Cell Signaling and Neuronal Death

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Abstract

The past few decades have revealed that cell death can be precisely programmed with two principal forms, apoptosis and necrosis. Besides pathophysiological alterations, physiologic processes, such as the pruning of neurons during normal development and the involution of the thymus, involve apoptosis. This review focuses on the role of inter- and intracellular signaling systems in cell death, especially in the nervous system. Among neurotransmitters, glutamate and nitric oxide have been most extensively characterized and contribute to cell death in excitotoxic damage, especially in stroke and possibly in neurodegenerative diseases. Within cells, calcium, the most prominent of all intracellular messengers, mediates diverse forms of cell death with actions modulated by many proteins, including IP3 receptors, calcineurin, calpain, and cytochrome *c*.

INTRODUCTION

Bcl-2 family: a family of proteins that regulate a critical intracellular checkpoint in the intrinsic pathway of apoptosis. They are usually categorized into different groups based on the presence of certain sequence motifs, Bcl-2 homology (BH) domains

Modulation of cell death can impact many diseases; for example, as an oversimplification, drugs that augment cell death might treat cancer, whereas drugs that inhibit cell death may prevent neurotoxicity in stroke and neurodegenerative diseases. For most of the twentieth century, few paid heed to molecular mechanisms of cell death, assuming that the cellular machinery simply falls apart following toxic insults. The elucidation of a specific system of programmed cell death, apoptosis, has radically changed thinking (1). An elaborate series of apoptotic cascades has been illuminated, with particular focus on the caspase enzymes (2) as well as apoptotic factors, especially those of the Bcl-2 family (1). Besides its role in disease, apoptosis has physiologic roles, such as eliminating the excess neurons formed in embryonic development and causing physiologic involution of the thymus gland. In the adult, apoptosis plays a role in maintaining cells that turn over frequently, such as epithelial populations. Disrupting apoptosis in this case contributes to cancer. Apoptosis has often been viewed as a reflection of moderate levels of cell stress, which afford the luxury of a gradual, programmed death, in contrast to necrosis, which, following overwhelming insults, less specifically disrupts cells. However, recent evidence has shown that necrosis may also involve systematic alterations, such as overactivation of poly(ADP-ribose)polymerase (PARP) (3). Agents that facilitate or inhibit various molecular targets in apoptotic and necrotic pathways are in clinical development. Because of the very extensive literature available (1, 2, 4, 5), this review does not address general mechanisms of cell death.

Therapeutic approaches to neurotoxicity have developed only recently. It was previously thought impossible to reduce brain damage associated with vascular stroke. During stroke, neurons die of hypoxia following blood vessel occlusion or hemorrhage, an essentially irreversible process. In the case of neurodegenerative diseases, it had long been assumed that until one knew the exact molecular causes of Alzheimer's, Parkinson's, and Huntington's diseases and could develop an "antidote," therapeutic intervention was not feasible. In Alzheimer's disease, neurotoxicity is thought to be elicited primarily by the amyloid-β_{1-42} peptide, which can augment *N*-methyl-D-aspartate (NMDA) receptor transmission and intracellular Ca^{2+} (6).

A major change in thinking about clinical neurotoxicity came from the gradual appreciation that most of the neuronal death following a stroke occurs over a period of days following the initial insult and reflects excitotoxicity elicited by glutamate, the major excitatory transmitter in the brain (7, 8). Proof of principle came from studies in rodents showing that glutamate receptor antagonists reduce stroke damage whether administered before or after ligating a cerebral artery. For neurodegenerative diseases, regardless of the fundamental cause of toxicity, neuronal damage would likely increase the sensitivity of cells to excitatory neurotransmission so that agents that diminish such excitation could be therapeutic.

Many neurotransmitters likely participate in signaling events that influence neurotoxicity, but glutamate appears to be the principal actor. Nitric oxide (NO), formed in response to glutamate activation of NMDA receptors, has also been implicated in neurotoxicity. Among intracellular messengers, Ca^{2+}, whose concentration within the cell is augmented by NMDA receptor activation, has been most associated with

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neuronal cell death. This review focuses on ways in which glutamate, NO, and Ca^{2+} contribute to neuronal cell death, with implications for potential therapeutic agents.

GLUTAMATE

Glutamate was first identified as a major excitatory neurotransmitter in the 1960s when a variety of agents that blocked glutamate actions were also shown to prevent physiologic synaptic excitation (9). However, identifying the pool of glutamate that is reserved for neurotransmission has, to this day, been a challenge. Glutamate is the most abundant amino acid in the brain, with ambient concentrations of approximately 10 mM. It participates in protein synthesis and interfaces closely with carbohydrate metabolism via transamination to alpha-ketoglutarate so that glucose is the physiologic precursor of most glutamate in the brain. Indeed, the turnover rate of glucose, about 1 $\mu mol\ g^{-1}\ min^{-1}$, resembles glutamate's turnover, implying that most of the brain's glucose metabolism is devoted to maintaining glutamate synthesis. Synaptic actions of glutamate are terminated by reuptake into nerve terminals and/or astrocytic glia that ensheath the synapse, with glial uptake being the predominant mechanism. Despite glutamate's ubiquity, immunohistochemical analysis reveals selective high densities of neuronal staining, presumably reflecting glutamatergic synapses.

Because of their nonspecificity, glutamate synthesis and degradation have not been the targets of effective therapeutics, with the following exception (further discussed below): glutamate generated from the dipeptide *N*-acetyl-L-aspartyl-L-glutamate (NAAG) by glutamate carboxypeptidase II (GCPII). The bulk of possible drugs stimulates or blocks various subtypes of glutamate receptors, which include ionotropic and metabotropic types.

In contrast to the high millimolar total concentration of brain glutamate, peripheral extracellular glutamate levels are only approximately 0.6 μM. Because cerebrocortical neurons in cultures lacking glia are killed by 2–4 μM glutamate, the margin for error in glutamate transmission/cytotoxicity is relatively modest.

N-acetyl-L-aspartyl-L-glutamate (NAAG): NAAG is one of the most prevalent neurotransmitters in the mammalian brain. NAAG acts as an agonist at Group II metabotropic glutamate receptors on neurons and glia

Glutamate carboxypeptidase II (GCPII): a metalloprotease that hydrolyses NAAG to *N*-actyl-L-aspartate (NAA). It also occurs in the prostate as prostate-specific membrane antigen (PSMA) and is released into the circulation in prostate cancer

Ionotropic glutamate receptors: receptors that are also ion channels and pass electric current in response to glutamate binding

NMDA Receptors

Ionotropic glutamate receptors, which pass electric current in response to glutamate binding, come in two classes that were first distinguished by the differential actions of various glutamate analogs on receptor activation. One class, known as NMDA receptors, is activated by the glutamate analogue, NMDA. Although NMDA receptor channels can conduct Na^+ and Ca^{2+}, under basal conditions the channel is blocked by Mg^{2+}. The Mg^{2+} blockade is relieved by cellular depolarization, with implications for synaptic plasticity, especially long-term potentiation (LTP). Continuous strong stimulation optimally activates NMDA receptors and plays an important role in LTP. With neurotoxic insults, disruption of energy metabolism diminishes the driving force for the Na^+ pump that maintains the resting membrane potential of cells so that neurons become depolarized, relieving the Mg^{2+} block of NMDA receptors. Excess Ca^{2+} entry then leads to diverse events that elicit cell death.

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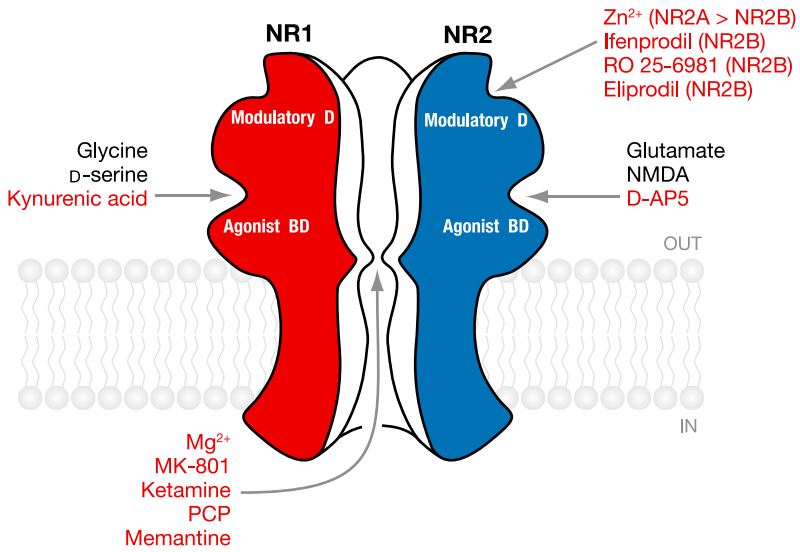

Figure 1

NMDA receptor model showing binding sites for agonists and antagonists. The extracellular portions of NR1 and NR2 consist of two domains, the modulatory domain (Modulatory D) and the agonist binding domain (Agonist BD) (20). Zn^{2+} is an endogenous ligand for NR2A- and NR2B-modulatory D (NR2A>NR2B stands for nanomolar affinity for NR2A and micromolar affinity for NR2B) (149), and the synthetic neuroprotectant ifenprodil and its derivatives bind at the same domain in an NR2B-selective manner (150). The binding sites for noncompetitive antagonists Mg^{2+}, MK-801, ketamine, PCP, and memantine are within the ion channel pore region. Antagonists are in red and agonists in black.

NMDA receptors contain a number of distinct binding sites of pharmacologic relevance (**Figure 1**). Besides the recognition site for glutamate, the receptors possess a binding site for glycine or D-serine, whose occupancy is required for NMDA receptor transmission. Glycine, as the first identified ligand for this site (10), was proposed as a fail-safe "second key" for the receptor to avoid accidental "overdose" stimulation by dietary and other sources of glutamate. However, glycine is also an abundant dietary amino acid. Recent studies indicate that the primary physiologic ligand for the "glycine" site of the receptor is the rare D-isomer of serine, D-serine (10a). Serine racemase mediates formation of D-serine from L-serine primarily in astrocytes that ensheath NMDA receptor synapses, but it may also function in neurons (11). Selective degradation of D-serine greatly reduces NMDA receptor neurotransmission (12) and excitotoxicity (13). Drugs that block the glycine site, such as kynurenic acid derivatives, reduce NMDA receptor neurotransmission and stroke damage (14) (**Figure 1**).

A site within the NMDA receptor channel binds phencyclidine (PCP) and related noncompetitive antagonists, such as the animal anesthetic, ketamine, and the potential antistroke drug, MK-801. These drugs act most effectively when the receptor is activated, a phenomenon referred to as open channel voltage-dependent blockade.

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Besides the Mg^{2+} binding locus, a site near the mouth of the channel binds Zn^{2+} to elicit a voltage-independent block (15, 16). A polyamine regulatory site activated by spermine and spermidine facilitates NMDA receptor transmission. These agents act in an allosteric fashion, augmenting NMDA receptor currents even with saturating concentrations of glycine, a phenomenon called glycine-independent stimulation (17). However, unlike D-serine/glycine, these agents are not required for NMDA receptor transmission.

NMDA receptors typically comprise four subunits. The glycine-binding NR1 subunit is present in all, whereas there are four subtypes of glutamate-binding NR2 subunits (NR2A–NR2D), and in some cases glycine-binding NR3 subunits (NR3A and NR3B) (reviewed in Reference 18). The NR2B subunits predominate in extrasynaptic areas, whereas NR2A tends to be confined to synapses. Excitotoxicity is thought to involve extrasynaptic receptors (19) such that selective NR2B antagonists, such as ifenprodil and eliprodil (SL 82075), are neuroprotective in animal models of stroke (20) (**Figure 1**).

The first compelling evidence of a role for glutamate in vascular stroke damage and the potential therapeutic value of antiglutamatergic agents came from the observation in the 1980s that MK-801, first developed as an antiepileptic drug, was a very potent and selective NMDA receptor antagonist (21) and could reduce stroke damage. MK-801 acts at the PCP site and elicits psychotomimetic effects, which interfere with therapy. Additionally, it has hypertensive actions. Ketamine, first developed as a general anesthetic and still used in veterinary medicine for anesthesia, also acts at this site. Accordingly, MK-801, like ketamine, is heavily sedating.

These limitations of MK-801 explain its lack of success in advanced clinical trials as well as the failure of numerous other noncompetitive antagonists acting at the PCP site. Lipton and associates (6, 22) have given evidence that uncompetitive channel blockade may be more clinically effective, leading to the therapeutic application of memantine. Whereas a noncompetitive antagonist such as MK-801 acts at an allosteric site, an uncompetitive antagonist may or may not act at such a site but, most importantly, its action is contingent on prior activation of the receptor by agonist. Memantine is a low-affinity, open channel blocker that appears to enter the channel selectively at times of pathophysiological activation but dissociates rapidly, preventing the drug from accumulating and blocking physiologic transmission. Memantine, a derivative of the antiinfluenza drug amantadine, possesses the three-ring adamantane structure. Whatever the exact pharmacology, memantine is clinically effective with a modest side effect profile that includes akathisia and dizziness. Memantine is approved for the treatment of mild/moderate Alzheimer's disease and is also effective in vascular dementia. In rodents, memantine reduces brain damage by approximately 50%, even when administered two hours after vascular occlusion, mimicking clinical settings (6).

AMPA Receptors

Besides NMDA receptors, a second class of ionotropic glutamate receptors was pharmacologically identified that respond selectively to the glutamate derivatives AMPA

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(α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and kainate, and molecular cloning revealed distinct AMPA and kainate receptors (reviewed in Reference 18). AMPA receptors are tetramers comprised of combinations of four subunits, GluR1–4, and they mediate most physiologic glutamate transmission as well as synaptic plasticity (23). Scaffolding proteins such as GRIP (glutamate receptor interacting protein) maintain AMPA receptors at synaptic sites (24). TARPs (transmembrane AMPA receptor regulatory proteins) regulate both AMPA receptor trafficking and channel opening (25).

Desensitization of AMPA receptors involves their internalization by replacement of GRIP with PICK1 following receptor phosphorylation by protein kinase C (26, 27), a mechanism required for expression of cerebellar long-term depression (LTD) (28). Internalized AMPA receptors are returned to the cell membrane by binding to *N*-ethylmaleimide sensitive factor (NSF) (29, 30) which provides a link to NMDA receptor transmission. NMDA receptor activation generates NO, which *S*-nitrosylates NSF (31), facilitating its binding to AMPA receptors and recycling to the plasma membrane (32). This feed-forward amplification of glutamatergic transmission seems well suited for a role in excitotoxicity, as activation of AMPA receptors is required to relieve the voltage-dependent Mg^{2+} blockade of NMDA receptors (33). The recycling of AMPA receptors affords several steps at which pharmacologic intervention might be possible. For instance, to diminish AMPA receptor transmission, one might develop drugs that block the binding of GRIP or of *S*-nitrosylated NSF to AMPA receptors.

Ca^{2+}-impermeability of AMPA receptors is regulated by editing of the GluR2 subunit mRNA, leading to an amino acid change at a critical residue in the pore region. Thus, AMPA receptors that lack GluR2 or are comprised of an RNA-edited, defective GluR2 are Ca^{2+}-permeable (34, 35) and cause excitotoxicity. Mice that have defective GluR2 show early onset epilepsy and premature death (36). Recent reports further reveal defective editing of the GluR2 mRNA in the spinal motor neurons of individuals affected by amyotrophic lateral sclerosis (ALS) (37). A selective channel blocker against GluR2-lacking AMPA receptors protects neurons against ischemia-induced death (38).

Metabotropic Glutamate Receptors

Metabotropic glutamate receptors (mGluR) are G-protein coupled and have been categorized into three groups based on their sequence homologies and G-protein coupling (39) (**Table 1**). Group I (mGluR1 and mGluR5) are coupled to the excitatory G_q protein. Both mGluR1 and mGluR5 are typically postsynaptic and act by stimulating phospholipase C (PLC) to generate inositol 1,4,5-trisphosphate (IP3), with associated Ca^{2+} release. mGluR5 receptors are physically linked to NMDA receptors by a chain of anchoring proteins, including PSD-95 (postsynaptic density-95), Shank, and Homer (40). Therefore, the activation of NMDA receptors can potentiate mGluR5 receptor signaling by limiting phosphorylation-induced desensitization through the activation of Ca^{2+}-dependent phosphatases (41). Reciprocally, activation of Group I receptors potentiates NMDA receptor transmission (42). Group II

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Table 1 Classification of the metabotropic glutamate receptors (mGluRs)

Receptor family	Subtypes	Effects on signaling	Selective agonist/antagonist
Group I: Excitatory (G_q)	mGlu1	Activate AC (mGluR1) → ⇑ cAMP	DHPG, 1*S*,3*R*-ACPD, quisqualate, LY393675
	mGlu5	Activate PLC → ⇑ IP3 → ⇑ [Ca^{2+}] Inhibits K^+ channels	DHPG, 1*S*,3*R*-ACPD, quisqualate, CHPG, MPEP, SIB-1757, SIB-1893
Group II: Inhibitory (G_i/G_o)	mGlu2	Inhibit AC → ⇓ cAMP	NAAG, 2*R*,4*R*-APDC, 1*S*,3*R*-ACPD, LY354740, LY379268, LY341495
	mGlu3	Inhibit VGCC → ⇓ [Ca^{2+}] Activates K^+ channels	NAAG, 2*R*,4*R*-APDC, 1*S*,3*R*-ACPD, LY354740, LY379268, LY341495
Group III: Inhibitory (G_i/G_o)	mGlu4	Inhibit AC → ⇓ cAMP	L-SOP, ACPT-1, L-AP4, PHCCC, MSOP, MAP4, CPPG
	mGlu6	Inhibit VGCC → ⇓ [Ca^{2+}]	L-SOP, L-AP4, MSOP, MAP4
	mGlu7		L-SOP, L-AP4, MSOP, MAP4
	mGlu8		L-SOP, L-AP4, 3,4-DCPG, MSOP, MAP4

Abbreviations: AC, adenylyl cyclase; [Ca^{2+}], intracellular calcium; VGCC, voltage-gated calcium channels; DHPG, (*S*)-3,5-dihydroxyphenylglycine; ACPD, 1-aminocyclopentane-1,3-dicarboxylic acid; LY393675, 2-(*S*)-amino-2-(3-*cis*-carboxycyclobutyl)-3-(9H-thioxanthen-9-yl) propionic acid; CHPG, (*RS*)-2-chloro-5-hydroxyphenylglycine; MPEP, 2-methyl-6-(phenylethylnyl)-pyridine; SIB-1757, 6-methyl-2-(phenylazo)-pyridinol; SIB-1893, (E)-2-methyl-6-(2-phenylethylnyl)-pyridine; NAAG, *N*-acetyl-L-aspartyl-L-glutamate; APDC, (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate; LY354740, (1*S*,2*S*,5*R*,6*S*)-(+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate; LY379268, (-)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate; LY341495, (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3(xanth-9-yl)propanoic acid; L-SOP, L-serine-O-phosphate; ACPT-1, (1*S*,3*R*,4*S*)-1-aminocyclopentane-1,3,4-tricarbocylic acid; L-AP4, L-2-amino-4-phosphono-butanoate; PHCCC, N-phenyl-7-(hydroxylimino)cyclopropa[b]chromen-1a-carboxamide; MSOP, (*RS*)-α-methylserine-O-phosphate; MAP4, (*S*)-2-amino-2-methyl-4-phosphonobutanoic acid; CPPG, (*RS*)-α-cyclopropyl-4-phosphonophenylglycine; 3,4-DCPG, (*S*)-3,4-dicarboxyphenylglycine.

receptors (mGluR2 and mGluR3) are linked to the inhibitory G_i/G_o proteins. The mGluR2 receptors are located on terminals of glutamatergic neurons, whereas the mGluR3 receptors are principally glial. Group III receptors (mGluR4, mGluR6, mGluR7, and mGluR8), like the group II class, are coupled to G_i/G_o proteins. Both Group II and Group III receptors inhibit adenylyl cyclase.

The greatest body of research on drug development has involved Groups I and II, with a particular focus on Group I antagonists and Group II agonists. The Group I subtypes, mGluR1 and mGluR5, are reciprocally localized in the brain and are almost invariably postsynaptic. mGluR5 antagonists elicit increased survival of dopaminergic neurons and relieve Parkinsonian symptoms (43). Antagonists of mGluR1/5 receptors are also anxiolytic, presumably reflecting the blockade of glutamate excitation (39). mGluR2 receptors are most often found at extrasynaptic sites on nerve terminals and inhibit the release of glutamate (44, 45), whereas mGluR3 receptors are more ubiquitous. A substantial body of work has been done with the mGluR2/3 agonist LY354740, which inhibits glutamate release but also blocks release of other numerous neurotransmitters, including dopamine, norepinephrine, GABA, and several neuropeptides (45), thus leading to anxiolytic actions in numerous animal

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models (39). mGluR2/3 agonists also protect dopaminergic neurons against toxicity (43).

Inositol 1,4,5-trisphosphate (IP3): a second messenger that is produced primarily by phospholipase C (PLC) metabolism of phosphatidylinositol-4,5-bisphosphate (PIP_2) in response to the stimulation of G protein–coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs)

Metabotropic glutamate receptors (mGluRs): seven transmembrane receptors that are coupled to second-messenger cascades via G-proteins

NAAG

The millimolar concentrations of NAAG in the brain make it one of the most abundant neurotransmitter-related molecules (46). Although first identified in the mid 1960s, NAAG's role as a putative neurotransmitter has only recently been well characterized (47). It functions as an agonist at Group II mGluRs, especially mGluR3, to reduce cyclic AMP (cAMP) production (48) (**Table 1**). NAAG is converted to glutamate and *N*-acetyl-L-aspartate by the well-characterized GCPII and the more recently identified GCPIII (49, 50). These enzymes are Zn^{2+}-activated metalloproteases localized to glia in the brain, but they are also abundant in parts of the gastrointestinal tract, kidney, and the human prostate (51). GCPII was independently discovered as prostate-specific membrane antigen (PSMA), whose concentrations are more selectively associated with metastatically active prostate cancer than the classic prostate-specific antigen (PSA) (52, 53).

The physiologic role of cerebral NAAG has been best elucidated by studies of GCPII inhibitors. In rats with middle cerebral artery occlusion, glutamate release into the extracellular space increases approximately 50-fold (54). Selective inhibitors of GCPII abolish this augmentation and markedly reduce stroke damage (55). However, these drugs do not affect the basal release of glutamate. Thus, basal release of glutamate does not involve NAAG stores, but during a stroke, almost all of the augmented release reflects the breakdown of NAAG by GCPII. Thus, GCPII inhibitors offer promise as antistroke therapy. GCPII inhibitors also relieve the neuropathic pain (56, 57), decrease in nerve conduction, and neuropathologic alterations associated with diabetic neuropathy (58). By contrast, drugs that are presently clinically employed in the treatment of neuropathic pain, such as gabapentin, relieve neuropathic pain but do not alter nerve conduction (59).

NITRIC OXIDE

NO is a noxious free radical gas, which, in the late 1980s, was discovered to exist physiologically in mammalian systems. Studies by Furchgott and collaborators (60) have shown that relaxation of blood vessels elicited by acetylcholine and other agents requires generation by the endothelium of an unstable substance that diffuses to the smooth muscle layer, which was subsequently identified as NO (reviewed in Reference 61). At about the same time, NO was also shown to mediate the bactericidal and tumoricidal actions of macrophages (62, 63). Based on the fact that NO relaxes blood vessels by activating soluble guanylyl cyclase to generate cyclic GMP and that glutamate through NMDA-subtype receptors can also stimulate cyclic GMP formation, we (64) and others (65) showed that inhibitors of NO synthase (NOS) prevent NMDA-induced augmentation of cyclic GMP in brain slices. We also observed that NMDA elicits a tripling of NOS activity, monitored by the conversion of arginine to citrulline, which is formed stoichiometrically with NO (64). The ability of neuronal

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depolarization to markedly augment NOS activity in a matter of seconds was at first perplexing but became clarified when we purified (66) and cloned (67) what is now designated as the neuronal form of the enzyme (nNOS). We found that nNOS binds calmodulin such that Ca^{2+} influx associated with depolarization activates the enzyme. There are three distinct forms of NOS reflecting three different genes: inducible NOS (iNOS), the macrophage form, which also exists in almost all tissues of the body; endothelial NOS (eNOS), which is predominantly in blood vessels; and nNOS. nNOS is localized in discrete neuronal populations in the brain and in numerous peripheral autonomic nerves. Evidence for NO neurotransmitter function is well established in the myenteric nervous system of the intestine, as nonadrenergic-noncholinergic (NANC) neurotransmission is markedly reduced by NOS inhibitors and in nNOS knockout ($nNOS^{-/-}$) mice (68–70). Penile nerves are enriched in nNOS, and NOS inhibitors prevent erection, establishing a neurotransmitter role for NO in penile erection (71). The therapeutic actions of phosphodiesterase-5 inhibitors in erectile dysfunction reflect their elevation of cyclic GMP levels, which are stimulated by the transmitting actions of NO.

Insight into physiologic functions of neuronal NO comes from studies of $nNOS^{-/-}$ mice (72). These mice have markedly dilated stomachs with hypertrophy of the pyloric sphincter, reflecting a physiologic role of NO in dilating the sphincter and providing a model of human infantile pyloric stenosis. Moreover, gastroparesis with dilated stomachs is a frequent complication of diabetes and resembles the phenotype of $nNOS^{-/-}$ mice (72). Diabetic mice display a loss of nNOS in their myenteric neurons, which can be reversed with insulin treatment and evidently reflects an insulin regulatory site in the promoter region of nNOS (73). Male $nNOS^{-/-}$ mice are hyperaggressive and display excessive sexual mounting activity on females, suggesting a role for neuronal NO in regulating aggressive and sexual behavior (74).

Penile erection is maintained in the $nNOS^{-/-}$ mice, which were developed by deletion of exon-2, and which still possess approximately 10%–15% residual NOS activity in the brain (72). The residual activity reflects alternatively spliced nNOSβ, which is catalytically active and retained in the knockout rodents (75). Subsequent studies have established that nNOSβ suffices to maintain penile erection (76). Recent studies with total deletion of nNOS, including nNOSβ, reveal hypogonadism and infertility (77).

A role for NO in neurotoxicity follows from the known activation of nNOS by NMDA receptor stimulation. In brain cultures, NMDA-induced neurotoxicity is markedly reduced by NOS inhibitors (78) and in $nNOS^{-/-}$ animal cultures (79). Stroke damage following middle cerebral artery occlusion is diminished by treatment with NOS inhibitors (80) and in $nNOS^{-/-}$ mice (81). NO can damage tissues by multiple mechanisms. The combination of NO with superoxide, which leaks from damaged mitochondria, leads to the formation of peroxynitrite, which degrades to the highly reactive hydroxyl free radical that can damage DNA, proteins, and lipids. Moncada and associates (82, 83) reported that NO impairs cytochrome *c* oxidase, disrupting the mitochondrial membrane potential and leading to energy depletion (**Figure 2**). In some studies, NO can be cytoprotective (84–86). These discrepancies may relate to different influences at high and low concentrations of NO as well as

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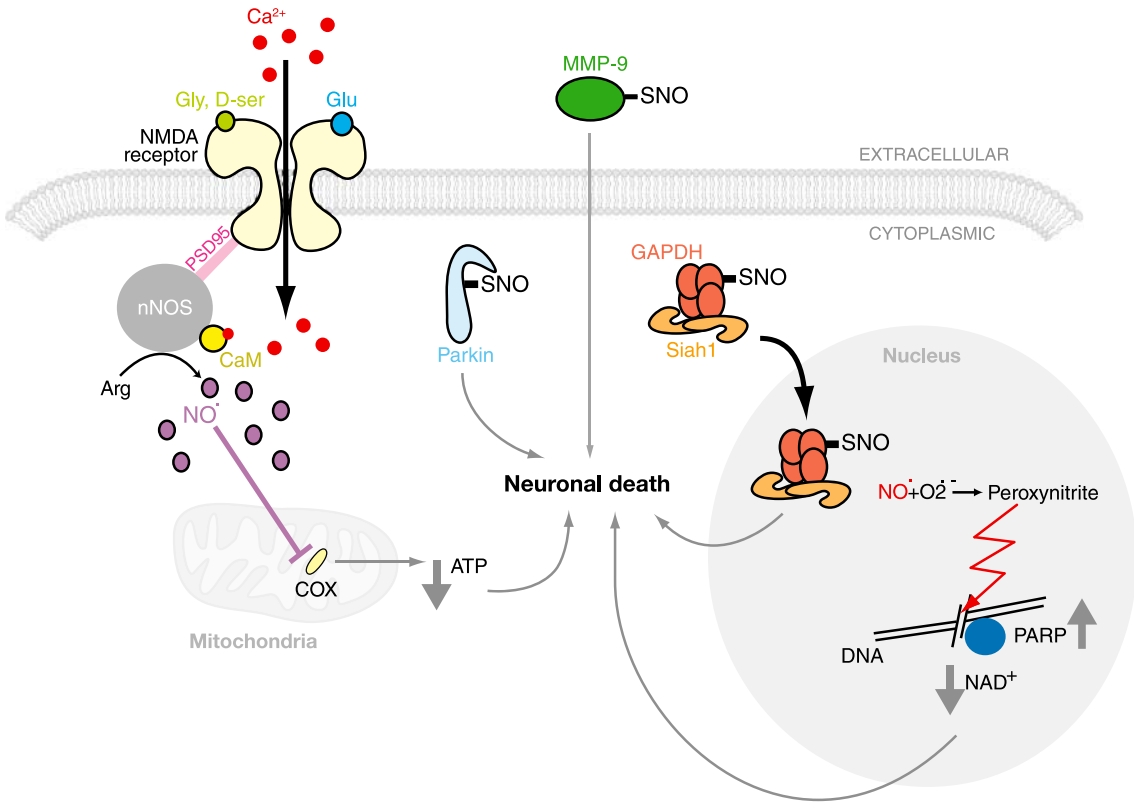

Figure 2

Excess NO production mediates neuronal death. Activation of NMDA receptors results in the influx of Ca^{2+}, which binds calmodulin (CaM) and activates nNOS, to convert L-arginine (Arg) to citrulline and NO. nNOS colocalizes with NMDA receptor via PSD-95. Although NO has many roles as a signaling molecule in neurons, generation of excess NO can be neurotoxic. NO inhibits cytochrome *c* oxidase (COX) in the mitochondria, which can cause ATP depletion (↓ATP) and neuronal death (82, 83). Neurotoxic actions of NO are also mediated by peroxynitrite, a reaction product from NO and superoxide anion ($O_2^{\cdot -}$). Peroxynitrite causes severe damage to DNA, which results in overactivation of PARP (PARP↑), depletion of NAD^+ (↓NAD^+), and neuronal death (3). NO *S*-nitrosylates many proteins, including MMP-9, parkin, and GAPDH. *S*-nitrosylation (SNO) activates MMP-9 and induces neuronal apoptosis (89). *S*-nitrosylation of parkin inhibits its E3-ubiquitin-ligase activity and protective function (91, 92). *S*-nitrosylated GAPDH initiates neuronal apoptosis by nuclear translocation, following Siah1 binding (98).

the differential formation of NO free radical ($NO^{\cdot}$), the nitrosonium cation (NO^+), nitrite (NO_2^-), or nitrate (NO_3^-) (85).

NO was first thought to act exclusively by stimulating cyclic GMP formation. Elegant studies by Stamler and associates (86, 87) provided evidence that NO might *S*-nitrosylate a variety of target proteins. Direct evidence that such *S*-nitrosylation occurs physiologically came with the development of the biotin-switch assay,

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permitting a simple approach to detecting endogenously *S*-nitrosylated proteins (88). In the brain, numerous major proteins are physiologically *S*-nitrosylated, including tubulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the sodium pump ATPase, and NMDA receptors (88). Moreover, basal *S*-nitrosylation of these proteins is abolished in the brains of $nNOS^{-/-}$ mice, establishing that physiologic neuronally derived NO mediates this modification.

S-nitrosylation: the coupling of an NO moiety to a cysteine thiol (-SH) to form an *S*-nitrosothiol (-SNO)

There is evidence that neurotoxicity may involve excess *S*-nitrosylation. For instance, cerebral ischemia augments *S*-nitrosylation of matrix metalloprotease-9 (MMP-9), stimulating its activity and leading to the formation of stable sulfinic or sulfonic acid derivatives whose irreversible activation triggers neuronal apoptosis (89) (**Figure 2**). Parkin is an E3-ubiquitin-ligase whose mutation is associated with some forms of genetically determined Parkinson's disease (90). In Parkinson's disease, *S*-nitrosylation of parkin regulates its E3-ubiquitin-ligase activity and causes cytotoxicity (91, 92) (**Figure 2**). Other *S*-nitrosylated proteins that may promote cell death include IκB kinase beta (IKKβ) (93), nuclear factor κB (NF-κB) (94), and MDM2 (95). A link between *S*-nitrosylation of these proteins and neuronal cell death remains to be clarified.

Based on evidence linking GAPDH to cell death (96), a novel cell death cascade has been identified whereby diverse cell stressors in multiple tissues activate NO formation, which leads to nuclear events that mediate apoptosis (97, 98). Those stressors activate iNOS in a wide range of tissues, whereas NMDA receptor overactivation leads to NO accumulation in neurons. The generated NO *S*-nitrosylates GAPDH at its catalytic cysteine (C152 for human GAPDH), abolishing catalytic activity but conferring upon GAPDH the ability to bind to Siah1, an E3-ubiquitin-ligase. Siah1, which possesses a nuclear localization signal, escorts GAPDH to the nucleus. Within the nucleus, GAPDH stabilizes the rapidly turning over Siah1, enabling its E3-ubiquitin-ligase activity to degrade diverse nuclear proteins that lead to cell death (**Figure 2**).

The neurotoxicity seen in Huntington's disease may involve the NO-GAPDH-Siah1 cascade (99–101). The neurotoxicity of mutant Huntingtin, which accounts for cytotoxicity, requires nuclear translocation of its N-terminal fragment, which lacks a nuclear localization signal (102). Mutant Huntingtin occurs in a ternary complex with GAPDH and Siah1, which mediates its nuclear translocation and cytotoxicity (101). There may be pharmacotherapeutic consequences of the NO-GAPDH-Siah1 pathway. The monoamine oxidase-B inhibitor *R*-(−)-deprenyl (deprenyl) has been employed for many years in the therapy of Parkinson's disease, based on the notion that it elevates dopamine levels and therefore provides symptomatic benefits (103). However, clinical and animal studies indicate that deprenyl is neuroprotective and slows the progression of disease, presumably by retarding the loss of dopamine neurons (104). Deprenyl derivatives that lack monoamine oxidase inhibitory activity, such as TCH346 (CGP3466), are potent neuroprotective agents (105). As TCH346 acts at low nanomolar concentrations, Waldmeier and colleagues (106) covalently linked it to tissue extracts and found a single labeled protein, GAPDH. We observed that as little as 1 nM deprenyl or TCH346 block the *S*-nitrosylation of GAPDH, its binding to Siah1, and nuclear translocation (107). Thus, the neuroprotective actions of

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these drugs evidently involve the NO-GAPDH-Siah1 system. Recent work reports a genetic association between the *GAPDH* locus on chromosome 12 and late-onset Alzheimer's disease (108). Agents that inhibit GAPDH-Siah1 interaction may provide a useful approach to cytoprotective therapy.

PARP, a well-characterized DNA repair enzyme, may also play a role in cell death associated with NO. Of the multiple forms of the enzyme, PARP1 is predominant and localized exclusively to the nucleus where it is activated by DNA strand breaks (3). PARP1 attaches long stretches of ADP-ribose to nuclear targets such as histones and PARP1 itself, utilizing NAD^+ as the substrate, which triggers chromatin-structure relaxation and increases the access of DNA-repair enzymes to the break. However, overactivation of PARP by excess DNA damage depletes NAD^+ and hence ATP, leading to cell death from energy loss (**Figure 2**). Presumably, with massive DNA damage, it is safer to destroy cells rather than to attempt DNA repair, which may be faulty and lead to mutational damage. Neurotoxicity in brain cultures is markedly diminished by treatment with PARP inhibitors (109), and stroke damage following middle cerebral artery occlusion is reduced up to 80% in $PARP1^{-/-}$ mice (110). In rodents, PARP inhibitors substantially reduce stroke damage (111).

Iron can be extremely cytotoxic, and it accumulates in the brains of patients with Parkinson's disease as well as Alzheimer's disease (112). Recent studies implicate glutamate-NMDA receptor-NO transmission in iron uptake (113). NO is delivered to most, if not all, of its targets by the binding of NOS to such targets either directly or through scaffolding proteins such as CAPON, which binds nNOS selectively (114). CAPON in turn binds to Dexras1, a member of the Ras family of small G-proteins, so named based on its augmented expression following stimulation by the glucocorticoid-analogue dexamethasone (115). NO *S*-nitrosylates and activates Dexrasl, serving as guanine nucleotide exchange factor (GEF) (115). Physiologic activation of Dexras1 requires its interaction with nNOS, as Dexras1 activation declines in $nNOS^{-/-}$ mice. Dexras1 physiologically binds to PAP7 (peripheral benzodiazepine-associated protein-7), a scaffolding protein, which in turn, binds to DMT1 (divalent metal ion transporter-1), the principal iron import protein in cells (113). NMDA receptor activation thus activates nNOS, which *S*-nitrosylates and activates Dexras1, which then simulates iron entry via DMT1. These findings raise the possibility that iron influx provides a signaling response to glutamate neurotransmission analogous to Ca^{2+} entry. The system may also participate in NMDA receptor neurotoxicity.

CALCIUM

Ca^{2+} is likely the most prominent intracellular messenger molecule. Its multiple cellular roles are much too extensive to describe here in full, so we review only selected functions that mediate cell death. Ca^{2+} enters cells by at least three separate types of channels: voltage-sensitive Ca^{2+} channels, store-operated channels, and receptor-operated channels such as the NMDA receptor. Within the cell, Ca^{2+} is sequestered in mitochondria, in the endoplasmic reticulum (ER), or via one of numerous Ca^{2+}-binding proteins. The SR (sarcoplasmic reticulum in muscle) provides the largest stores of intracellular Ca^{2+}. Ca^{2+} is retained within the ER by the sarco(endo)plasmic

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reticulum Ca^{2+}-ATPase (SERCA) pump. It is released from ER stores in response to neurotransmitter/hormone receptor activation of PLC, generating IP3, which acts upon its receptors (IP3R) in ER. Release of intracellular Ca^{2+} via IP3R is typically followed by Ca^{2+} entry into cells via a channel that had been thought to be related to the store-operated Ca^{2+} channel but appears to be distinct and involves one or more of a family of TRP (transient receptor potential) Ca^{2+} channels (116). Although depletion of Ca^{2+} stores by IP3R release of Ca^{2+} can activate Ca^{2+} entry, IP3R regulation of entry is largely independent of its ability to release Ca^{2+} (117). Moreover, PLC-γ itself is critical for agonist-induced Ca^{2+} entry independent of its lipase activity (118). Ca^{2+}-induced Ca^{2+} release involves discrete ryanodine receptors (119).

Inositol 1,4,5-trisphosphate receptor (IP3R): receptors that release Ca2+ into the cytosol from internal stores in response to IP3

Free cytosolic Ca^{2+} concentrations are maintained at approximately 100 nM, orders of magnitude less than extracellular levels, by the SERCA pump as well as by extrusion through the plasma-membrane Ca^{2+}-ATPase. Mitochondria accumulate Ca^{2+} via a uniporter and release it by reversal of the uniporter, a Na^{+}-H^{+} dependent exchanger, or via a mitochondrial permeability transition pore (mPTP). Although the mPTP may have an important physiologic function in regulating intracellular Ca^{2+} dynamics, it has been studied most extensively as a channel that participates in cell death via release of Ca^{2+} and other substances (120).

ER Ca^{2+} disposition may play a direct role in apoptosis. Besides regulating Ca^{2+} signaling, the ER participates in the modification and movement of newly synthesized proteins. Pathologic increases or decreases in ER Ca^{2+} markedly alter protein folding and lead to cell death following ER stress (121). Abnormal protein folding can be counteracted by the unfolding protein response pathway, but following ER stress this pathway may elicit apoptosis (122). BI-1 (Bax inhibitor-1) is localized to ER and suppresses ER stress-induced apoptosis (123). Bap31 (Bcl-2-associated protein-31), an ER membrane protein, is cleaved by caspase-8, and the generated fragment leads to apoptosis associated with ER Ca^{2+} release (124).

Mitochondria have long been known to accumulate Ca^{2+} via a relatively low affinity uniporter (125). Mitochondria operate in conjunction with ER in this process as revealed by the studies of Pozzan and collaborators (126), who established an intimate physical interaction between ER and mitochondrial membrane with ER Ca^{2+} directly entering mitochondria at selected sites. We elucidated a novel signaling system whereby ER and mitochondria interact in causing apoptosis via IP3R–cytochrome *c* interactions (127) (**Figure 3**). IP3R is an extremely large protein, approximately 2800 amino acids, with the IP3 binding site occupying only a few hundred amino acids at the N terminus and the Ca^{2+} channel only a few hundred amino acids at the C terminus. The large intervening area is presumed to provide sites for the binding of the large number of substances that can regulate IP3R function (128). Yeast two-hybrid analysis revealed selective binding of cytochrome *c* to IP3R (127). Release of Ca^{2+} by IP3R is well known to be regulated by endogenous Ca^{2+}. Low concentrations of Ca^{2+} augment release, whereas concentrations above the physiologic 100 nM concentrations inhibit release in a feedback that presumably prevents excessive Ca^{2+} release by IP3. As little as 1 nM cytochrome *c* markedly and selectively inhibits this feedback system leading to uncontrolled, excessive Ca^{2+} release. The Ca^{2+} enters the adjacent mitochondria, triggering cytochrome *c* release, which binds to IP3Rs

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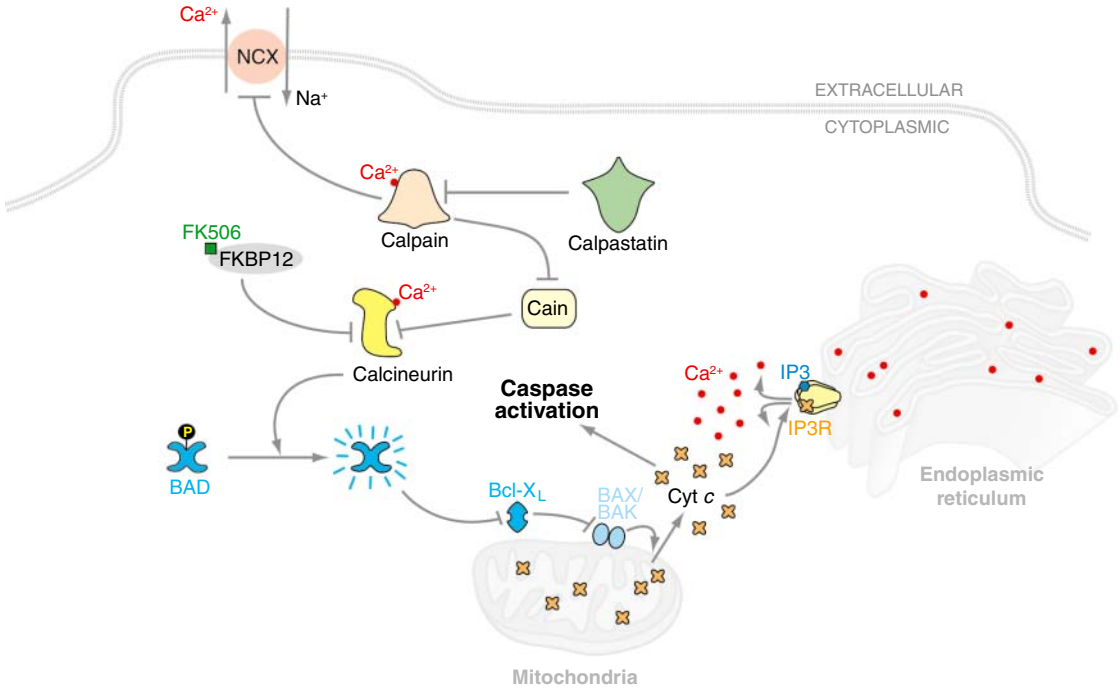

Figure 3

Excess Ca^{2+} release in neurons causes neurotoxicity. Calpain, a Ca^{2+}-dependent protease, cleaves NCX (Na^+-Ca^{2+} exchanger) during ischemia, resulting in increased [Ca^{2+}] which causes neuronal death. Overexpression of calpastatin, an inhibitor of calpain, reverses the effect (144). FK506 binds to FKBP12, a complex that inhibits calcineurin, a Ca^{2+}-dependent phosphatase (135). Cain, an inhibitor of calcineurin (147), is cleaved by calpain (148). Calcineurin dephosphorylates Bad which heterodimerizes with Bcl-X_L and causes Ca^{2+}-induced apoptosis (138). Cytochrome *c* (cyt *c*) released from mitochondria (5) activates caspases and mediates apoptosis (130). IP3R, bound with cyt *c* and IP3, is resistant against Ca^{2+}-dependent inhibition of Ca^{2+} release which leads to uncontrolled Ca^{2+} release (127).

on the ER, further augmenting Ca^{2+} release. This feed-forward cycle is transmitted throughout the cell causing synchronized Ca^{2+} release and apoptotic cell death. Evidence consistent with this model includes the finding that apoptotically released cytochrome *c* binds to IP3Rs on the ER, whereas cytochrome *c* in cells lacking IP3R passes into the supernatant fraction rather than associating with the ER membrane containing fraction. A dominant-negative peptide that selectively blocks the binding of IP3R and cytochrome *c* prevents cell death suggesting that drugs with this activity might be therapeutic agents (129).

Besides releasing ER Ca^{2+}, cytochrome *c* activates the caspase cascade, which has been extensively reviewed (2, 130). Permeabilization of the outer mitochondrial membrane to permit cytochrome *c* release involves the opening of pores via several mechanisms, including the actions of the proapoptotic Bcl-2 family of proteins, most notably Bax and Bak (131) (**Figure 3**). Cytochrome *c* may also exit via a rupture of the

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outer mitochondrial membrane initiated by mPTP, which comprises the mitochondrial benzodiazepine receptor, the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), and the mitochondrial cyclophilin D (120). As these proteins include components of both the outer and inner mitochondrial membrane, mPTP occurs at sites of fusion between these membranes. mPTP is triggered by a variety of factors, including Ca^{2+} and reactive oxygen species (132). Studies with cyclophilin D knockout mice implicate mPTP in necrosis but not apoptosis (133, 134). These mice display marked resistance to cardiac ischemia/reperfusion injury but are susceptible to apoptosis.

Calcium-Dependent Enzymes

Calcineurin is a Ca^{2+}-dependent phosphatase that mediates the immunosuppressant actions of cyclosporin A and FK506. These drugs bind to their respective receptor proteins, cyclophilin and FK506-binding protein (FKBP12), with the drug-receptor complex binding to and inactivating calcineurin (135) (**Figure 3**). The immunosuppressant cyclosporin A blocks Ca^{2+}-dependent apoptosis in some cells, implicating calcineurin in this process (136). However, cyclosporin A also blocks cyclophilin D, a key component of mPTP involved in neuronal cell death (137). Calcineurin also dephosphorylates phospho-Bad, a proapoptotic member of the Bcl-2 family. Dephosphorylation of Bad by calcineurin causes it to translocate from the cytosol to mitochondria, enhancing heterodimerization with Bcl-X_L to promote neuronal apoptosis (138) (**Figure 3**). Overexpression of calcineurin in apoptosis-resistant cells dephosphorylates Bad and restores the apoptotic phenotype (139, 140).

Calpains are a family of several Ca^{2+}-stimulated proteases whose inhibition blocks cell death (141). Calpain activity is regulated by calpastatin, an endogenous inhibitor (142, 143) (**Figure 3**). Because calpain can cleave multiple proteins, including caspases themselves (122), it is difficult to determine which of its actions participates in apoptosis. A recent study has shown that calpain cleaves the plasma membrane Na^+-Ca^{2+} exchanger (NCX) during brain ischemia, and overexpression of calpastatin rescues neurons from excitotoxic death (144) (**Figure 3**). Calpain also cleaves antiapoptotic proteins such as Bcl-2 and Bcl-X_L, and the cleaved products promote cell death (145). Regardless of the exact target of calpain, its importance is evident by the substantial antiapoptotic actions of calpain inhibitors, with several under clinical investigation as cytoprotectants (146).

An interesting intersection of calpain and calcineurin in cell death is evident in calpain's cleavage of cabin/cain, an endogenous calcineurin inhibitor (147, 148) (**Figure 3**). This cleavage activates calcineurin and leads to Ca^{2+}-mediated cell death (148).

CONCLUSIONS

The literature on molecular mechanisms of cell death is vast so that this review perforce is delimited. We focused on intercellular and intracellular signaling systems that are physiologic mediators of the actions of neurotransmitters in the nervous

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system. When activated in excess, they participate in cell death mechanisms, and agents that block these systems are neuroprotective. By contrast, other cascades such as those involving caspases and members of the Bcl-2 family have been predominantly characterized as "killers." Conceivably, such systems also participate in physiologic intracellular signaling. If so, then drugs influencing caspases and members of the Bcl-2 family might be therapeutic in diverse conditions unrelated to cell death.

SUMMARY POINTS

1. Glutamate, a major excitatory neurotransmitter in the brain, causes excitotoxicity when released in excess.
2. Overactivation of NMDA receptors by glutamate causes excess Ca^{2+} entry, which leads to diverse events that elicit cell death. Thus, pharmacologic blockade of NMDA receptors via memantine, for example, has therapeutic benefits.
3. Activation of AMPA receptors relieves the voltage-dependent Mg^{2+} blockade of NMDA receptors, implicating links to excitotoxicity. AMPA receptors, which lack GluR2 or are comprised of an RNA-edited, defective GluR2, are Ca^{2+}-permeable and cause excitotoxicity.
4. Group I mGluR antagonists and Group II mGluR agonists are anxiolytic, presumably reflecting the blockade of glutamate excitation.
5. Selective inhibitors of GCPII, which convert NAAG to glutamate and *N*-acetyl-L-aspartate, reduce stroke damage.
6. Glutamate binds to NMDA receptors leading to Ca^{2+} influx and activation of nNOS. NO can cause neurotoxicity via inhibition of cytochrome *c* oxidase in mitochondria, overactivation of PARP, or *S*-nitrosylation of proteins, including MMP-9, parkin, and GAPDH, all of which lead to neuronal death.
7. Calcium, the most prominent of all intracellular messengers, mediates diverse forms of cell death, with actions modulated by many proteins, including IP3 receptors, calcineurin, calpain, and cytochrome *c*.

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