Brain tissue responses to ischemia

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The brain is particularly vulnerable to ischemia. Complete interruption of blood flow to the brain for only 5 minutes triggers the death of vulnerable neurons in several brain regions, whereas 20–40 minutes of ischemia is required to kill cardiac myocytes or kidney cells. In part, the prominent vulnerability of brain tissue to ischemic damage reflects its high metabolic rate. Although the human brain represents only about 2.5% of body weight, it accounts for 25% of basal metabolism, a metabolic rate 3.5 times higher even than that of the brains of other primate species. In addition, central neurons have a near-exclusive dependence on glucose as an energy substrate, and brain stores of glucose or glycogen are limited. However, over the last 15 years, evidence has emerged indicating that energetics considerations and energy substrate limitations are not solely responsible for the brain's heightened vulnerability to ischemia. Rather, it appears that the brain's intrinsic cell-cell and intracellular signaling mechanisms, normally responsible for information processing, become harmful under ischemic conditions, hastening energy failure and enhancing the final pathways underlying ischemic cell death in all tissues, including free radical production, activation of catabolic enzymes, membrane failure, apoptosis, and inflammation. Since these common pathways are explored in other accompanying *JCI* Perspectives, we will emphasize the role of injury-enhancing signaling mechanisms specific to the central nervous system (CNS) and discuss potential therapeutic approaches to interrupting these mechanisms.

Mechanisms of injury after ischemia

Cerebral ischemia may be either transient and followed by reperfusion, or essentially permanent. A region of the brain may be affected, as occurs during an arterial or venous stroke, or the entire brain may become globally ischemic, as occurs during a cardiac arrest. In addition to such settings where ischemia is the primary insult, ischemia may also contribute secondarily to brain damage in the setting of mass lesions, hemorrhage, or trauma.

Within seconds of cerebral ischemia, local cortical activity as detected by electroencephalography ceases; if the ischemia is global, unconsciousness rapidly ensues (witness the Stokes-Adams attack). This massive shutdown of neural activity is induced by K^+ efflux from neurons, mediated initially by the opening of voltage-dependent K^+ channels and later by ATP-dependent K^+ channels, leading to transient plasma membrane hyperpolarization. A few minutes later, despite this energy sparing response, an abrupt and dramatic redistribution of ions occurs across the plasma membrane, associated with membrane depolarization (efflux of K^+ and influx of Na^+, Cl^-, and Ca^{2+}). This "anoxic depolarization" results in the excessive release of neurotransmitters, in particular, glutamate, promoting further spatial spread of cellular depolarization, depletion of energy stores, and advancement of injury cascades (see below).

Neurotransmitter-induced toxicity

Glutamate-induced neuronal death. The main excitatory neurotransmitter throughout the CNS is the dicarboxylic amino acid, glutamate. Reflecting this ubiquitous role in cell-cell signaling, average whole brain concentrations are on the order of 10 mM, with presumably much higher concentrations within synaptic vesicles. Under ischemic conditions, transmitter glutamate is massively released (initially mediated by vesicular release from nerve terminals, and later by reverse transport from astrocytes), reaching near-millimolar concentrations in the extracellular space. Unfortunately, such concentrations of glutamate are neurotoxic, and substantial evidence now implicates the toxicity of glutamate (excitotoxicity) in the pathogenesis of neuronal death after ischemia and other acute insults.

Extracellular glutamate accumulating under ischemic conditions overstimulates *N*-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate-type glutamate receptors, promoting Na^+ influx and K^+ efflux through glutamate receptor-activated membrane channels. NMDA receptor–gated ion channels are additionally highly permeable to Ca^{2+} and mediate Ca^{2+}

influx into neurons. The gating of glutamate receptor–activated channels effectively achieves membrane shunting, which spreads in waves (spreading depression) from the ischemic core out toward the margins of the ischemic zone (ischemic penumbra). Spreading depression increases metabolic demand and energy failure, thus further enhancing glutamate release. Marked neuronal cell body swelling and dendrite swelling occur, hallmarks of necrosis death, as Na^+ and Ca^{2+} entry is joined by the influx of Cl^- and water. Elevations in neuronal intracellular free Ca^{2+} ($[Ca^{2+}]_i$), mediated both directly by NMDA receptors and indirectly via membrane depolarization–activated voltage-gated Ca^{2+} channels and reverse operation of the Na^+-Ca^{2+} exchanger, bear particular responsibility for promoting spreading depression and triggering deleterious cytotoxic cascades.

In neuronal cell cultures, selective NMDA receptor blockade prevents most of the Ca^{2+} influx and cell death induced by brief intense glutamate exposures (1). NMDA antagonists also markedly attenuated the death of cultured neurons induced by oxygen and/or glucose deprivation, observations that fit well with studies conducted with selective agonists. Exposure to NMDA for as little as 3–5 minutes is sufficient to trigger widespread cultured cortical neuronal death ("rapidly triggered excitotoxicity"), whereas exposure to even saturating concentrations of kainate typically requires hours to do the same ("slowly triggered excitotoxicity"). This difference in time course fits with a higher rate of Ca^{2+} influx mediated directly by NMDA receptor–gated channels, compared with a slower rate of Ca^{2+} influx mediated by the voltage-gated channel and exchanger routes activated by AMPA or kainate receptors. NMDA receptor antagonists are also highly neuroprotective in animal models of focal brain ischemia, as well as hypoglycemia or trauma (2), although not transient global ischemia (3). In this latter setting, NMDA receptor–mediated excitotoxicity may be less prominent than AMPA receptor–facilitated Zn^{2+} entry in inducing lethal neuronal injury (see below). Reasons for this shift in prominence are presently not well-defined, but a contributing factor may be extracellular acidity due to accumulation of lactic acid during global ischemia, an event less prominent in the penumbra of focal ischemia where perfusion is partially maintained. This acid shift selectively downregulates NMDA receptors and NMDA receptor–mediated excitotoxicity but enhances AMPA receptor–mediated excitotoxicity (4); it may also enhance toxic Zn^{2+} entry through voltage-gated Ca^{2+} channels (5).

Other signaling messengers and growth factors. In addition to glutamate, other neurotransmitters released to the extracellular space during ischemia can significantly influence resultant brain injury. Dopamine, which increases 500-fold in the extracellular space following global ischemia, may contribute to striatal neuronal death. Moreover, experimental reduction of dopamine release, which can be accomplished by creating lesions in the dopaminergic neurons projecting from the substantia nigra or by using the tyrosine hydroxylase inhibitor alpha-methyl-*p*-tyrosine to deplete endogenous stores of dopamine, attenuates striatal injury in rodent global ischemia models (6). Contributing to dopamine-induced potentiation of ischemic injury may be its ability to enhance glutamate receptor currents.

Neurotransmitters do not all act to promote injury; several, including serotonin, gamma-aminobutyric acid (GABA; see below), and adenosine, are neuroprotective. Adenosine, which accumulates rapidly during ischemia due to breakdown of ATP, has beneficial effects in many tissues. The activation of adenosine A_{2a} receptors on vascular smooth muscle cells and neutrophils enhances blood flow and decreases inflammation, respectively (7). Adenosine also has nervous system–specific beneficial effects, mediated by the ability of neuronal adenosine A_1-receptors to reduce neurotransmitter release and membrane excitability. In addition, the expression of several growth factors increases in ischemic tissues, likely as a protective response. Exogenous administration of growth factors has shown therapeutic promise in several experimental models of organ ischemia, including in liver, kidney, heart, and brain. Examples of growth factors whose administration reduces brain damage in rats subjected to cerebral ischemia are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins 4/5 (NT-4/5), basic fibroblast growth factor, and IGF-1, which apparently blocks neuronal apoptosis (see below). Some growth factors may also enhance nerve fiber sprouting and synapse formation after ischemic injury, thereby promoting functional recovery.

Despite their overall salutary effects, certain growth factors may also enhance neuronal vulnerability to excitotoxic and free radical–induced death. Acute exposure to BDNF, NT-3, or NT-4/5 reduces the vulnerability of cultured neocortical neurons to apoptosis, but exacerbates the cellular necrosis of the same cells after exposure to oxygen-glucose deprivation or NMDA. The deleterious consequences of the neurotrophins as well as IGF-1 may be explained in part by enhanced NMDA receptor–mediated Ca^{2+} influx, enhanced production of free radicals, or possibly acute proexcitatory effects that could increase excitotoxicity (8). These deleterious consequences are not restricted to embryonic or in vitro systems, as free radical–mediated tissue damage induced by direct intraparenchymal injection of iron into the adult rat spinal cord was increased by pretreatment with BDNF, NT-3, or NT-4/5 (J. McDonald et al., unpublished data). If these growth factors have an injury-enhancing component effect in the ischemic brain, perhaps masked by other survival-promoting

effects, interventions aimed specifically at blocking this component may uncover higher levels of net neuroprotective effects.

Zinc toxicity. Zinc, the second most abundant transition metal in the human body, is present in all cells, for the most part tightly bound to proteins, such as metalloenzymes and transcription factors, where it serves catalytic and structural roles. In the brain, there is an additional substantial pool of chelatable Zn^{2+} localized to synaptic vesicles in excitatory (glutamatergic) nerve terminals, which is released in a Ca^{2+}-dependent fashion with depolarization and can alter the behavior of several transmitter receptors and voltage-gated channels (9). While the normal functional significance of this presumptive signaling Zn^{2+} pool is not presently understood, growing evidence suggests that it contributes to nerve cell death under pathological conditions such as ischemia or seizures or following head trauma (10).

Following transient global ischemia, chelatable Zn^{2+} translocates from nerve terminals into the cell bodies of vulnerable neurons (11). This translocation precedes neuronal degeneration, and its interruption by the intracerebroventricular (icv) injection of a chelator, ethylenediaminetetraacetic acid saturated with equimolar Ca^{2+} (CaEDTA), reduces subsequent neuronal death. Furthermore, exposure to the high micromolar concentrations of Zn^{2+} likely to occur in brain extracellular space after synchronous cellular depolarization is sufficient to kill cultured neurons, especially if the neurons are depolarized, which facilitates entry of Zn^{2+} across the plasma membrane through voltage-gated Ca^{2+} channels (10). Recent observations from our laboratory suggest that Zn^{2+} toxicity may also contribute to the development of cerebral infarction following mild transient focal ischemia (G.J. Zipfel et al., unpublished observations).

Downstream mediators

Intracellular signaling. The massive release of neurotransmitters and elevations in $[Ca^{2+}]_i$ induced by cerebral ischemia produce gross perturbations in intracellular signaling pathways that may contribute to resultant injury or death. Protein kinase C (PKC) is rapidly activated during ischemia as a common response in several organs including the brain, kidney, and heart, and it may enhance neuronal excitotoxicity by increasing vesicular glutamate release and neuronal excitability (12). Selective PKC inhibitors have not, to our knowledge, been tested to date in animal models of cerebral ischemia. However, pretreatment with broad spectrum protein kinase inhibitors, such as staurosporine or 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), has provided some therapeutic potential by decreasing neuronal cell death in a global model of cerebral ischemia and attenuating the extracellular accumulation of glutamate induced by ischemia in rodent brains, respectively (13, 14). Following PKC activation triggered by cerebral ischemia, a persistent drop in PKC levels occurs that may enhance susceptibility to apoptosis.

The highly conserved mitogen-activated protein (MAP) kinases, including c-Jun NH_2-terminal kinases (JNKs), p38 kinases, and extracellular signal–regulated kinases (ERKs) are activated in many cells by stress and may modify processes relevant to cellular injury and programmed cell death (15). In the brain, all three MAP kinase pathways may be activated following the induction of ischemia, and the p38 and ERK pathways have been implicated in enhancing ischemic neuronal death. Pretreatment with the selective p38 inhibitor SB203580 reduced both activity of the p38 pathway and neuronal death after transient global ischemia (16). In another study, pretreatment with the ERK inhibitor PD98059, but not SB203580, reduced infarction after transient focal ischemia (17). ERK signaling has also been suggested to have neuroprotective effects, either by attenuating apoptosis, or by mediating the development of resistance to subsequent oxygen-glucose deprivation (18) (ischemic tolerance; see below). Reflecting limitations in current pharmacology, contributions of the JNK pathway have not yet been identified in cerebral ischemia studies, but the possibility of such a role is raised by the finding that mice lacking Jnk3, an isoform with restricted expression in the brain, heart, and testes, exhibit resistance to seizure-induced neuronal death (19).

Injury effectors: free radicals and catabolic enzymes. Adding to the injury occurring during a given ischemic insult, postischemic reperfusion appears to induce further tissue damage in virtually all organs, likely mediated by the accelerated formation of several reactive oxygen species including superoxide, hydroxyl, and nitric oxide (NO) radicals. One particularly damaging consequence of reactive oxygen species formation in several cell types may be single-strand DNA breakage, leading to activation of the repair enzyme poly(ADP-ribose) polymerase (PARP) and PARP-mediated depletion of cellular NAD^+ and energy stores (20). NO generated by inducible NO synthase (iNOS or type II NOS), expressed in macrophages, neutrophils, and microglia following immunological challenge, may also contribute to late tissue injury. In contrast, a second isoform of NO synthase present in endothelial cells (eNOS or type III NOS) may play a protective role, relaxing vascular smooth muscle cells and helping to preserve blood flow (21).

In the CNS, free radical production is likely a specific downstream mediator of glutamate-induced neuronal death. Neurons have a special ability to respond to increases in $[Ca^{2+}]_i$ with increases in NO production via neuronal NO synthase (nNOS or type I NOS, a Ca^{2+} calmodulin–dependent enzyme); inhibiting nNOS either pharmacologically or genetically (via gene deletion) renders cultured neurons

resistant to NMDA-induced death, and also reduces infarct volume in rodent models of transient focal ischemia (22). NMDA receptor activation may also stimulate oxygen radical production by uncoupling neuronal mitochondrial electron transport (23). Another link between brain signaling and free radical generation in the ischemic brain may be neuronal Zn^{2+} overload (24).

Free radical–mediated cytotoxicity in the ischemic brain is likely augmented by damage mediated by the excessive activation of Ca^{2+}-dependent catabolic enzymes. Phospholipase A_2 and C (PLA_2 and PLC) are activated following NMDA receptor stimulation and promote membrane phospholipid breakdown (which itself enhances free radical formation and inflammation). The Ca^{2+}-activated proteases, or calpains, likely contribute to destruction of structural and regulatory proteins. Genetic ablation of the cytoplasmic form of PLA_2 (25), or pharmacological inhibition of PLC (26) or calpains, reduces brain injury in animal models of cerebral ischemia (27).

Necrosis or apoptosis?

Tissue ischemia is a defining example of a violent "environmental perturbation" capable of producing necrosis, fulminant cell death associated with plasma membrane failure, and swelling of cell body and internal organelles (28). In the nervous system, the notion that ischemic insults cause neurons to undergo necrosis is strengthened by the implication of excitotoxicity in ischemic neuronal death. As noted above, glutamate receptor overactivation typically leads to prominent swelling of cell body and dendrites. Despite this intuitive link between ischemic insults and necrosis, growing evidence indicates that ischemia may additionally induce programmed cell death in many tissues, including the heart, kidney, and brain.

While the classical morphological features of apoptosis are not prominent following cerebral ischemia, the parallel triggering of programmed cell death and excitotoxicity may induce a mixture of features. Said another way, there is no reason to believe that the onset of excitotoxicity and even some cellular swelling is incompatible with subsequent progression down an apoptotic cascade. Supporting this contention, several recent studies have identified molecular signatures of apoptosis in the ischemic brain, including the translocation of cytochrome c from mitochondria to cytosol, and activation of caspase-3. Furthermore, icv infusion of the caspase-3 inhibitor [*N*-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone or z-DEVD.FMK] decreased infarct size after transient focal ischemia and reduced hippocampal CA1 cell death in transient global ischemia (29). Transgenic overexpression of the antiapoptotic gene *bcl-2*, as well as its delivery via herpes virus vector, have been found to reduce infarct volume in mice subjected to focal cerebral ischemia, and survival of hippocampal CA1 neurons following transient global ischemia also was enhanced in transgenic mice overexpressing *bcl-2* (30).

Inflammation

Ischemia and reperfusion in the brain, as in other organs, induces an inflammatory response which may exacerbate initial levels of tissue injury. Elevation of mRNA levels of the cytokines TNF-α and IL-1β occurs as early as 1 hour after the induction of ischemia. In addition, adhesion molecules on the endothelial cell surface (e.g., intercellular adhesion molecule 1 [ICAM-1], P-selectins, and E-selectins) are also induced, enhancing neutrophil adhesion and passage through the vascular wall into brain parenchyma, an event followed by invasion of macrophages and monocytes. There are several possible mechanisms by which postischemic inflammation could contribute to injury, including microvascular obstruction by neutrophils or production of toxic mediators such as NO by activated inflammatory cells (31).

Specific implication of postischemic inflammatory responses in the pathogenesis of ischemic brain injury is provided by studies indicating that this injury can be attenuated by preischemic induction of systemic neutropenia, pharmacological block of adhesion molecules or their receptors, deletion of the *Icam-1* gene, or interfering with the action of inflammatory mediators such as IL-1β or IFN regulatory factor 1, a transcription factor coordinating the expression of inflammation-related genes (32).

Endogenous neuroprotective responses

While ischemia triggers a multitude of cytotoxic pathways in the brain, it also triggers some endogenous protective responses capable of limiting injury. Some of these responses, present in the brain as well as other organs, enhance vascular blood flow, thereby limiting the insult itself (for example, thrombolysis or NO-mediated vasodilation); we will not discuss these here. Other responses reduce the intrinsic vulnerability of brain parenchyma to further ischemic damage. Several such parenchymal responses act acutely to attenuate circuit excitability and hence excitotoxicity, whereas other parenchymal responses act in a lasting fashion to downmodulate both excitotoxicity and general cellular vulnerability to injury. Examples of acute modulatory responses include: the activation of interneurons, leading to release of the inhibitory neurotransmitter GABA and reduced circuit excitability; downmodulation of NMDA receptor function due to Zn^{2+} block, or oxidation of a redox regulatory site on the receptor; and depletion of extracellular Ca^{2+} and Na^{+}, leading to reduction in the membrane gradient favoring influx of these ions.

Lasting reduction of vulnerability to ischemic injury

has been best studied utilizing the paradigm of "ischemic tolerance" or "ischemic preconditioning," a general tissue phenomenon first described in the heart (see Williams and Benjamin, this Perspective series, ref. 33). Murry et al. (34) found that a series of brief sublethal ischemic insults rendered the heart resistant to a more severe ischemic insult. In 1990, Kitagawa et al. (35) described a similar phenomenon in the gerbil brain and emphasized that the protective response lasted several days. Strong evidence that brain ischemic tolerance partly reflects parenchymal changes has been provided by several in vitro demonstrations of the phenomenon. For example, neocortical cell cultures exposed to sublethal oxygen-glucose deprivation exhibit reduced neuronal death when rechallenged with more severe oxygen-glucose deprivation 24–48 hours later (36, 37).

Mechanisms that mediate ischemic neuronal death have been implicated in triggering the development of ischemic tolerance, in particular glutamate release, activation of NMDA receptors, and the formation of reactive oxygen species (38). Ischemic tolerance can be induced by preconditioning stresses other than ischemia, including spreading depression (38) or inhibition of mitochondrial electron transport (39).

What changes underlie the development of brain ischemic tolerance? Since it takes hours to develop after preconditioning both in vivo and in vitro, and it can be blocked by cycloheximide (40), involvement of new protein synthesis is plausible. Brain cells, like other types of cells, respond to sublethal ischemic challenge by mobilizing a host of cellular defenses such as heat shock proteins, free radical scavengers, calcium buffers, antiapoptotic factors, and growth factors. Activation of adenosine A_1 receptors leading to enhanced activation of K_{ATP} channels has been implicated in ischemic tolerance in myocardial cells and central neurons (38). Recent studies have suggested that brain ischemic tolerance may also strongly reflect changes in CNS-specific processes such as the presynaptic release of neurotransmitters (Figure 1). Preconditioned neocortical cultures exhibited increased GABA and reduced glutamate release compared with controls; the former change alone when mimicked pharmacologically was sufficient to explain observed tolerance (M.C. Grabb and D.W. Choi, unpublished results). Reduced extracellular glutamate accumulation during an ischemic insult was also found in preconditioned rat brains compared with controls (41).

Neuroprotective interventions

Currently, the only Food and Drug Administration–approved treatment for patients presenting with an acute ischemic stroke is tissue plasminogen activator, a thrombolytic agent that limits the ischemic insult itself by lysing arterial thrombus and restoring blood flow. As with treatment of myocardial infarction, one can anticipate refinements in cerebral vascular thrombolysis, aiming to reduce distant bleeding complications by improvements in drug specificity or by spatial restriction of drug delivery. A second major strategy is to reduce the vulnerability of brain tissue to a given ischemic insult, an approach that, unlike thrombolytic agents, may someday be administered by paramedics in the field without need for a CT or MRI scan to exclude hemorrhage.

In part, these neuroprotective efforts are similar to other ongoing organ-protective efforts, for example in the setting of coronary artery occlusion or organ transplantation. These efforts target common mechanisms of ischemic tissue injury such as cellular Ca^{2+} overload, the generation of reactive oxygen species, the activation of catabolic or energy-depleting enzymes, apoptosis, or inflammation. However, the larger part of efforts to

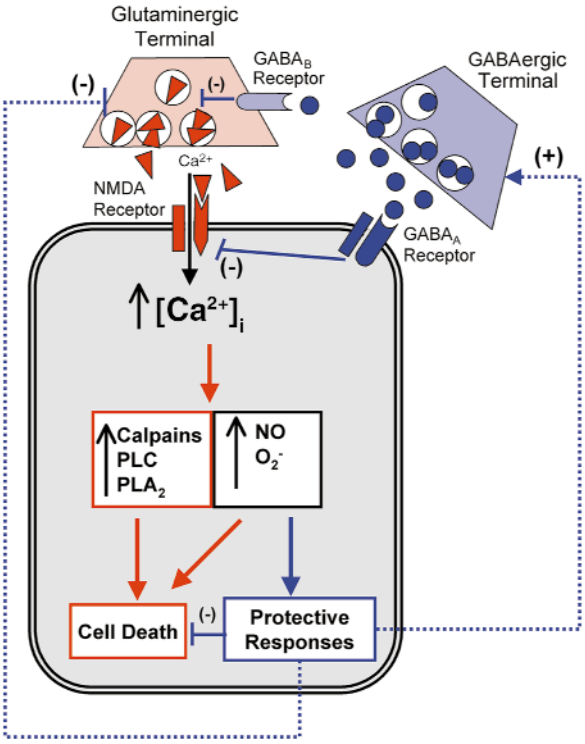

Figure 1
Schematic of mechanisms implicated in ischemia-induced neuronal death (in red) and the development of ischemic tolerance (in blue) in the brain. During ischemia, glutamate is released into the synaptic cleft and activates NMDA receptors, increasing calcium entry. Calcium activates multiple pathways, promoting cellular injury via the generation of oxygen and NO radicals and the activation of catabolic enzymes. Sublethal insults may induce cytoprotective tolerance, in large part through similar NMDA receptor– and calcium-mediated pathways. Contributing prominently to the development of ischemic tolerance may be alterations in nerve terminals that increase release of GABA and reduce release of glutamate. GABA likely acts both presynaptically through GABA$_B$ receptors (and G-proteins) to decrease glutamate release and postsynaptically through GABA$_A$ receptors (and the gating of Cl^- channels to counter membrane depolarization and to limit calcium entry).

reduce brain vulnerability to ischemic injury has primarily focused on attenuating excitotoxicity. While the results of neuroprotective clinical trials to date have been discouraging (see below), there are many promising cytoprotective and antiexcitotoxic approaches to reducing ischemic brain damage still in the development pipeline.

General cytoprotective approaches

Some attractive downstream targets in the ischemic cascade include calpains and oxygen free radicals. MDL 28,170, a potent inhibitor of calpain, has been found to decrease infarct volume after transient focal ischemia even when administered 6 hours after ischemia onset (27), and the free radical scavenger phenyl-*N-tert*-butyl nitrone (PBN) can reduce infarct volume even when administered up to 3 hours after ischemia onset (42). Two phase III clinical trials involving free radical scavengers in acute ischemic stroke were conducted recently. One trial testing the lipid peroxidation inhibitor tirilazad mesylate was prematurely terminated due to concerns about the safety of the drug. The second trial involved treatment with ebselen, a seleno-organic compound with antioxidant activity, within 48 hours of stroke onset. While patients acutely treated with ebselen as a whole did not show a better outcome measured 3 months after their stroke, subgroup analysis suggested that patients treated within 24 hours fared better than the placebo group (43).

The final pathways leading to apoptosis represent another possible target for therapeutic intervention in a variety of organs subjected to ischemic insults. Following mild transient cerebral ischemia, infarction develops in a surprisingly delayed fashion over days (44), suggesting that antiapoptotic therapies could potentially have a prolonged therapeutic window. Supporting this notion, delayed administration of the caspase inhibitor zDEVD-fluoromethyl-ketone was effective at decreasing infarct size even if given 9 hours after mild transient focal ischemia in mice (29). On one hand, this approach proves to have a long therapeutic window after the onset of ischemia; on the other hand, it risks saving some cells too badly damaged to be functionally useful.

Interference with inflammatory cascades is another general approach likely to aid neural cell survival. A key early event amenable to therapeutic intervention may be the adherence of leukocytes to blood vessels in the ischemic region shortly after insult. Inhibition of this step may not only limit the release of proinflammatory cytokines, but also reduce the ischemic insult itself by limiting microvascular occlusion. Although a phase III clinical trial using anti–ICAM-1 antibodies failed (possibly due to murine antibody–induced complications), a second trial using humanized antibodies directed against leukocyte integrins CD11/CD18 is now underway (43). An alternative approach may be to inhibit the release or action of proinflammatory cytokines from microglia or astrocytes. For instance, administration of the IL-1 receptor antagonist (which is a naturally occurring inhibitor in the brain) reduces ischemic death (31). In addition, iNOS inhibition decreased infarct volume after focal ischemia, even when given 24 hours after permanent middle cerebral artery occlusion (45), a remarkably delayed intervention.

Brain-specific antiexcitotoxic approaches

Based on the prominent role of excitotoxicity in experimental ischemic brain injury, several antiexcitotoxic strategies have been brought to clinical trials in recent years. In most cases, the underlying goal has been the reduction of glutamate release, glutamate receptor stimulation, or associated cellular Ca^{2+} overload. For example, several drugs have been developed to block glutamate receptor subtypes, including AMPA receptors (YM872, MPQX), and NMDA receptors (Selfotel®) or their channels (cerestat, CP101,606, dextrorphan, NPS 1506, remacemide). In addition, other drugs may attenuate excitotoxicity indirectly by limiting neuronal depolarization (Na^+ channel blockers, e.g., fosphenytoin, or GABA mimetics, e.g., clomethiazole), or reducing Ca^{2+} influx into neurons through voltage-gated Ca^{2+} channel blockers (e.g., nimodipine) (43). Although some trials are still ongoing, the results from several completed trials have been disappointing (Table 1). In part, the explanation for these failures may lie in the difficulty of conducting clinical trials with stroke patients. Heterogeneity in study populations, in particular the comingling of patients with large cortical infarcts and small white matter lacunes, or complex relationships between lesion size and clinical deficits as measured by clinical assessment scales, can make detection of neuroprotective effects difficult. Alternatively, currently attempted broad attacks on excitotoxicity may be too limited by side effects or may even promote injury (see below). It may be necessary therefore to refine antiexcitotoxic approaches to improve therapeutic indices, or to combine antiexcitotoxic approaches with other strategies.

Refinement of glutamate receptor antagonist approaches. A major limitation in past clinical trials of glutamate receptor antagonists has been dose ceilings imposed by drug side effects. Not unexpectedly, interfering with the brain's major excitatory transmitter system can lead to alterations in motor or cognitive function (prominent with NMDA antagonists), or sedation (prominent with AMPA antagonists). It seems plausible that the therapeutic index of NMDA antagonist therapy might be improved by the utilization of subtype-selective agents, such as ifenprodil, an antagonist selective for the NR2B subtype of NMDA receptors. NR2B receptors are preferentially expressed in forebrain relative to hindbrain, so blocking these receptors may produce greater neuroprotection in forebrain with less interference with motor function than subtype-

unselective NMDA antagonists. In addition, ifenprodil inhibition of NR2B receptors increases with increasing agonist stimulation, a "use dependency" that might increase drug effect at overactivated synapses relative to normal synapses (46).

The neuroprotective efficacy of NMDA antagonist therapy might also be enhanced by combination with AMPA or kainate receptor antagonists, both to increase overall antiexcitotoxic efficacy on ischemic neurons, as well as specifically to extend protection to GABAergic neurons expressing Ca^{2+}-permeable AMPA receptors, and oligodendrocytes. Indeed, failure to rescue GABAergic neurons while successfully rescuing nearby excitatory neurons might lead to an increase in local circuit excitation and seizure activity in stroke survivors. High-level pan-blockade of both NMDA and AMPA receptors could have problematic side effects, for example, respiratory depression, but these difficulties might be surmountable through the use of subtype-selective drugs. An alternative approach to blocking NMDA and AMPA receptors concurrently might be to reduce glutamate release, for example, through hypothermia or reduction of circuit excitability with GABA agonists or blockers of voltage-gated Na^+ channels.

Zinc-directed therapies. While current putative antiexcitotoxic therapies have focused on glutamate receptor activation and resultant Ca^{2+} overload, the pathological role of neuronal Zn^{2+} overload suggests additional targets for therapeutic intervention. Indeed, variable reduction of toxic Zn^{2+} influx may underlie some of the inconsistent beneficial effects of voltage-gated Ca^{2+}-channel antagonists observed in animal models of transient global ischemia (47). Further delineation of the precise routes responsible for toxic Zn^{2+} may permit greater reduction in this toxic Zn^{2+} overload. Another possible approach would be to reduce Zn^{2+} release from nerve terminals. In settings where ischemia is anticipated, it may even prove possible to accomplish this via acute dietary zinc reduction, as anecdotal evidence in humans has suggested that such reduction profoundly disturbs brain function, likely due to reduction of transmitter Zn^{2+} release (48). Further off, one can envision

Table 1
Agents recently tested as acute treatments for brain ischemia (43)

Drug category	Drug name	Mechanism	Trial status
Glutamate antagonists	YM872	AMPA antagonists	Phase II: ongoing
	ZK-200775 (MPQX)		Phase IIa: abandoned
	CGS 19755 (Selfotel®)	Competitive NMDA antagonists	Phase III: no efficacy
	aptiganel (Cerestat®)	NMDA channel blockers	Phase III: no efficacy
	dextrorphan		Phase II: abandoned
	dextromethorphan		abandoned
	magnesium		Phase III: ongoing
	NPS 1506		Phase Ib/IIa: suspended
	remacemide		Phase III in cardiopulmonary bypass: borderline efficacy
	ACEA 1021 (Licostinel®)	NMDA glycine site antagonist	Phase I: abandoned
	GV 150526		Phase III: no efficacy
	SL 82-0715 (eliprodil)	NMDA polyamine site antagonist	Phase III: abandoned
GABA agonists	clomethiazole (Zendra®)	↓ excitation, ↓ glutamate release	Phase III: ongoing
Opiate antagonists	nalmefene (Cervene®)	↓ glutamate release	Phase III: no efficacy
Serotonin agonists	Bay x 3702 (Repinotan®)	↓ glutamate release	Phase III: results pending
Voltage-gated calcium channel antagonists	nimodipine (Nimotop®)	↓ Ca^{2+} influx	Phase III: no efficacy
	flunarizine (Sibelium®)		Phase III: no efficacy
Voltage-dependent potassium channel agonists	BMS-204352	↓ Ca^{2+} influx	Phase III: ongoing
Sodium channel antagonists	Fosphenytoin (Cerebryx®)	↓ excitation, ↓ glutamate release	Phase III: no efficacy
	BW619C89		Phase II: abandoned
Free-radical scavengers	tirilazad mesylate (Freedox®)	↓ free radical–mediated injury	Phase III: abandoned
	ebselen		Phase III: borderline efficacy
Phosphatidylcholine precursor	citicoline (Ceraxon®)	Membrane stabilizer	Phase III: no efficacy
Growth factors	Fibroblast growth factor (Fiblast®)	Antiapoptotic?, ↑ NMDA receptor inactivation	Phase II / III: abandoned
Leukocyte adhesion inhibitor	anti-ICAM antibody (Enlimomab®)	Reduction of leukocyte infiltration	Phase III: no efficacy
	Hu23F2G		Phase III: ongoing
Unknown	lubeluzole (Prosynap®)	↓ glutamate release, ↓ neuronal excitability, or ↓ NO-mediated injury	Phase III: no efficacy

All of the above putative therapeutic agents, with the exception of the anti-inflammatory agents, share, at least in part, a common rationale: reduction of excitotoxic neuronal injury.

strategies for modifying neuronal Zn^{2+} transporters to improve the extrusion or sequestration of intracellular Zn^{2+}, or for upregulating intracellular Zn^{2+}-binding proteins such as metallothioneins.

Combination therapies. Recent implication of apoptosis in the pathogenesis of ischemic neuronal death raises an unsettling possibility that current efforts to block NMDA receptor-mediated Ca^{2+} influx may go too far, achieving the desired reduction of toxic calcium overload and excitotoxicity in some neurons, but then promoting apoptosis in other neurons through Ca^{2+} starvation (4). It is plausible that different neurons might sustain different levels of $[Ca^{2+}]_i$ at different times, with neurons further from the ischemic core or at later time points after ischemia onset sustaining less calcium influx than counterparts in the acute ischemic core. These neurons may be damaged badly enough to trigger apoptosis, but their $[Ca^{2+}]_i$ levels may fall below the "set point" optimal for promoting survival (49), such that broad and sustained NMDA receptor blockade promotes apoptosis, reducing the benefits to be had by attenuating calcium overload in other neurons.

If this scenario proves valid, it may be possible to enhance the benefits and reduce the dangers of NMDA antagonists by concurrently administering antiapoptotic treatments. Dual inhibition of excitotoxic necrosis and ischemic apoptosis has shown promise in two experimental studies to date. Coadministration of the NMDA antagonist dextrophan with cycloheximide produced greater than 80% reduction in infarct volume following transient focal ischemia in rats, better than either agent alone (50); and Ma et al. (51) observed neuroprotective synergy between MK-801 and the caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD.FMK) on both infarct size and therapeutic window. The combination of antiexcitotoxic strategies with thrombolysis has also been shown to provide additive protection in a rodent model of embolic stroke (52). On theoretical grounds, antioxidant drugs might be especially valuable in reducing reperfusion-induced injury, for example in association with thrombolytic therapy, or the deleterious component of certain growth factor actions.

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