95 selectively reduces Ca^{2+}-activated NO production without affecting nNOS expression. Thus, PSD-95 appears to be required for efficient coupling of NMDAR activation to NO signalling and toxicity.

Intracellular mechanisms of neuronal injury in ischaemic neurotoxicity

As described above, elevated extracellular glutamate levels result in the prolonged activation of glutamate receptors and, thus, increased intracellular Ca^{2+} levels. Mitochondria have the ability to sequester large amounts of intracellular Ca^{2+} and are therefore important for cell survival; however, abnormal mitochondrial Ca^{2+} accumulation is also a primary mediator of mitochondrial dysfunction (Ref. 96), affecting ATP levels, ROS and apoptosis.

Reduced ATP production

Ca^{2+} is sequestered into the mitochondrial matrix via a proton electrochemical gradient that is generated by the electron transport chain, depolarising the mitochondrial potential (Refs 97, 98, 99). This influx of Ca^{2+} results in a reduction in the electrochemical gradient and subsequently reduces ATP synthesis. In response to elevated Ca^{2+}, cells also utilise much of the ATP energy to drive the plasma membrane Ca^{2+} pump so that Ca^{2+} can be extruded. The concurrent accumulation of intramitochondrial Ca^{2+}, reduced ATP synthesis and increased ATP usage are suggested to have predominant roles in mediating early ischaemic cell death (Ref. 100).

Increased ROS production

In addition to the Ca^{2+}-mediated reduction in mitochondrial ATP production, deficits in mitochondrial electron chain functioning can result in excessive ROS production, leading to neurotoxicity (Refs 101, 102, 103). It has been shown that there is an increase of superoxide anion production throughout both ischaemic and reperfusion intervals (Ref. 104). Rises in intracellular Ca^{2+} result in futile mitochondrial Ca^{2+} cycling that, in turn, also results in increased ROS production (Ref. 105). ROS production exceeding neuronal buffering capacity can impinge on neuronal integrity.

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ROS production is also produced in the cytoplasm following elevations in intracellular Ca^{2+}. Under hypoxic conditions, the Ca^{2+}-induced activation of proteases has been suggested to mediate the conversion of xanthine dehdrogenase (XDH) into xanthine oxidase (XOD) (Refs 106, 107). XOD activity incorporates molecular oxygen as a final electron acceptor, and therefore, following prolonged activity, has the potential to elevate intracellular superoxide anion levels substantially (Refs 108, 109, 110, 111). Ischaemia-induced rises in intracellular Ca^{2+} also activate phospholipases, in particular phospholipase A2, which release arachadonic acid (Ref. 112). Arachadonic acid metabolism by oxidases results in the production of oxygen free radicals (Refs 113, 114). Glutamatergic-induced ROS production can also be mediated by a pathway specifically associated with the NMDAR subtype. Neuronal NO production is linked with NMDA activation through the PSD scaffolding protein PSD-95 and nNOS (Ref. 115) (see above).

The accumulation of intracellular ROS by these mechanisms can mediate cytodestructive, and ultimately lethal, processes (Fig. 3). It is argued that glutamate-mediated neurotoxicity results from the overproduction of both NO and superoxide anion. The reaction product of these two species, peroxynitite ($ONOO^-$), is chemically complex, as it has the activity of both the hydroxyl radical and the nitrogen dioxide radical (Ref. 116). $ONOO^-$ is a potent oxidant that reacts with sulphydryls (Ref. 117) and with zinc-thiolate moieties (Ref. 118). It can also nitrate and hydroxylate aromatic rings on amino acid residues (Ref. 119), and oxidise lipids (Ref. 120), protein and DNA (Refs 121, 122). Both NO and $ONOO^-$ can damage DNA, leading to the activation of the nuclear DNA repair enzyme poly (ADP ribose) synthetase (PARS). Although PARS activity is focused on DNA reparative processes, this enzyme is highly promiscuous and energy dependent. Thus, prolonged PARS activity can impinge on cell survival by rapidly depleting cellular energy reserves. If prolonged PARS activity occurs together with deficits in mitochondrial ATP production, the collective result might bias the cell to death over survival. In addition to causing DNA damage, NO/$ONOO^-$ has been shown to inhibit mitochondrial respiratory chain enzymes (Ref. 123).

Apoptosis

Following an ischaemic insult, neurons can die either almost immediately following the injury or as many as 10 days later (Ref. 124). Delayed cell death is typically seen in the penumbra region, whereas immediate cell death occurs within the ischaemic core (Ref. 125). Immediate cell death follows the uncontrolled rise in glutamate-induced intracellular Ca^{2+} described above (Refs 126, 127, 128, 129), whereas delayed mortality occurs via a programmed cell death mechanism (apoptosis) (Refs 130, 131, 132, 133, 134, 135, 136) (Fig. 3).

Among the various classical apoptotic pathways, mitochondrial release of cytochrome c, a component of the mitochondrial electron transport chain, initiates apoptosis via caspase-associated pathways (Ref. 137). Cytochrome c release from the mitochondria is, in part, mediated through both free-radical-dependent mechanisms and/or mitochondrial pores triggered by processes such as Bax oligomerisation (for review see Ref. 138). Cytoplasmic cytochrome c binds to apoptosis protease-activating factor 1 (Apaf-1), a cytosolic protein containing a caspase recruitment domain (CARD), a nucleotide-binding domain, and many WD-40 repeats (Ref. 139). Once cytochrome c binds to Apaf-1, the nucleotide dATP or ATP binds to the complex and triggers its oligomerisation to create an apoptosome (Ref. 139). The CARD domain then becomes exposed in the apoptosome, subsequently recruiting multiple procaspase 9 molecules to facilitate their autoactivation. Once activated, caspase 9 cleaves procaspase 3 into caspase 3, which is both the active from of the enzyme and the primary effector enzyme in neuronal apoptosis (Refs 137, 140). The hallmarks of apoptosis include internucleosmal DNA cleavage, somal shrinkage and neuronal condensation, nuclear membrane breakdown, externalisation of phosphatidylserine, and the formation of apoptotic bodies (Ref. 141; for review see Ref. 142).

Several lines of evidence suggest that p53 is a salient upstream initiator of apoptosis following neuronal injury. p53 is a tumour suppressor that has been shown to be upregulated in response to excitotoxins, hypoxia and ischaemia (Refs 143, 144, 145, 146). In a p53-knockout murine model, neuronal brain damage induced by ischaemic injury is significantly reduced (Refs 147, 148). Following oxidative DNA damage, p53 levels increase, and trigger apoptosis by upregulating several

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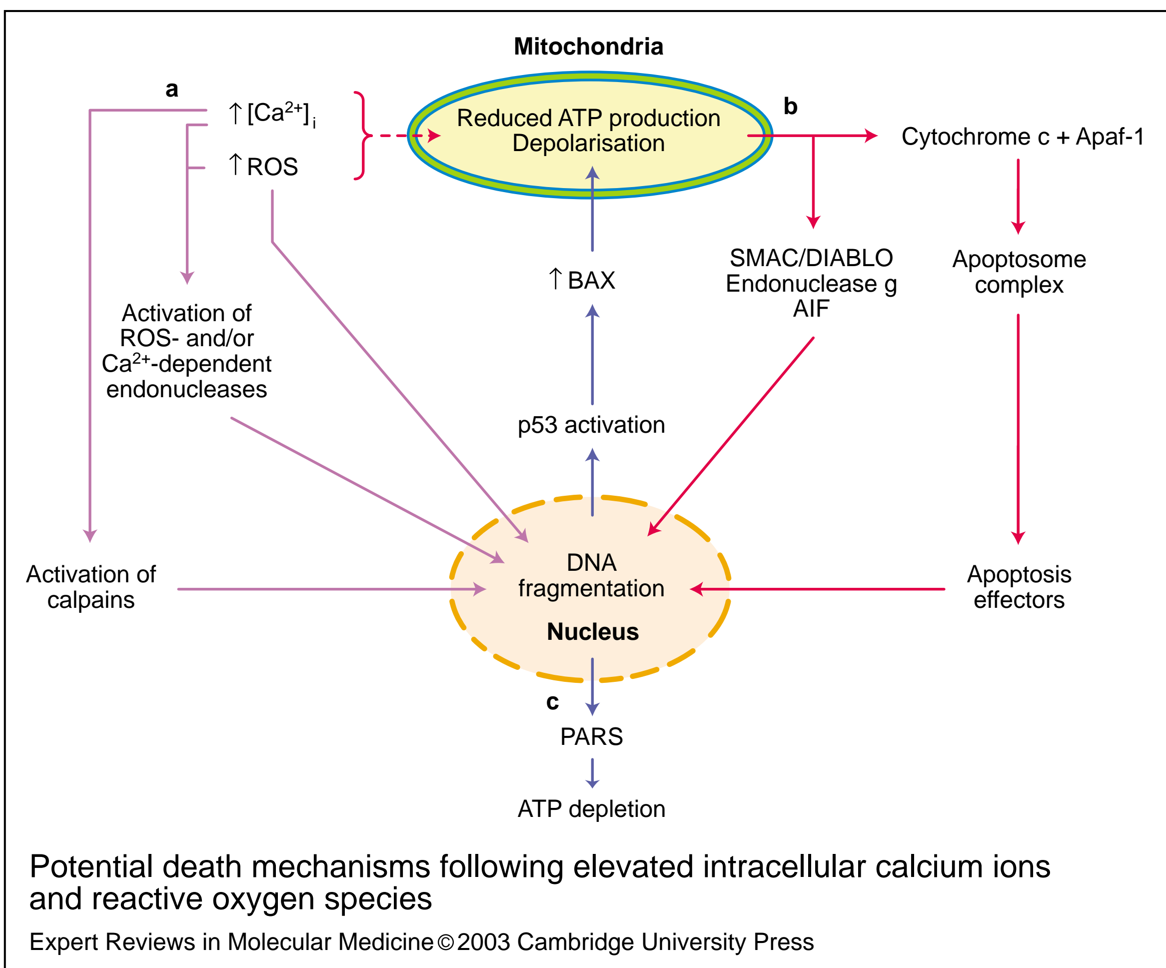

Figure 3. Potential death mechanisms following elevated intracellular calcium ions and reactive oxygen species. Neurons have endogenous buffering systems to circumvent the consequences of elevated calcium ions (Ca^{2+}) and reactive oxygen species (ROS) levels. However, following injury to these buffering mechanisms or if the Ca^{2+} and ROS levels are too high for buffering systems, the cells succumb to their injury via multiple mechanisms. (a) Increases in intracellular Ca^{2+} and ROS can activate DNA-damaging endonucleases, as well as introduce DNA damage directly. In addition, increased intracellular Ca^{2+} can activate Ca^{2+}-dependent proteases including calpains; uncontrolled calpain activity damages many cytosolic proteins salient for cell survival (not shown), as well as damaging DNA. (b) Elevated Ca^{2+} and ROS can also affect mitochondrial function, thereby reducing ATP production, and, if uncontrolled, lead to mitochondrial permeability transition (MPT). MPT results in the release of intermitochondrial membrane proteins including cytochrome c, SMAC/DIABLO, endonuclease g, and apoptosis-inducing factor (AIF). These molecules, once released into the cytosol, mediate toxicity by introducing DNA damage and/or activating intracellular cascades leading to apoptosis. (c) The net effect of DNA damage, either via direct oxidation or by enzymatic cleavage, is the activation of energy-consuming DNA repair enzymes such as poly (ADP ribose) synthetase. DNA damage is also detected by checkpoint sensors such as p53 that, upon activation, result in the increased expression of pro-apoptotic molecules such as Bax (pro-apoptotic molecule of the Bcl-2 family). Rises in intracellular Bax levels have been proposed to mediate the formation of mitochondrial membrane pores, thereby triggering MPT. Abbreviations: Apaf-1, apoptosis protease-activating factor 1; SMAC/DIABLO, second mitochondrial activator of caspases (promote apoptosis by relieving the inhibition of caspases by the apoptosis inhibiting proteins; AIF, apoptosis-inducing factor.

regulatory enzymes including Bax, CD95 and DR5 (a receptor for the death ligand TRAIL), which are all classical members of the core apoptosis pathways (Ref. 149).

In addition to the caspase-mediated cell death mechanisms, researchers have recently discovered caspase-independent forms of programmed cell death. These mechanisms involve the release of

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mitochondrial proteins such as endonuclease g (endo g) or apoptosis-inducing factor (AIF) subsequent to mitochondrial depolarisation (Ref. 150; for review see Ref. 138). Endo g is a nuclease that is capable of inducing nuclear DNA cleavage (Refs 151, 152, 153). Although AIF-mediated cell death in ischaemia is not yet fully understood, recent work by Zhang et al. suggests that neuronal death subsequent to experimental brain injury involves AIF translocation from the mitochondria to cell nuclei (Ref. 154). Interestingly, they also found that the direct application of $ONOO^-$ to cortical neurons leads to the mitochondrial release of AIF (Ref. 154). These findings suggest that NMDAR-mediated excitotoxicity might also share direct associations with mitochondrial AIF release, as $ONOO^-$ is a potent free radical specifically associated with both superoxide anion generation and NMDAR-mediated NO production.

Clinical implications and applications

In theory, glutamate-receptor-mediated excitotoxicity during stroke has the potential to be treated by blocking ion-channel function. To this end, numerous specific NMDAR and AMPAR channel blockers have been studied in the search for a neuroprotective stroke therapeutic. However, antagonism of glutamate receptor activity has not proven clinically useful, partly as a result of pharmacokinetic difficulties and adverse side effects. The high levels of NMDAR antagonists needed to treat excitotoxic damage can induce hallucinations, centrally mediated hypo- or hypertension, catatonia and sometimes anaesthesia. AMPAR blockers have shown more potential for therapeutic use, especially in the protection of CA1 hippocampal neurons during global ischaemia, yet their clinical use has been limited by poor solubility and renal toxicity (Ref. 155).

At physiological levels, glutamatergic communication and the resulting Ca^{2+} influx are vital to neuronal survival. Too much or too little glutamatergic activity might result in adverse sequelae. For example, NMDAR blockade has been shown to cause extensive apoptosis in perinatal rats (Ref. 156). Alternative strategies for treating excitotoxic damage have been proposed including the use of: (1) antagonists selective for particular receptor subunits (ifenprodil for NMDAR NR2B) (Ref. 157); (2) partial blockers of receptor activity, such as glycine antagonists (Ref. 158); (3) low-affinity blockers whose binding is more easily displaced, allowing some glutamatergic communication (Ref. 159); and (4) drugs that indirectly modulate excitotoxic signalling, such as mGluR antagonists (Ref. 160).

Yet another approach in the search for a stroke therapeutic is the targeting of the specific intracellular signal pathways that propagate excitotoxic signals from glutamate receptors. This strategy targets specific protein–protein interactions in order to uncouple glutamate receptors from their downstream effectors. Targeting the PDZ interaction with the C-terminal tSXV motif of NR2B represents a therapeutic strategy that could circumvent the negative consequences of blocking NMDAR function. Interfering with the NR2B–PSD-95 interaction could suppress excitotoxicity in a manner similar to knockdown of PSD-95 expression. To this end, a targeted peptide has been designed (Ref. 161) comprising the nine C-terminal residues of NR2B (NR2B9c), including the tSXV motif, which is anticipated to bind the second PDZ domain of PSD-95. In order to allow for efficient delivery of the peptide into cells in vitro and across the blood–brain barrier in vivo, NR2B9c has been conjugated to the cell membrane transduction domain of the human immunodeficiency virus 1 (HIV-1) Tat protein (Ref. 161). The Tat transduction domain is able to transport proteins of variable size across membranes in a rapid, dose-dependent manner independent of receptors or transporters (Refs 162, 163, 164). Studies in vivo have shown that the Tat–NR2B9c peptide protected cultured neurons from NMDA-mediated excitotoxicity without affecting NMDAR Ca^{2+} signalling or electrophysiology (Ref. 161). Most importantly, the peptide dramatically reduced cerebral infarction and improved neurological function in rats subjected to transient focal cerebral ischaemia. The peptide treatment was effective both one hour before and one hour after the onset of excitotoxicity and cerebral ischaemia. Figure 4 illustrates the dissociation of NMDAR NR2B subunits from PSD-95 in vitro using Tat peptides and fusion proteins.

Research in progress and outstanding research questions

The strategy outlined above of a targeted disruption of protein–protein interactions based on a molecular understanding of excitotoxic mechanisms might provide practical future

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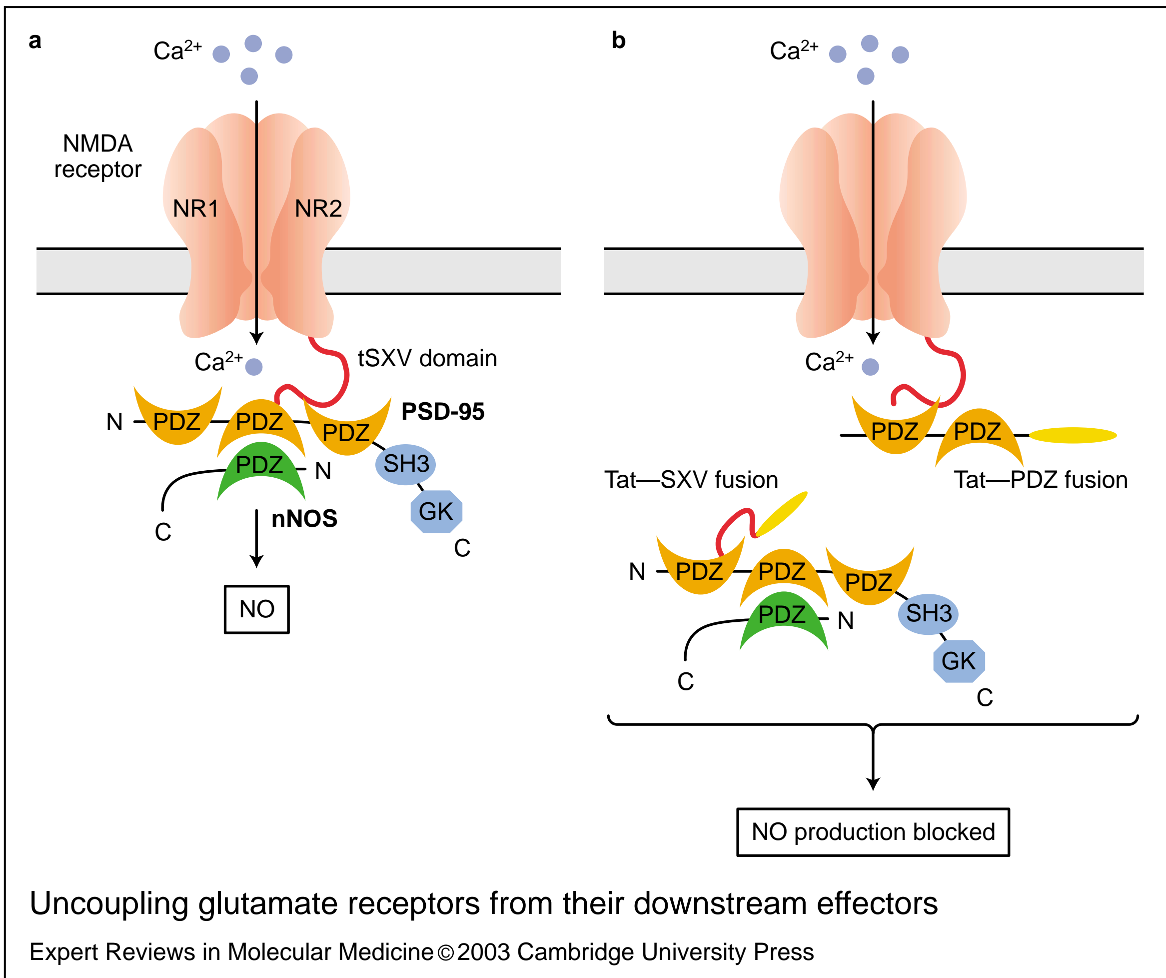

Figure 4. Uncoupling glutamate receptors from their downstream effectors. (a) The NMDA receptor (NMDAR) is linked to downstream neurotoxic molecules such as nitric oxide synthase (nNOS) through PSD-95: through its first and second second PDZ domain, PSD-95 forms a ternary complex by binding to both the tSXV motif of NMDAR NR2 subunit and to the PDZ domain in nNOS (Ref. 115). (b) Disrupting NMDAR–PSD-95 complexes can reduce the efficiency by which calcium ions (Ca^{2+}) activate excitotoxic signalling molecules such as nNOS. This disruption is achieved by the intracellular introduction of HIV-1 Tat peptides (including the transduction domain for efficient transfer across membranes) fused either to the first and second PDZ domains of PSD-95 or to the C-terminus (containing the SXV motif) of NR2B; these would be expected to bind to NMDAR NR2 and PSD-95, respectively (Ref. 161). Abbreviations: GK, guanylate kinase domain; HIV-1, human immunodeficiency virus 1; NMDA, *N*-methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PSD-95, postsynaptic density 95 protein; SH3, Src homology domain 3.

treatments for human neurological disorders. As our understanding of protein-binding domains has grown, so has the potential for therapeutic intervention. SH2, SH3 and ligand-binding domains, enzyme active sites and protein dimerisation sites have all been investigated as targets for therapeutic intervention (Refs 165, 166). The coupling of Ca^{2+}-dependent nNOS signalling to NMDAR activation is but one possible pathway in glutamate-mediated excitotoxicity. As we expand our knowledge of the signalling machinery attached to glutamate receptors, new potential therapeutic targets might also arise. For instance, little is known yet about the role of GRIP-1/ABP multimers in clustering synaptic proteins with AMPARs at the synapse. It is possible that specific enzymes or signal molecules, clustered with AMPARs, are deregulated upon glutamate overactivity or cell stress and mediate a toxic second messenger cascade. In addition,

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nNOS might not be the only signal pathway influenced by the Tat–NR2B9c peptide described above that disrupts the NR2B–PSD-95 interaction. Indeed, PSD-95 binds and clusters a wide variety of enzymes and modulators that could be important in NMDAR signalling and/or neurotoxicity. Further investigation into the organisation of the excitatory PSD and of the molecular mechanisms of glutamate-mediated excitotoxicity should reveal additional targets for pharmacological intervention in the treatment of stroke and other neurodegenerative conditions.

Acknowledgements and funding

M.M.A. is a fellow of the Heart and Stroke Foundation of Canada (HSFC) and the Canadian Institutes of Health Research (CIHR). M.A. is supported by a doctoral studentship from the CIHR. M.T. is a CIHR Clinician-Scientist supported by operating grants from the CIHR, the Canadian Stroke Network and NIH Grant 39060. We thank Dr Ramesh Raghupathi (Department of Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, PA, USA) and an anonymous peer reviewer for their comments.

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Figures

Figure 1. Calcium ions in trauma and ischaemia.
Figure 2. Interactions of glutamate receptors with postsynaptic density proteins.
Figure 3. Potential death mechanisms following elevated intracellular calcium ions and reactive oxygen species.
Figure 4. Uncoupling glutamate receptors from their downstream effectors.

Table

Table 1. Glutamate receptor family subunits.

Citation details for this article

Michelle M. Aarts, Mark Arundine and Michael Tymianski (2003) Novel concepts in excitotoxic neurodegeneration after stroke. Expert Rev. Mol. Med. Vol. 5, 16 December, DOI: 10.1017/S1462399403007087

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