Mechanisms of neuronal cell injury/death and targets for drug intervention

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The loss of neurons is responsible for many acute neurological disorders, as well as many chronic neurodegenerative diseases. Drug discovery research has concentrated on blocking necrosis produced by activation of one or more excitatory amino acid (EAA) receptors. Both receptor antagonists and compounds reducing EAA release are currently in clinical trials. With the recent advances in the understanding of apoptosis new strategies for neuroprotection are also emerging. It is anticipated that clinically useful neuroprotective drugs will soon be available for treatment of a range of CNS disorders. This article reviews some of the mechanisms responsible for neuronal cell death and outlines strategies to block calcium overload and free radical production.

As post-mitotic cells, neurons are incapable of dividing and regenerating. This inability magnifies the importance of maintaining neuronal wellbeing, and the impact of brain injury or disease. The manifestations of neurological and neurodegenerative disease are generally attributable to either a selective or a generalized loss of neurons. Therefore, understanding the processes that lead specifically to the death of neurons is critical for developing pharmacological strategies to treat these disorders. Several of the major mechanisms that have been implicated as initiating or sustaining the pathways to neuronal cell death are discussed in this review.

Traditionally, neuronal death has been thought of as necrotic: some extraneuronal stimulus triggers a pathway that leads to the cell's injury or demise. For many acute neurological disorders, such as stroke or head trauma, necrosis is the primary cause of cell death. Ischemia, for example, initiates a well characterized cascade of events that elicits cell swelling and eventually cell death[1]. Glial cells, which contribute to the wellbeing and function of neurons, also die in many acute neurological disorders, but the mechanisms leading to glial death may be different from those for neurons. From the perspective of pharmacological intervention necrotic neuronal death is well studied and good models exist to assess novel treatments. Progress in specific strategies is discussed in greater detail below and current therapeutic drugs are summarized in Table 1.

A second distinct pathway of neuronal cell death is apoptosis or programmed cell death (PCD)[2]. The role of PCD in neurological and neurodegenerative disorders is harder to elucidate than necrotic death, and strategies for intervention of apoptosis are more problematical. However, the potentially critical role of apoptosis in mediating a variety of neurological/neurodegenerative diseases has recently become widely appreciated[3–5]. In practice, it is difficult to differentiate between necrosis and apoptosis in brain tissue because the criteria that distinguish between them are based upon transient events. In the CNS it is important to remember that biochemical markers are not always indicative of processes occurring in neurons. An absence of the hallmarks of apoptosis,

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Table 1. Neuroprotective agents in clinical trials[a]

	Company	Stage	Indication[f]
NMDA antagonists[b]			
Competitive antagonists			
SDZ-EAA-494 (d-CPP-ene)	NIH	Phase III	TBI
CGS19755 (Selfotel)	Ciba-Geigy	Phase III (abandoned)	Stroke, TBI
Noncompetitive antagonists			
CNS1102 (Aptiganel)	Cambridge Neurosciences	Phase II, III	Stroke, TBI
FPL12924AA (Remacemide)	Astra (Fisons)	Phase II	Stroke, epilepsy
Glycine site antagonists			
Acea1021[c]	CoCensys (Ciba-Geigy)	Phase II	Stroke
ZD9379	Zeneca	Phase I	Stroke
GV150526	Glaxo Wellcome	Phase II	Stroke
ACPC	NIH	Phase I	Stroke
Subtype-selective antagonists			
SL820715 (Eliprodil)[d]	Synthelabo	Phase III	Stroke
CP101606	Pfizer	Phase I	PD
AMPA antagonists			
YM90K	Yamanouchi	Phase I	Stroke
LY293558	Eli Lilly	Phase I	Stroke
PD152247 (PNQX)[c]	Parke-Davis	Preclinical	Stroke
Excitatory amino acid (glutamate) release inhibitors			
Sodium channel modulators			
BW619C89	Glaxo Wellcome	Phase III (abandoned)	Stroke, TBI
Fosphenytoin (Cerebyx®)	Parke-Davis	Phase II	Stroke
Lubeluzole[e]	Janssen	Phase III	Stroke
Riluzole (Rilutek®)	Rhone-Poulenc	Marketed	ALS
Lamotrigine (Lamictal®)	Glaxo Wellcome	Marketed	Epilepsy
Kappa opiate			
CI977 (Enadoline)	Parke-Davis	Phase II	TBI
Neuronal calcium channel blockers			
SNX111	Neurex (Parke-Davis)	Phase II	Stroke, TBI
AE0047	Green Cross	Phase III	Stroke
Calpain inhibitors			
AK275	Alkermes	Preclinical	Stroke
MDL104903	Hoechst, Marion, Roussel	Preclinical	Stroke
Free radical inhibitors			
Tirilazad (Freedox®)	Upjohn-Pharmacia	Phase III (abandoned)	TBI
Idebenone	Takeda	Phase III	Alzheimer's
YM737	Yamanouchi	Phase II	Stroke
OPC14117	Otsuka	Phase II	Alzheimer's
Growth factors			
Basic fibroblast growth factor (bFGF)	Scios Nova Synergen	Phase I	Stroke
Glial cell-line derived neurotrophic factor (GDNF)	Amgen	Phase I	ALS

[a] This is not a comprehensive list and may not accurately reflect the clinical status of all compounds. It attempts to be representative of the mechanisms currently under investigation as neuroprotective agents.
[b] Nitroglycerin (a marketed drug) modulates NMDA receptor function by action at the 'redox site', and may be neuroprotective.
[c] Compound has activity at both GLY_N receptors and AMPA receptors for a mixed EAA antagonist profile.
[d] Prolongation of the QT interval in the heart may put some patients at risk.
[e] Other mechanisms may contribute to neuroprotective action.
[f] TBI, traumatic brain injury; PD, Parkinson's disease; ALS, amyotrophic lateral sclerosis.

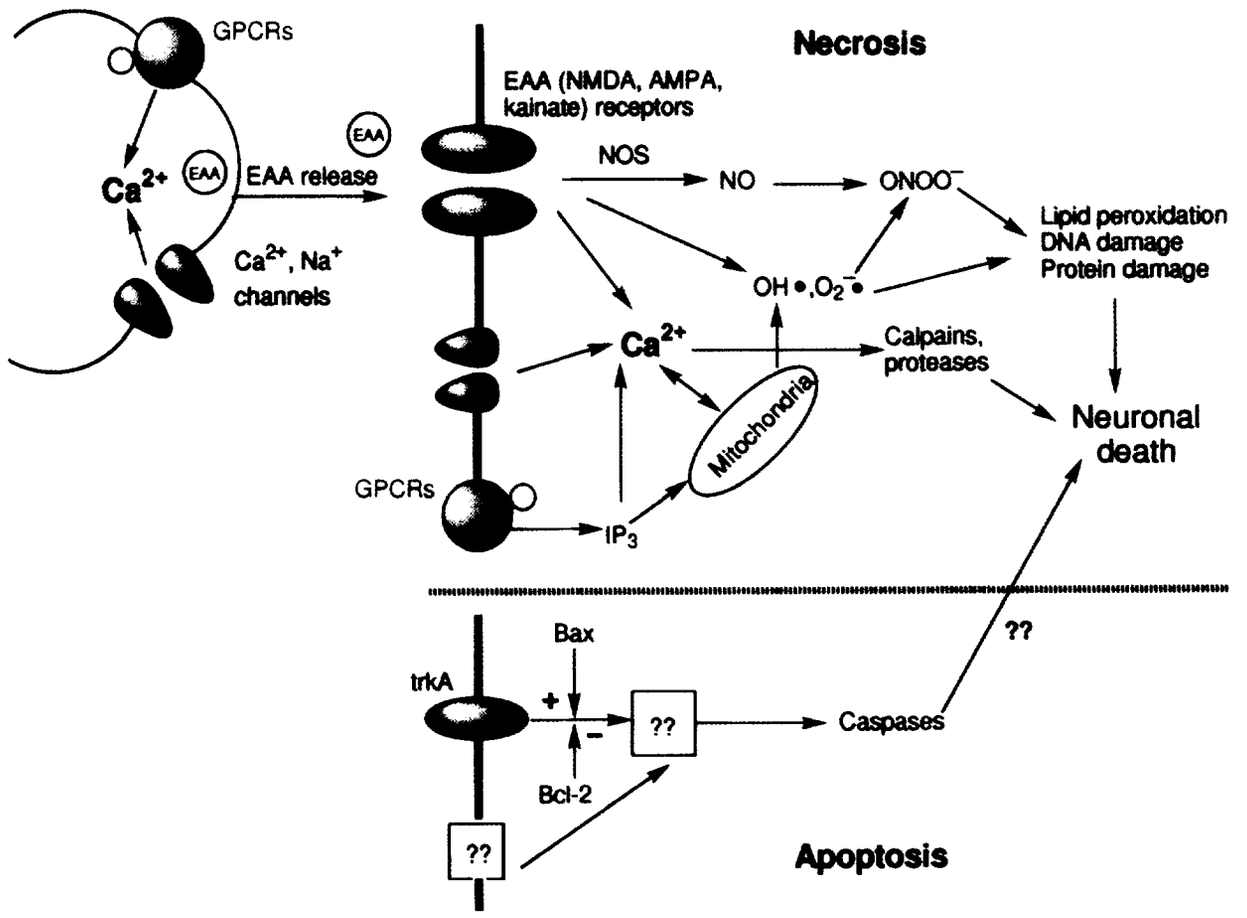

Figure 1. *Schematic representation of a neuron indicating the relationship between many of the mechanisms involved in producing cell death (EAA, excitatory amino acids; GPCRs, G-protein-coupled receptors; NOS, nitric oxide synthase; trkA, tyrosine kinase linked receptors).*

such as DNA laddering or chromatin condensation, does not preclude the involvement of apoptosis. Similarly, the presence of these hallmarks does not mean that all the resultant neuronal death is mediated via apoptosis. Under severe stress some neurons may initiate apoptotic processes, but in fact die from simultaneous necrosis. As a strategy for therapeutic intervention, the pharmacology of apoptosis is poorly characterized. Although several exciting findings regarding specific genes involved in apoptosis have been identified, the substrates and role of these genes are not yet well understood. When considering the blockade of apoptosis as a therapeutic target, it is essential to recognize that apoptosis is a normal and necessary process in other parts of the body. Blocking neuronal apoptosis may adversely affect other organs or the immune system. For example, T cells are regulated by apoptosis, and interruption of their turnover may provide a permissive state and allow growth of cancerous cells. Figure 1 provides a schematic summary of many of the mechanisms for necrotic and apoptotic neuronal death.

In aged individuals, neurons and other cells in the body may die because of their reduced ability to compensate for progressive DNA damage. Evidence suggests that genetic materials in cells are also subjected to stresses that cause cumulative damage to the DNA (Ref. 5). Although a variety of cellular mechanisms exist that repair DNA damage, repeated stresses or a reduction in the capacity of the repair mechanisms may lead to permanent alterations in the DNA and consequently to missense translation of key cellular proteins and enzymes. Faulty DNA repair and subsequent damage may be an inevitable result of aging[6]. In the CNS this damage may lead to neuronal loss and to concomitant functional consequences.

For drugs that work within the CNS, an additional obstacle for a viable therapeutic treatment of neuronal cell death is the problem of delivering the drug to its target. The blood–brain barrier (BBB) is the principal deterrent to penetration into the CNS. Intracellular and intranuclear mechanisms increase the complexity of selective action and offer additional cellular barriers to be overcome. Because more is known about the structure and function of extracellular receptors, they are obviously the primary target of many current strategies for neuroprotective agents and include both ligand-gated neuronal receptors and various ion channels that regulate neuronal function.

Excitatory amino acid (EAA) receptors

There is overwhelming evidence that EAAs can induce selective neuronal death both *in vitro* and *in vivo*[7–10]. The original theory of excitotoxicity proposed by Olney is that overactivation of EAA receptors leads to an increase in the cytosolic Ca^{2+} concentration to pathological levels, which causes enhanced lipolysis and membrane disruption, proteolysis and altered gene expression[11]. Excitotoxicity is clearly one of the contributing factors leading to necrotic cell death that is due to ischemia, traumatic brain injury, hypoglycemia and epileptic seizures, but it is unlikely that glutamate receptor activation is the major etiological factor in specific chronic neurodegenerative diseases (Parkinson's disease, Alzheimer's disease, Huntington's disease)[12]. Although overactivation of EAA receptors has been implicated in these disorders, this is probably secondary to some other primary deficit.

Since the first description of excitotoxicity an immense amount of research has gone into defining the receptors and the signal transduction pathways that are involved in neuronal cell death. EAA receptors can be broadly classified as ionotropic, linked to an ion channel, or metabotropic, linked to intracellular second messengers such as inositol trisphosphate (IP_3). Molecular cloning has confirmed earlier pharmacology and has been extensively reviewed[13–15]. This suggested that three classes of ionotropic receptors exist based upon their agonist sensitivity and molecular make-up:

- *N*-methyl-D-aspartate (NMDA) receptors, which are composed of at least one NR1 subunit and one or more NR2 subunits (four distinct NR2 subunits named NR2A–D have been identified along with numerous splice variants);
- AMPA receptors, which respond to α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) and are composed of an unknown stoichiometry of homomeric or heteromeric combinations of GluR1 to GluR4 (also described as GluRA–D); and
- kainate receptors, which bind kainic acid with higher affinity than AMPA and are composed of GluR5–7 and KA1–2, again in both homomeric (for GluR5–7) or heteromeric combinations.

NMDA receptors

The mechanism(s) by which activation of EAA receptors induces neuronal death has not been completely elucidated, but has been dominated by the 'calcium overload' hypothesis. Application of EAAs on neurons causes an increase in intracellular calcium[16]. This occurs by direct entry of calcium as the permeant ion, or indirectly by depolarization of the membrane potential and the opening of voltage-dependent calcium channels. The fact that the ion-channel complex of NMDA receptors is directly permeable to calcium has provided considerable impetus for producing NMDA receptor antagonists[17]. The vast complexity of the NMDA receptor and its modulatory sites has been thoroughly reviewed elsewhere[13–15]. Table 1 provides a summary of some of the key compounds that currently are or have been in clinical trials for the treatment of either stroke or traumatic brain injury based upon their ability to inhibit NMDA receptors[18–21].

The first class of compounds to be identified were competitive antagonists containing a terminal phosphonic acid moiety. Several of these derivatives (for example CPPene, CGS19755) showed significant neuroprotection both *in vitro* and in animal models of stroke and trauma. Clinical experience with these agents suggests a restricted utility in conscious patients, because they produce a number of unacceptable central side effects[19]. The results showed that significant behavioral side effects occurred at or near doses that were expected to provide neuroprotection. A role for this class of compounds may still exist for the treatment of head or spinal cord injury where patients are unconscious.

Similar psychotomimetic side effects are well characterized for the prototypical NMDA channel blocker, phencyclidine (PCP)[22]. Potent NMDA channel blockers, such as dizolcipine (MK801), appear to have no dose separation between side effects and efficacy[23]. However, aptiganel (CNS1102), another NMDA channel blocker with different kinetic properties, has been ushered through to Phase III clinical trials in traumatic brain injury and stroke in spite of these

reservations. Theoretically, channel blockers with kinetics that more closely resemble the normal physiological state may be neuroprotective at doses that produce relatively minor side effects. This is also the rationale behind the clinical trials of remacemide[24].

The co-agonist glycine site (Gly_N) of the NMDA receptor complex offers an alternative target to limit the side effect liability of NMDA antagonists[25]. It is unclear why blocking Gly_N produces fewer side effects than the competitive antagonists or channel blockers, but this has been demonstrated in both animals and man[26]. Initial efforts to characterize the *in vivo* pharmacology of Gly_N antagonists were hampered by the lack of accessibility of the compounds to the CNS. Recently, potent Gly_N antagonists with good access into the CNS were identified including the quinoxalinediones[27] (e.g. ACEA1021 and SM18400), several 4-hydroxy-2-quinolones[28] (e.g. L701423), benz[*b*]azepine-2,5-diones (e.g. ZD9379), and indole-2-carboxylic acids (e.g. GV150526A). Several of these are currently undergoing clinical evaluation (Table 1).

The NMDA antagonists discussed above represent a first generation of neuroprotective drugs. Most were synthesized before the molecular cloning of NMDA receptors, and in general inhibit all subunit combinations of NMDA receptors. By analogy to other receptor families, it is reasonable to assume that subtype-selective inhibitors of the NMDA receptor complex can be identified. Ifenprodil and eliprodil were originally described as noncompetitive NMDA receptor antagonists that interact at the polyamine modulatory site[29]. Recently, these compounds were shown to be selective and relatively potent inhibitors of the NR1/2B subtype of NMDA receptors[30]. Subtype-selective compounds, such as eliprodil and CP101606, represent a second generation of NMDA antagonists that do not induce the psychotomimetic side effects observed with nonselective antagonists, significantly improving their therapeutic index in animals[31].

AMPA receptors

Like NMDA receptors, certain combinations of AMPA receptors are also directly permeable to calcium (those containing GluR2)[32,33]. Independent of this direct permeation pathway, depolarization of neurons by AMPA and kainate receptors can also induce calcium entry via secondary activation of NMDA receptors and voltage-dependent calcium channels. The prototypical competitive AMPA antagonist is NBQX (Ref. 34). Numerous studies have demonstrated that NBQX is neuroprotective in both focal and global ischemia models[35]. Despite its remarkable *in vivo* activity NBQX has a number of shortcomings that preclude its clinical development, which include a short duration of action, poor water solubility and crystallization in the kidney. Related compounds such as YM90K and PNQX may represent better drug candidates[36,37]. Noncompetitive AMPA receptor antagonists, as exemplified by GYKI52466, offer an alternative strategy[38]. These drugs appear to provide some separation between doses that are neuroprotective and those that produce side effects in animals (typically ataxia and sedation). A potential advantage of a noncompetitive AMPA antagonist is that it would be expected to be more effective in blocking receptors in the presence of excitotoxic glutamate concentrations than a competitive antagonist (Table 1).

Kainate receptors

To date no potent, bioavailable kainate antagonists have been identified, and so it is uncertain that a kainate antagonist will be neuroprotective. NS102 is one of the few examples of a selective kainate receptor antagonist that has demonstrated any *in vivo* activity[39]. As with NMDA receptors, it is reasonable to postulate that inhibitors of specific subtypes of AMPA and kainate receptors can be discovered. It remains to be seen whether non-NMDA subtype-selective antagonists will be safe and effective neuroprotective agents.

Metabotropic glutamate receptors

There are at least eight metabotropic glutamate receptors, which are either positively linked to IP_3 production or negatively coupled to cAMP (Refs 40,41). Because the generation of IP_3 induces release of Ca^{2+} from intracellular stores, stimulation of these receptors may contribute to increased intracellular calcium and the downstream consequences of high Ca^{2+}. Several selective antagonists of metabotropic glutamate receptors have been identified, but in general these have not shown sufficient *in vivo* activity to assess the neuroprotective potential of metabotropic glutamate antagonists[42]. Furthermore, there is some evidence that agonists may also have benefit in preventing neuronal cell death[43]. One selective metabotropic glutamate agonist is in clinical trials for anxiety and relief of nicotine addiction[44].

Excitatory amino acid (glutamate) release inhibitors

Because EAAs can lead to neuronal death by activating multiple receptors, another strategy for drug intervention is to block the release of EAAs. Several drugs, many of which are modulators of sodium channels, have been shown to reduce

glutamate release in ischemic tissue[45]. Phenytoin, an anticonvulsant, is the prototype of this class of drugs and is known to stabilize the inactivated conformation of the Na^+ channel and thereby prevent rapid firing of the neuron. Additionally, phenytoin blocks prolonged membrane depolarization and sodium loading, and reduces the release of EAAs (and other neurotransmitters). Each of these events is likely to contribute to the neuroprotective activity of phenytoin seen in animal models of stroke[46]. Clinical practice has demonstrated that this class of drugs has a good separation between doses that produce efficacy and those that produce side effects. Consequently, fosphenytoin (a water-soluble prodrug of phenytoin) is currently in clinical trials for the treatment of ischemic disorders[47]. Lamotrigine and the related compounds BW1003C87 and BW619C89 have shown efficacy in animal models, but have been dropped from clinical development[48,49]. Another sodium channel modulator in clinical development is lubeluzole. This drug also modulates nitric oxide activity, which may contribute to its neuroprotective effect (see below)[50]. Other agents that may act presynaptically to reduce the release of neurotoxic EAAs, and thus inhibit the resulting excitotoxic cascade, include kappa agonists, such as enadoline, adenosine agonists and the metabotropic receptor modulators mentioned above (Table 1)[51,52].

Calcium channel antagonists

If high intracellular calcium leads to neuronal cell death, then an obvious strategy is to modulate voltage-sensitive calcium channels (VSCCs). Because vesicular release of neurotransmitter is Ca^{2+}-dependent, blockade of certain VSCCs may reduce EAA release. However, in acute neurological syndromes, such as ischemia, much of the EAA release is probably non-vesicular and not affected by this mechanism[53]. There are at least four different VSCCs found in the brain, but the exact function of each has not been well elucidated[54]. Antagonism of the L-type VSCC with dihydropyridines or other drugs has shown mixed efficacy both *in vitro* and *in vivo*[55]. Because L-channels are slowly inactivating, they should contribute significantly to Ca^{2+} loading. *In vivo*, systemic hypotension clearly limits the utility of these drugs and nimodipine was not found to be effective in clinical trials of stroke[56]. However, nimodipine is approved for the treatment of subarachnoid hemorrhage, and recent clinical data suggest it may be useful in certain cases of traumatic brain injury[57]. On the other hand, blockade of the N-type (class B) calcium channels with the highly potent and selective conopeptide SNX111 has demonstrated excellent *in vivo* efficacy in models of focal and global ischemia[58,59]. Interestingly, N-type VSCC blockers are not effective at blocking hypoxic-ischemic cell death *in vitro*, and are less effective at blocking EAA release than conopeptides that were not neuroprotective[59,60]. The lack of *in vitro* efficacy of peptide inhibitors of P/Q Ca^{2+} channels suggests that they may not be neuroprotective. Similarly, several anticonvulsants inhibit T-type Ca^{2+} channels, but these have not shown neuroprotection. No small molecules with efficacy similar to SNX111 have been reported, suggesting that it may be difficult to find or design selective inhibitors of specific neuronal calcium channels.

Intracellular targets

The mechanisms mentioned so far have all involved activation or blockade of cell-surface receptors. A number of intracellular targets also exist for blocking neuronal death. Ideally an intracellular target for therapeutic intervention should not be required for normal cellular processes, but be evoked during or following the pathological condition. Because most potential intracellular targets are also found in other cells, the issue of neuronal specificity arises.

Calpain inhibitors

Perhaps the most attractive intracellular targets for neuroprotection are the calcium-activated proteases or calpains. These proteases are activated only by high levels of calcium, and structural proteins are their primary targets[61]. Therefore, the blockade of calpains should affect only those cells involved in pathophysiological conditions. Calpain inhibitor I and several synthetic calpain inhibitors have been shown to be neuroprotective both *in vitro* and *in vivo*[62]. In general, the degree of neuroprotection for a calpain inhibitor is less than that seen with EAA receptor antagonists. The first calpain inhibitors, such as leupeptin (Ac-Leu-Leu-Arg-H) were peptidyl aldehydes, which are intrinsically labile and reactive, and also susceptible to enzymatic cleavage. Second generation peptidyl aldehyde inhibitors, such as calpeptin (Z-Leu-Leu-H) and MDL28170 (Z-Val-Phe-H) have improved cellular permeability, but still have similar deficiencies *in vivo*. Nonpeptide small-molecule inhibitors have been described, but a viable therapeutic agent has not yet been identified[62]. This illustrates one of the major limitations to this strategy: once high levels of intracellular calcium have occurred, a variety of parallel pathways are activated. Therefore, even complete blockade of one pathway, in this

case calpains, will probably provide only partial neuroprotection. On the other hand, calpain inhibitors remain attractive because they may be able to rescue neurons even when administered with a significant delay after a neurotoxic insult.

Nitric oxide

The blockade of nitric oxide (NO) is another appealing mechanism related to the excitotoxic cascade. NO has been shown to be cytotoxic[63]. One consequence of elevated intracellular Ca^{2+} is the stimulation of NO synthase (NOS) and the generation of NO from L-arginine. Although the exact mechanism of cytotoxicity is unknown, it may involve the generation of peroxynitrite following reaction with superoxide anions (see below)[64,65]. Conflicting results have been observed with NO synthase inhibitors in neuronal cell culture, which probably reflects subtle differences in the culture conditions and/or excitotoxic challenge[66]. Mice in which the gene encoding neuronal NOS (nNOS) has been deleted have reduced damage in response to focal and transient global ischemia[67,68]. This experiment provides convincing evidence for the importance of NO in mediating ischemic injury. Most NOS inhibitors are not specific to the brain and also inhibit endothelial NO, which produces undesirable effects on systemic blood pressure and cerebral blood flow[69]. The relatively specific inhibitor of nNOS, 7-nitro-indazole, has been shown to reduce both excitotoxic injury and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced damage *in vivo*[70,71]. One of the actions of lubeluzole may be its ability to reduce NO production in the brain[50]. The clinical utility of NOS inhibitors awaits compounds with the appropriate specificity and bioavailability.

Free radical inhibitors

Perhaps the most universal mechanism for inhibiting neuronal death is to scavenge reactive oxygen species (ROS). The CNS is particularly vulnerable to oxidative damage caused by free radical attack because high concentrations of polyunsaturated fatty acids and proteins with sulfur-containing amino acids, which are particularly susceptible, are found in the CNS. ROS or free radicals are produced in small amounts by normal cellular processes including respiration, which generates the superoxide anion ($O_2^{-}\bullet$) and the prooxidant hydrogen peroxide (H_2O_2). These species are only moderately reactive, but are easily transformed in the presence of iron into highly reactive hydroxyl radicals (OH•), which react readily with both proteins and lipids. Furthermore, superoxide can react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), another highly reactive compound[63]. Several naturally occurring defenses exist that limit the production and the damage produced by free radicals, including α-tocopherol (vitamin E), ascorbic acid (vitamin C), glutathione, and enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase.

The role of free radicals in a number of degenerative neurological disorders is becoming more apparent. ROS mediated processes play a central role in producing neuronal damage following transient cerebral ischemia. A recent study measured the increase of OH• formation following cerebral ischemia, and demonstrated that its effect upon ultimate brain damage is temperature-dependent[72]. Free radicals may contribute to the neuronal degeneration in Downs Syndrome, which leads to mental retardation in early life and predisposes adults to Alzheimer's disease[73]. Oxidative stress may also be involved in the etiology of neurodegeneration in the chronic neurodegenerative disorders, amyotrophic lateral sclerosis (ALS), Parkinson's disease and Alzheimer's disease[74]. One of the theories for the etiology of Alzheimer's disease involves the overproduction of β-amyloid from its precursor. *In vitro* data suggest that β-amyloid may kill neurons via generation of ROS (Ref. 75). Depletion of glutathione has also been observed in Parkinson's disease and in familial ALS (Ref. 76). In addition, a mutation of the superoxide dismutase gene that limits metabolism of superoxide anion radicals has been identified in a familial form of ALS (Ref. 77). Because oxidative stress is thought to be involved in the etiology of these disorders, antioxidant therapy or neurotrophic factors are expected to have utility for attenuating the damage[6,78].

Antioxidants such as vitamin E, β-carotene and vitamin A are essential nutrients with potential as prophylactic treatment to relieve oxidative stress by enhancing intracellular radical scavenging potential[79]. Compounds that may increase cellular glutathione levels, such as lipoic acid, *N*-acetyl cysteine and YM737 are also of interest[79]. Transforming growth factor-β1 (TGF-β1) protects neurons in culture against both Ca^{2+} and free radical-mediated degeneration *via* preservation of mitochondrial potential and function[80]. Several other strategies to reduce free radical formation or to increase the radical quenching potential of the cells have been proposed[6,79]. Efficacy with the lipid peroxidation inhibitor, tirilazad, was disappointing in clinical trials for both the treatment of cerebral ischemia and traumatic brain injury[81]. As a class, lipid peroxidation inhibitors are

still being considered for their potential in traumatic brain injury and other indications for neuroprotective agents, although several compounds have been dropped from clinical trials[82]. Compounds such as idebenone and OPC14117 inhibit lipid peroxide formation, but have shown greater promise in enhancing memory in Alzheimer's disease than as neuroprotectants[83,84]. A free radical spin trapping reagent, *N*-t-butyl-α-(2-sulfophenyl)nitrone (S-PBN), reduced striatal lesions up to six hours after intrastriatal injection of malonate, a mitochondrial toxin[85]. In a similar manner, striatal lesions and hydroxyl radical concentrations produced by 1-methyl-4-phenylpyridinium (MPP^+), malonate and 3-acetylpyridine were attenuated by either S-PBN or α-phenyl-*N*-t-butyl-nitrone (PBN)[86]. Neurotrophic factors [for example nerve growth factor (NGF), basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF)] attenuate glutamate-induced peroxides and increase antioxidant enzymes (upregulating glutathione reductase and SOD) and thus protect cells from oxidative stress[87]. ALS, Parkinson's disease and Alzheimer's disease may all result from an inability of the brain to prevent free radical damage or oxidative stress. Neurotrophic factors are still being considered as promising treatments for neurological diseases[6]. Glial cell-line derived neurotrophic factor (GDNF) is being evaluated in ALS and Parkinson's disease because of its survival-promoting effects on developing motor neurons and dopaminergic neurons[88].

Mitochondrial dysfunction

Another key component of oxidative stress may be mitochondrial dysfunction. Mitochondria are the organelles responsible for generating the energy required to function and maintain homeostasis in the brain. Mitochondrial dysfunctions may contribute to the pathogenesis of chronic neurodegenerative disorders, such as Parkinson's disease, and to the consequences of aging on mental acuity[89,90]. Chronic poisoning of the oxidative phosphorylation pathways ultimately disturbs the function of the mitochondrial respiratory chain, and in turn decreases production of ATP. Striatal and nigral degeneration have been induced by mitochondrial toxins, such as MPP^+, a specific irreversible inhibitor of mitochondrial complex I, producing symptoms in animals as well as humans that resemble the pathology seen in Huntington's disease and parkinsonism[91].

Electron transport by neuronal mitochondria may be an important source of glutamate-induced ROS and contribute to oxidative stress initiated by glutamate exposure[92]. An NMDA-induced increase in ROS can be blocked by rotenone or antimycin, both inhibitors of mitochondrial electron transport, and mimicked by the electron transport uncoupler, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone[93]. Striking changes in mitochondrial function occur during and immediately after intense glutamate receptor activation. Accumulation of Ca^{2+} and a subsequent change in mitochondrial membrane permeability may be a critical early event specific to NMDA-mediated excitotoxicity[94].

Programmed cell death (apoptosis)

Programmed cell death (PCD) is a gene-directed process that is morphologically defined by chromatin condensation, internucleosomal DNA fragmentation and generation of membrane enclosed cellular fragments. PCD may be an abortive attempt for neurons in distress to proliferate and divide; postmitotic neurons die rather than divide when given a proliferative signal. An inappropriate activation of apoptosis may lead to pathologies related to stroke, heart attack, Alzheimer's disease, AIDS dementia and aging.

The most universal blocker of neuronal apoptosis is the gene product of *bcl-2* (Ref. 95). Although the mechanism of *bcl-2* protection is not understood, expression of this gene inhibits neuronal death from serum withdrawal, free radicals, glutamate and β-amyloid[2]. Several homologs of *bcl-2* have been identified including *bcl-*x_S, which is anti-apoptotic, and *bcl-*x_L and *bax*, which are proapoptotic. Because *bcl-2* and *bax* homodimerize and heterodimerize, the relative balance of these two gene products determines the propensity of the neuron to undergo apoptosis. This mechanism suggests a potential therapeutic strategy, because mimicking *bcl-2* and/or inhibiting *bax* should be neuroprotective. It is unlikely that therapeutic compounds will be successfully developed in this area until more is understood about the role of *bcl-2* and *bax* in cellular homeostasis, and/or detailed structural information of the gene products and their targets is known.

Based upon a sequence analogy to the well characterized death genes, *ced-3* and *ced-4*, in the nematode *Caenorhabditis elegans*, the cysteine protease interleukin-1β-converting enzyme (ICE) has been implicated as a trigger of the cell suicide program[96]. Several ICE homologs have been identified and are also implicated in producing apoptosis; recently it has been suggested that these proteases form a family called caspases[97]. ICE is particularly interesting as a therapeutic target, because an upregulation of ICE expression may be neurotoxic for two reasons: first, its

ability to induce apoptosis, and secondly, the product of the enzyme, IL-1β, is a pro-inflammatory agent that could contribute to neuronal injury. The IL-1β receptor antagonist peptide has been shown to protect against both excitotoxic and stroke-induced injury *in vivo*[98]. Therefore inhibiting ICE may be effective for inhibiting neuronal death by a variety of mechanisms. The cowpox virus protein CrmA has been shown to block the actions of ICE (as well as a number of its homologs) and several small peptide inhibitors containing aspartate residues have been used successfully to block apoptosis *in vitro*[99]. Although these compounds have been useful for elucidating the role of caspases in mediating apoptotic cell death, no compound has emerged with therapeutic potential.

Withdrawal of growth factors generally leads to apoptotic death of neurons. This mechanism is probably critical in the developing nervous system, but in the adult can lead to inappropriate cell loss. Therefore, another potential therapeutic strategy would be to provide trophic support for neurons. Growth factors are relatively large proteins and therefore are unlikely candidates as therapeutic entities. An exception is bFGF, which has been shown to be neuroprotective in models of excitotoxicity/ischemia and is currently in clinical trials[100].

The signal transduction cascade activated by neurotrophic factors involves tyrosine phosphorylation of receptor tyrosine kinases (trkA) and subsequent activation of intermediate kinases and transcription factors[101]. Consequently, small molecules capable of activating tyrosine kinase phosphorylation cascades may represent another neuroprotective strategy. Similarly, some cytokines, such as tumor necrosis factor, utilize similar signal transduction pathways. This may explain their ability to protect cells from glutamate-induced excitotoxicity[102].

It is postulated that mechanisms that link neuronal excitation to gene expression are likely to exist because neuronal cells can be persistently altered following a brief exposure to an extracellular stimulus. Cellular immediate-early genes (cIEG) are found to be one link between extracellular stimulus and later changes in gene expression. A large body of evidence has shown that cIEG expression is associated with both necrotic and apoptotic neuronal death[103]. c-*fos* and c-*jun* are both expressed at elevated levels before PCD and necrotic death caused by neurotoxins, ischemic events and trauma. c-*fos* has other circumstantial links to metabolic stress and may be involved in gene metabolism or free radical scavenging. We do not yet have a temporal understanding of the biochemical processes *in vivo* that are associated with and lead to programmed neuronal cell death. Once the sequence of molecular and cellular events is determined, strategies to influence their outcome will be more accessible.

Conclusion

Neuronal cell death is unlikely to have a single, discrete pathway, and is under most circumstances likely to be multifactorial. This implies that several strategies may be useful for neuroprotection and the likelihood for successful intervention may be dependent upon action at more than one target site. Preclinical evidence exists that combinations of drugs might be more effective than a single mechanistic agent. Combinations that have demonstrated this neuroprotective synergy include NMDA antagonists with AMPA antagonists[104], and AMPA antagonists with sodium channel blockers[105].

In spite of these findings, initial drug development strategy depends upon demonstrating a clinical benchmark of neuroprotection using a single pharmacological agent. Clinicians use conservative strategies of patient care because of their concern of doing more harm than good when treating either acute or chronic neurological disorders. It is essential that new therapies provide clear evidence of neuroprotection with a therapeutic index that instills confidence in the caregiver and a minimal risk of being contraindicated with their current practices. An agent that has a limited safety profile or suspected adverse interaction is unlikely to achieve widespread acceptance for use in chronic conditions.

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