Section I
Mechanic Approaches to CNS Neuroprotection

CHAPTER 1

Blocking Excitotoxicity

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A. Introduction

The major excitatory transmitter in the mammalian central nervous system
is glutamate, which exerts its signaling actions through the stimulation of
ionotropic and metabotropic receptors (Watkins et al. 1981; Mayer and
Westbrook 1987; Nakanishi and Masu 1994). Under pathological conditions,
glutamate receptor overactivation can trigger neuronal death, a phenomenon
known as excitotoxicity (Lucas and Newhouse 1957; Olney 1969). Incentive
for developing practical methods for blocking excitotoxicity arises from its
implication in several acute and chronic neurological disease states. While
recent clinical trials aimed at blocking excitotoxicity in stroke patients have
been disappointing, there are several plausible reasons for these trial failures,
including specific study design issues, treatment side effects, and a need to
achieve concurrent block of parallel injury pathways. In our view, the case for
antiexcitotoxic approaches in stroke remains open, and there are other possi-
ble disease targets yet to be explored. Ongoing delineation of the cellular and
molecular underpinnings of excitotoxicity has led to the progressive unveil-
ing of countermeasures, aimed at attenuating presynaptic glutamate release,
postsynaptic receptor activation, the movement or action of cation second
messengers, or downstream intracellular injury cascades. The excitotoxicity
concept itself may need to be expanded, to encompass the death of oligoden-
drocytes as well as neurons, and ionic derangements besides Ca^{2+} overload.

B. Contributions to Disease

The ability of glutamate receptor overactivation to cause neuronal death in
humans is demonstrated most directly by the toxicity of several naturally occur-
ring glutamate receptor agonists (Ludolph et al. 2000), including domoic acid,
produced by a phytoplankton that occasionally contaminates blue mussels
(Perl et al. 1990; Teitelbaum et al. 1990), the amino acid β-oxalyl-amino-L-
alanine from seeds of the chickling pea (Ludolph et al. 1987), and the mush-
room poisons acromelic acid and ibotenic acid (Leonhardt 1949). These toxins
all activate ionotropic glutamate receptors and induce a variety of distur-
bances, including seizures and cognitive alterations, as well as neuronal death.

Beyond dietary exposure to exogenous excitotoxins, excitotoxicity may also be induced by the endogenous neurotransmitter glutamate. Endogenous glutamate-mediated excitotoxicity has been hypothesized to play a fundamental pathogenic role in the neuronal death associated with a wide variety of acute neurological insults, including brain ischemia (both the transient, global interruption of blood supply experienced during cardiac arrest with resuscitation, as well as the focal ischemia associated with thromboembolic stroke), seizures, mechanical trauma, and isolated hypoxia or hypoglycemia (COYLE et al. 1981; ROTHMAN and OLNEY 1986; CHOI 1988b). Elevations in extracellular glutamate concentrations have been observed in the context of ischemia (BENVENISTE et al. 1984), seizures (MELDRUM 1994), and head trauma (KATAYAMA et al. 1990). In the context of excessive extracellular accumulation of glutamate, the movement of cations, including Ca^{2+}, through overactivated glutamate receptors can lead to multiple toxic consequences (see Sect. E.III.).

Glutamate may become lethal even when its synaptic release and extracellular concentration are not especially elevated, in settings where the ability of postsynaptic neurons to maintain homeostasis is compromised by energy depletion (NOVELLI et al. 1988), for example, due to mitochondrial dysfunction (BEAL 2000; NICHOLLS and WARD 2000). It is thus plausible that excitotoxicity may contribute, at least in a secondary fashion, to some of the neuronal loss associated with certain neurodegenerative diseases such as Huntington's disease (COYLE and SCHWARCZ 1976; McGGEER and McGEER 1978), Alzheimer's disease, or Parkinson's disease. In amyotrophic lateral sclerosis, loss of transporter-mediated glutamate uptake has been postulated to induce the excitotoxic death of motor neurons (ROTHSTEIN et al. 1992).

C. Excitotoxicity in Brief

Glutamate kills central neurons by activating several subtypes of ionotropic receptors: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate (OLVERMAN et al. 1984; WISDEN and SEEBURG 1993; HOLLMANN and HEINEMANN 1994; KERCHNER et al. 1999). Much has been learned about the mechanisms underlying excitotoxic death through studies performed in vitro over the past 15 years. Intense exposure to either glutamate or NMDA for only a few minutes is sufficient to trigger widespread necrosis of cultured cortical neurons over the next hours, a phenomenon we have called "rapidly-triggered excitotoxicity" (CHOI 1992). Neurons swell acutely, due to the massive influx of Na^+ (through NMDA or AMPA/kainate receptors) followed by Cl^- and water, and then undergo delayed neurodegeneration several hours later. This latter component is dependent upon NMDA receptor activation and the presence of extracellular Ca^{2+} and is associated with a massive increase in cytoplasmic free Ca^{2+} concentrations (OGURA et al. 1988; CHENG et al. 1999). The importance of elevations in intracellular Ca^{2+} in mediating excitotoxicity is underscored by the ability of cell-

permeable Ca^{2+} chelators to attenuate glutamate-mediated cell death in neuronal culture as well as to decrease injury induced by experimental focal ischemia in rodents (TYMIANSKI et al. 1993).

Neuronal swelling consequent to Na^+, Cl^-, and water influx is not always lethal. Brief exposure to kainate induces marked cortical neuronal swelling in vitro but is followed by little delayed neurodegeneration. The high permeability of NMDA receptors to Ca^{2+} is crucial to the ability of brief glutamate exposure to trigger widespread cortical neuronal death, a hypothesis strengthened by the observation that a subset of cortical neurons containing Ca^{2+}-permeable AMPA receptors, formed when the critical mRNA-edited GluR2 (GluR-B) subunit is absent from heteromeric AMPA receptor complexes (BURNASHEV et al. 1992), is selectively vulnerable to brief, intense activation of those receptors (KOH and CHOI 1988; TURETSKY et al. 1994).

On the other hand, if the exposure time is lengthened from minutes to hours, AMPA/kainate receptor agonists can destroy most cortical neurons ("slowly-triggered excitotoxicity") (CHOI 1992; GWAG et al. 1997). AMPA or kainate toxicity in cultured hippocampal or cerebellar neurons is also dependent upon the presence of extracellular Ca^{2+} (GARTHWAITE and GARTHWAITE 1986; ROTHMAN et al. 1987). Prolonged activation of AMPA or kainate receptors will induce Na^+ influx and sustained depolarization, promoting Ca^{2+} entry via voltage-gated Ca^{2+} channels and reverse operation of the Na^+/Ca^{2+} exchanger (CHOI 1988a; YU and CHOI 1997). Additionally, Ca^{2+} release from intracellular stores may contribute to cytoplasmic Ca^{2+} accumulation (FRANDSEN and SCHOUSBOE 1991).

There are probably many potentially lethal derangements in cellular processes induced by profound elevations in cytoplasmic Ca^{2+}, but work in recent years has assigned particular responsibility for ensuing cellular necrosis to the activation of catabolic enzymes, generation of free radicals including nitric oxide, impairment of mitochondrial energy production, and excessive utilization of energy by the DNA repair enzyme poly(ADP-ribose) polymerase-1 (PARP-1). These downstream steps are discussed further in Sect. E.III.1.

Recent studies have also suggested that Na^+ and Ca^{2+} may not be the only cations whose excessive movement across neuronal membranes can mediate cell death; in particular, movements of Zn^{2+} or K^+ may also contribute. Concentrated in synaptic vesicles at excitatory terminals throughout the forebrain and in some other locations is a chelatable pool of Zn^{2+} (TIMM and NETH 1959; PEREZ-CLAUSELL and DANSCHER 1985; FREDERICKSON 1989). During transient global ischemia, this synaptic Zn^{2+} appears to translocate into postsynaptic neurons that later go on to die (TONDER et al. 1990). Preventing this translocation with an extracellular Zn^{2+} chelator reduced neuronal death (KOH et al. 1996). The ability of excessive exposure to extracellular Zn^{2+} to induce neuronal death has been demonstrated directly in neuronal cell cultures (YOKOYAMA et al. 1986; CHOI et al. 1988; MANEV et al. 1997; AIZENMAN et al. 2000; LOBNER et al. 2000). Zn^{2+}-induced death is potentiated by membrane

depolarization (induced by glutamate receptor agonists or elevated extracellular K$^+$ concentrations), likely reflecting enhancement of toxic Zn^{2+} entry via voltage-gated Ca^{2+} channels and the Na$^+$/Ca^{2+} exchanger (WEISS et al. 1993; YIN and WEISS 1995). The ability of Zn^{2+} to enter cortical neurons through voltage-gated Ca^{2+} channels was demonstrated directly in electrophysiological experiments, which also revealed a potentiation of Zn^{2+} permeation in conditions of lowered extracellular pH, as may be present during ischemia (KERCHNER et al. 2000).

Neuronal intracellular Zn^{2+} concentration attained during a toxic Zn^{2+} exposure was correlated to the extent of subsequent cell death, with substantial death occurring at intracellular Zn^{2+} concentrations exceeding 250–300 nM (CANZONIERO et al. 1999); astrocytes are more resistant than neurons to death induced by comparable elevations of [Zn^{2+}]$_i$ (DINELEY et al. 2000). At such elevated Zn^{2+} levels, many alterations in intracellular biology can be expected. One consequence may be disruption of glycolysis due to nicotinamide adenine dinucleotide (NAD$^+$) depletion and consequent secondary inhibition of glyceraldehyde-3-phosphate dehydrogenase, as cortical neurons exposed to toxic levels of extracellular Zn^{2+} exhibited loss of ATP and elevation of the upstream glycolytic substrates dihydroxy-acetone phosphate and fructose 1,6-bisphosphate (SHELINE et al. 2000). While neurons may normally have limited dependence upon glycolysis for energy production (MAGISTRETTI 2000), a crucial role during pathophysiological conditions such as ischemia is not implausible. Mitochondrial disturbances and free radical production may also contribute to Zn^{2+}-induced death (MANEV et al. 1997; KIM et al. 1999a,b).

While little attention has been paid historically to the functional importance of the K$^+$ permeability of glutamate receptor-gated channels, K$^+$ efflux has been identified as a potentially important component of the sequence of events leading to programmed cell death. Delayed rectifier K$^+$ channel current (I_K) is enhanced in neurons undergoing apoptosis (YU et al. 1997b), and blockade of these channels by TEA or clofilium attenuated neuronal death induced by oxygen-glucose deprivation in vitro or by transient focal ischemia in vivo (CHOI et al. 1998). In lymphocytes, loss of intracellular K$^+$ may be a critical step in the apoptotic cascade, perhaps because DNA fragmentation and proteolytic activation of caspase-3 are inhibited at normal levels of intracellular free K$^+$ (BORTNER et al. 1997; BORTNER and CIDLOWSKI 1998). While NMDA receptor overactivation typically induces neuronal necrosis mediated by Na$^+$ and Ca^{2+} influx, NMDA receptor activation could induce apoptosis dependent upon K$^+$ efflux when the extracellular concentrations of Na$^+$ and Ca^{2+} were reduced, as occurs in the ischemic brain (YU et al. 1999). Even in the presence of normal extracellular Na$^+$ and Ca^{2+}, the K$^+$ efflux mediated by glutamate receptor-gated channels may enhance the propensity of neurons to undergo apoptosis.

D. Extending Excitotoxicity to Glia

Excitotoxicity has conventionally been considered to be specific to neurons. Although Type I astrocytes express functional AMPA receptors (CONDORELLI

et al. 1993; MARTIN et al. 1993; MATUTE et al. 1994), they are highly resistant to death upon activation of those receptors by agonist exposure (COYLE et al. 1981; ROTHMAN 1984; CHOI et al. 1987). An ability of glutamate to kill immature oligodendrocytes in vitro was demonstrated by VOLPE and colleagues, but this toxicity appeared to be dependent upon interference with cellular cysteine uptake and consequent glutathione depletion, rather than upon glutamate receptor activation (OKA et al. 1993).

However, recent evidence has suggested that more mature oligodendrocytes may also be vulnerable to a true excitotoxic death mediated by glutamate receptor overactivation. Oligodendroglial lineage cells and oligodendrocytes cultured from rat optic nerve express multiple AMPA and kainate receptor subunits, and exposure to kainate or AMPA plus cyclothiazide (to inhibit AMPA receptor desensitization) can destroy these cells in a Ca^{2+}-dependent manner (YOSHIOKA et al. 1995; MATUTE et al. 1997). Differentiated forebrain oligodendrocytes appear even more sensitive to excitotoxicity, as 100–300 μM AMPA alone can trigger widespread cell death within 24 h (MCDONALD et al. 1998a). In vivo, injection of AMPA or kainate into white matter killed oligodendrocytes near the injection site (MATUTE et al. 1997), in a manner sensitive to coinjection of AMPA and kainate receptor antagonists (MCDONALD et al. 1998a).

While further studies are needed to determine why oligodendrocytes are far more vulnerable to AMPA and kainate receptor-mediated toxicity than astrocytes, one possible explanation might be the expression of Ca^{2+}-permeable AMPA receptors in the former cell type (HOLZWARTH et al. 1994; PUCHALSKI et al. 1994). Compared to cortical neurons bearing Ca^{2+}-permeable AMPA receptors, however, oligodendrocytes are killed by longer agonist exposure times, at least 2–3 h (MCDONALD et al. 1998a). This agonist exposure time appears intermediate between rapidly and slowly triggered excitotoxicity in neurons. Possibly, differences in AMPA receptor expression or behavior, means of buffering internal Ca^{2+}, or intrinsic differences in vulnerability to Ca^{2+} overload may account for this difference in susceptibility.

E. Points of Intervention

I. Reducing Extracellular Glutamate

1. Circuit Activity and Glutamate Release

One approach to decreasing excitotoxic injury may be to inhibit neuronal circuit activity and, therefore, to reduce vesicular glutamate release from presynaptic terminals. This might be accomplished by several means, including: (a) hypothermia; (b) increasing GABAergic tone; (c) K^+ channel openers; (d) modulating adenosine receptors; (e) blocking voltage-gated Na^+ channels, or (f) blocking voltage-gated Ca^{2+} channels. In the context of decreased energy substrate availability, as during ischemia, these strategies would have the additional benefit of reducing energy demand. Another effect of measures directed

at decreasing glutamate release would likely be reduction of synaptic Zn^{2+} release from the same nerve terminals (Assaf and Chung 1984; Martinez-Guijarro et al. 1991), although the suggestion that Zn^{2+} may be localized to a subset of synaptic vesicles raises the possibility of a differential modulation of glutamate and Zn^{2+} release (Perez-Clausell and Danscher 1985).

a) Hypothermia

Well recognized as a neuroprotective maneuver for decades, hypothermia has been proposed to be a "gold standard" against which other interventions should be measured (Buchan 1992). Both intra- and postischemic hypothermia produce lasting neuroprotective effects in animal cerebral ischemia studies (Barone et al. 1997), in large part due to inhibition of glutamate release (Busto et al. 1989). Neuroprotective effects of hypothermia can also be demonstrated in neuronal cell cultures, again reflecting a reduction in endogenous glutamate release, as well as probably other actions (Bruno et al. 1994).

At present, the clinical use of hypothermia is limited to surgical procedures that require concomitant cardiac arrest and neurosurgical procedures such as cerebral aneurysm clipping (Tommasino and Picozzi 1998). Although some benefits of moderate hypothermia have been demonstrated for traumatic brain injury (Marion et al. 1997), testing of hypothermic therapy in human stroke has been slowed by concerns of potential complications such as coagulopathies, arrhythmias, and myocardial infarction (Steen et al. 1979, 1980). However, hypothermia remains a promising therapeutic approach, especially if methods can be employed to localize cooling to the brain.

b) Increasing GABAergic Tone

GABA, the major inhibitory neurotransmitter in the mammalian brain, mediates its neuronal effects through three receptor subtypes, $GABA_A$, $GABA_B$, and $GABA_C$, all presumably pentameric complexes (Bormann 2000). $GABA_A$ and $GABA_C$ receptors are ligand-gated chloride channels, while $GABA_B$ receptors are coupled to G-proteins, usually in presynaptic terminals, where they mediate an increase in K^+ conductance and downmodulation of transmitter release (Karlsson and Olpe 1989; Gage 1992). $GABA_A$ receptor agonists, such as muscimol or benzodiazepines reduced brain injury following rodent cerebral (Sternau et al. 1989; Lyden and Hedges 1992; Shuaib et al. 1993; Schwartz-Bloom et al. 1998) or spinal cord (Madden 1994) ischemia. $GABA_A$ receptor stimulation by muscimol also reduced excitotoxicity in neuronal culture, presumably by hyperpolarizing membranes and reducing activation of voltage-gated Ca^{2+} channels, as well as enhancing the voltage-dependent Mg^{2+} block of NMDA receptors (Muir et al. 1996; c.f. Erdo and Michler 1990). However, a cautionary note was raised by the observation that $GABA_A$ receptor agonists paradoxically enhanced excitotoxicity induced by oxygen-glucose deprivation in vitro, possibly due to a contravening

effect of maintaining the driving force for Ca^{2+} in energy-depleted and depolarized neurons, thereby outbalancing neuroprotective actions (MUIR et al. 1996).

In contrast to $GABA_A$ receptor agonists, $GABA_B$ receptor agonists like baclofen have provided inconclusive results in animal cerebral ischemia, perhaps in part due to complications such as postischemic hypertension and hemorrhage (STERNAU et al. 1989; ROSENBAUM et al. 1990; JACKSON-FRIEDMAN et al. 1997); they have also been ineffective against excitotoxicity in cell culture (MUIR et al. 1996). $GABA_C$ receptors, which exhibit a predominantly retinal distribution in vertebrates, have not been exploited for antiexcitotoxic purposes (JOHNSTON 1996). Other approaches to increasing GABAergic tone for antiexcitotoxic effects in vivo include the use of GABA reuptake inhibitors such as tiagabine (SUZDAK and JANSEN 1995) and CI-966 (PHILLIS 1995), or blockers of GABA metabolism such as the GABA transaminase inhibitor vigabatrin (SHUAIB et al. 1992).

c) Opening K^+ Channels

Membrane excitability might also be reduced by increasing the opening of K^+ channels other than those gated after $GABA_B$ receptor activation. Mammalian neurons express multiple K^+ channel subtypes, including channels that are voltage-gated (BROWN 1993), ATP-sensitive (HADDAD and JIANG 1994), and Ca^{2+}- or Na^+-activated (DRYER 1994; SAH 1996). Various K^+ channel openers reduced endogenous glutamate release following brief ischemia in hippocampal slices (ZINI et al. 1993). Activators of ATP-sensitive K^+ channels attenuated excitotoxic death in neuronal cultures, at least in part by decreasing the magnitude of intracellular Ca^{2+} elevation (ABELE and MILLER 1990). Similar agents, including Y-26763 (TAKABA et al. 1997), cromakalim (HEURTEAUX et al. 1993), and nicorandil, have exhibited therapeutic value in animal cerebral ischemia studies. The identification of pharmacological openers of large-conductance Ca^{2+}-activated K^+ channels (BK channels) such as BMS-204352 may provide another potentially neuroprotective means of hyperpolarizing neuronal membranes during an excitotoxic insult; phase III clinical trials are underway for this drug as an acute treatment for stroke (BOZIK et al. 2000). However, a note of caution regarding this approach is raised by the potential of K^+ efflux to promote apoptosis (see Sect. C.).

d) Modulating Adenosine Receptors

Adenosine acts as an agonist at three major receptor subtypes, A_1, A_2 (A_{2A} and A_{2B}), and A_3, each of which transduces its signals through coupled G-proteins (OLAH et al. 1995). Stimulation of A_1 receptors leads to multiple circuit depressing effects, including enhancement of K^+ and non-GABAergic Cl^- conductances and reduction of pre- and postsynaptic Ca^{2+} conductances (MAGER et al. 1990; RIBEIRO 1995). Adenosine protected cortical neurons in vitro from oxygen-glucose deprivation (GOLDBERG et al. 1988), presumably

through activation of presynaptic A_1 receptors and a subsequent decrease in vesicular glutamate release (Corradetti et al. 1984). But results with A_1 receptor agonists in animal studies have been inconsistent, perhaps due to drug-induced bradycardia and hypotension resulting from activation of A_1 receptors in cardiovascular tissues (von Lubitz et al. 1995).

Work on the role of A_2 receptors in excitotoxic damage has focused mostly on the A_{2A} subtype, which enhances glutamate release when activated and reduces release when antagonized in ischemic cortex (O'regan et al. 1992; Simpson et al. 1992). However, available evidence suggests a more complicated role for this receptor in modulating excitotoxicity. Moderately selective A_{2A} receptor antagonists reduced injury subsequent to cerebral ischemia in the sensitive gerbil model (Gao and Phillis 1994; von Lubitz et al. 1995), and A_{2A} gene deletion provided moderate protection against injury induced by focal cerebral ischemia in mice (Chen et al. 1999). On the other hand, in contrast to the predicted ability of A_{2A} receptor stimulation to augment excitotoxicity, the selective A_{2A} receptor agonist CGS21680 inhibited hippocampal injury induced by systemic kainate injection (Jones et al. 1998). Similarly, the potential contribution of A_3 receptors to excitotoxicity appears complex, especially given the limitations of current pharmacology and highly species-dependent patterns of expression (von Lubitz 1999; Klotz 2000). Since a major obstacle for antiexcitotoxic drugs targeting adenosine receptors is the presence of these receptors in nonneural tissues, adenosine analogs with fewer cardiovascular effects have recently been developed. One such A_1 receptor-specific compound, adenosine amine congener (ADAC), reduced injury and improved functional recovery following rodent cerebral ischemia when administered as late as 6 h postischemia (von Lubitz et al. 1996a; von Lubitz et al. 1996b).

Like the adenosine and GABA receptor systems, stimulation of the group II and group III subtypes of metabotropic glutamate receptors may offer yet another modulator-based approach to attenuating transmitter glutamate release (see Sect. II.3.).

e) Blocking Voltage-Gated Na^+ Channels

Na^+ influx through voltage-gated Na^+ channels provides the electrical force for action potential generation and circuit excitation; furthermore, as noted above, intracellular Na^+ accumulation promotes Ca^{2+} influx via voltage-gated Ca^{2+} channels and reverse operation of the Na^+/Ca^{2+} exchanger. A favorable characteristic to consider when designing or screening Na^+ channel blockers for antiexcitotoxic potential may be the use-dependence of the agent since drugs with this property would be predicted to inhibit the most active neurons preferentially. The Na^+ channel blockers tetrodotoxin (Yamasaki et al. 1991; Lysko et al. 1994), phenytoin (Cullen et al. 1979; Taft et al. 1989), and riluzole (Pratt et al. 1992) decreased neuronal injury following cerebral ischemia in rodents. In culture, the antiexcitotoxic effect of local anesthetics and anticonvulsants alone, which exert their effects predominantly through use-dependent Na^+

channel blockade, has been variable (Koh and Choi 1987; Ogura et al. 1988; Mattson and Kater 1989).

White matter represents a particularly important therapeutic target for Na$^+$ channel blocking drugs. A series of elegant studies using the rat optic nerve has suggested that activation of voltage-gated Na$^+$ channels in the setting of anoxic injury is responsible for triggering toxic Ca^{2+} influx in axons, mainly through the reverse activity of the Na$^+$/Ca^{2+} exchanger (Stys et al. 1991). The prominence of this dependence of Ca^{2+} influx upon Na$^+$ channels in white matter may reflect the absence of glutamate receptor-mediated entry routes available in gray matter. Indeed, in cultured cortical neurons, once NMDA and AMPA receptors are pharmacologically blocked, an additional neuroprotective effect of tetrodotoxin or phenytoin against neuronal death triggered by oxygen-glucose deprivation can be unmasked (Lynch et al. 1995). These findings suggest that combined therapy targeting both postsynaptic glutamate receptors and axonal Na$^+$ channels may provide more effective neuroprotection than either alone.

f) Blocking Voltage-Gated Ca^{2+} Channels

At least seven subtypes of voltage-gated Ca^{2+} channels, each with distinct electrophysiological properties and cellular localization, have been identified in mammalian neurons (Miljanich and Ramachandran 1995; Perez-Reyes and Schneider 1995). Presynaptic N-type Ca^{2+} channels play a crucial role in vesicular neurotransmitter release (Kamiya et al. 1988; Dutar et al. 1989), and drugs selective for this channel subtype attenuated neuronal injury in rodent cerebral ischemia studies (Valentino et al. 1993; Buchan et al. 1994). P- and Q-type channels, also expressed presynaptically, regulate physiological glutamate transmission in hippocampal slices (Wheeler et al. 1994), and a peptide inhibitor of these channels reduced infarct volume following rodent cerebral ischemia (Asakura et al. 1997). While L-type Ca^{2+} channels are predominantly located on postsynaptic cell bodies, activation of these channels can in certain cases enhance neurotransmitter release, so that antagonists may offer the dual benefit of reducing postsynaptic Ca^{2+} influx as well as presynaptic glutamate release during an excitotoxic insult (Middlemiss and Spedding 1985). Consistent with these predictions, dihydropyridine antagonists reduced excitotoxic death in neuronal culture (Abele et al. 1990; Weiss et al. 1990). However, as with N-type channel blockers, the therapeutic value of neuronal L-type channel blockade in cerebral ischemia remains ambiguous due to complicating cardiovascular effects in vivo (Kobayashi and Mori 1998). Additionally, clinical trials with dihydropyridines in the context of subarachnoid hemorrhage or ischemic stroke have yielded inconsistent or disappointing results (Rosenbaum et al. 1991; American Nimodipine Study Group 1992; Murphy 1992; Kaste et al. 1994). More recently, broad-spectrum neuronal voltage-gated Ca^{2+} channel antagonists with minimal cardiovascular side effects, such as SB 201823A, have shown some promise in rodent cerebral ischemia studies (Barone et al. 1995).

2. Glutamate Transport

The Ca^{2+}-dependent, vesicular release of glutamate does not wholly account for the rise in extracellular glutamate concentrations during brain ischemia. A substantial contribution is also made in a Ca^{2+}-independent fashion, by reverse operation of glutamate transporters in both neurons and glia (NICHOLLS and ATTWELL 1990; SZATKOWSKI et al. 1990; ATTWELL et al. 1993; JABAUDON et al. 2000; ROSSI et al. 2000). These transporters normally function to remove glutamate from synapses and thus to terminate a synaptic signaling event. However, since the direction in which a glutamate transporter operates is governed by the gradients of the other ions that are co- or countertransported, perturbations in intra- and extracellular ionic conditions can induce release of glutamate from the cytoplasm of astrocytes and neurons into the extracellular space.

The transport of one glutamate anion is coupled to cotransport of three Na^+ ions, countertransport of one K^+ ion, and cotransport of one proton or counter-transport of one hydroxyl ion (KANNER and SHARON 1978; BARBOUR et al. 1988; BOUVIER et al. 1992; ZERANGUE and KAVANAUGH 1996). During brain ischemia, cells experience a shortage of high-energy phosphates; the Na^+-K^+ ATPase is inhibited, and extracellular K^+ and intracellular Na^+ concentrations rise. The magnitude of this run-down in ionic gradients predicts that glutamate transporters would operate in reverse until a new equilibrium is reached, with extracellular glutamate concentrations reaching beyond $100\,\mu M$ (SZATKOWSKI and ATTWELL 1994), levels that are potentially neurotoxic. Therefore, glutamate transporters may represent a useful pharmacological target in attenuating excitotoxic damage (VANDENBERG 1998), whether or not impairment of transporter function is involved in disease pathogenesis as has been suggested in the case of amyotrophic lateral sclerosis (see Sect. B). There might be particular value in developing agents that selectively inhibit reverse transport, in analogy with the recent development of selective blockers of reverse Na^+/Ca^{2+} exchange (IWAMOTO et al. 1996; HOYT et al. 1998). In addition, glutamate transporters activate a Cl^- conductance that is not directly coupled to glutamate transport (FAIRMAN et al. 1995; WADICHE et al. 1995). That the Cl^- conductance is decoupled from glutamate uptake is supported by the ability of Zn^{2+} to modulate these two activities differentially in certain transporter subtypes (SPIRIDON et al. 1998; VANDENBERG et al. 1998). By developing an agent that enhances this hyperpolarizing flow of Cl^-, it may be possible to decrease the magnitude of ischemic depolarization in cells that express transporters, thus favoring forward operation.

II. Manipulating Glutamate Receptors

1. NMDA Antagonists

Consistent with the prominent role of NMDA receptors in mediating glutamate-induced Ca^{2+} overload and rapidly-triggered excitotoxic neurodegener-

ation in vitro, NMDA antagonists can reduce the death of cultured cortical neurons induced by hypoxia, glucose deprivation, and trauma (Choi 1992), and a substantial literature indicates that NMDA antagonists can reduce neuronal death in multiple models of brain injury in vivo. These include animal models of ischemia (Simon et al. 1984; McCulloch 1992), hypoglycemia (Wieloch 1985), sustained seizures (Meldrum 1994), and trauma (McIntosh et al. 1989). Unfortunately, several recent clinical trials of NMDA antagonists in stroke patients have been disappointing; side effects including hallucinations, ataxia, or hypotension were prominent with several drugs (Kemp et al. 1999; Read et al. 1999). It remains to be seen whether efficacy can be established with this strategy, perhaps with the aid of enhancements in dosage regimens or drug characteristics, or whether utility in human stroke will prove to be fundamentally constrained (see below). Nonetheless, considering the high potential of the NMDA receptor system to contribute to excitotoxic neuronal death, we think it likely that NMDA antagonists will eventually find use as neuroprotective agents in one or another disease setting.

NMDA receptor blockade can be achieved in a variety of ways, using agents that act at distinct molecular sites within the heteromeric receptor complex. Competitive antagonists bind the glutamate recognition site; channel blockers, also termed uncompetitive antagonists, bind sites within the channel pore; glycine antagonists bind the glycine recognition site; and noncompetitive antagonists bind other sites on NMDA receptors, downmodulating receptor activation via remote actions, for example, via allosteric changes. The latter modulatory sites include those responding to polyamines (Ransom and Stec 1988), redox potential (Aizenman et al. 1989), hydrogen ions (Tang et al. 1990; Traynelis and Cull-Candy 1990), and Zn^{2+} (Peters et al. 1987; Westbrook and Mayer 1987; Christine and Choi 1990; Legendre and Westbrook 1990). Whereas NMDA receptor activation would be reduced by the free radicals and lactic acid produced by ischemia, the ischemic release of polyamines, including putrescine, spermine, and spermidine would be expected to upmodulate NMDA receptor activity (Paschen et al. 1992; Kerchner et al. 1999). Zn^{2+} effects might be complex, as acute direct NMDA receptor inhibition might be followed by more lasting Src kinase-mediated upregulation (Manzerra et al. 2001).

A well-recognized theoretical limitation of competitive NMDA receptor antagonists is that they are more effective when ambient glutamate concentrations are low and hence may be more effective against receptors operating physiologically than at the overactivated receptors contributing to acute excitotoxic damage. Channel blockers, glycine antagonists, and noncompetitive antagonists would not have this difficulty, but all of these antagonists are at risk for evoking what are probably mechanism-driven cognitive and motor side effects. NMDA antagonists also have the potential for inducing vacuolization or even death in small numbers of neurons in the cingulate or retrosplenial cortex, perhaps mediated by the paradoxical release of excitation in specific circuits (Olney et al. 1989).

How might the therapeutic index of NMDA antagonists be improved? Three approaches are currently being explored: (a) preferentially blocking overactivated NMDA receptors relative to physiologically-activated receptors, (b) limiting antagonism with partial or weak antagonists; and (c) enhancing target specificity with subtype selective antagonists. The first approach might be achieved by using activity-dependent channel blocking compounds that not only require channel opening to reach their binding site, but also exhibit a greater degree of blockade at higher levels of receptor activity. Memantine is such a compound (and it also has low affinity for its channel binding site – see below, this section); it has has shown promise in attenuating excitotoxic neuronal loss in vitro as well as brain damage in a rodent model of stroke, at concentrations that might permit near-normal levels of physiological NMDA receptor-mediated synaptic transmission (CHEN et al. 1992). In addition, the apparent affinity of ifenprodil and related antagonists for NMDA receptors increases at higher agonist concentrations (KEW et al. 1996), which, in addition to other useful properties (see below, this section), may contribute to a reduced side effect profile.

Low affinity channel blockers may represent one means to achieve moderate levels of NMDA receptor antagonism. Interestingly, despite the value generally attached to potency in drug development, an inverse relationship between drug affinity and toxicity is apparent for many NMDA channel blockers (ROGAWSKI 1993; PALMER and WIDZOWSKI 2000). In principle, because lower affinity compounds typically exhibit faster unblocking rates and require a higher concentration to achieve a given level of blockade, they equilibrate with their receptors more quickly, resulting in faster termination of NMDA receptor gating than is achieved by equieffective doses of higher affinity agents. At the same time, the faster unbinding of low affinity channel blockers should lead to less trapping of antagonist as receptor activity falls off and channels close. Such properties are attractive and may underlie reduced side effects.

A practical method for achieving limited antagonism of the NMDA receptor may be through the use of glycine site antagonists. While complete glycine site antagonism would be expected to generate a set of mechanism-driven side effects comparable to those produced by glutamate site antagonists and channel blockers, partial glycine site agonists, such as cycloserine (HOOD et al. 1989), by producing limited-efficacy blockade of NMDA receptor activity, may be able to strike an attractive balance between reduction of excitotoxicity and the downsides associated with high-level receptor blockade. Alternatively, levels of the endogenous glycine-site agonist, D-serine (SCHELL et al. 1995; SNYDER and KIM 2000), might be therapeutically reduced, hopefully still leaving enough ambient D-serine or glycine to keep receptors from shutting down completely. D-serine is synthesized by the enzyme serine racemase (WOLOSKER et al. 1999) within a discrete population of protoplasmic astrocytes that ensheath synapses (SCHELL et al. 1997); degradation of D-serine with

exogenously applied D-amino acid oxidase inhibits NMDA receptor-mediated synaptic transmission in hippocampal slices (MOTHET et al. 2000).

Another promising approach involves the use of NMDA receptor subunit-selective antagonists. Ifenprodil, originally recognized as an NMDA antagonist that interacts with the polyamine binding site (CARTER et al. 1989; REYNOLDS and MILLER 1989), acts as a noncompetitive NMDA receptor antagonist and reduces excitotoxic neurodegeneration following glutamate or NMDA exposure in vitro (GRAHAM et al. 1992) and focal ischemia in vivo (GOTTI et al. 1988). It turned out to be approximately 400-fold more potent at NMDA receptor complexes containing the subunit NR2B than those containing NR2A (WILLIAMS 1993), NR2C, or NR2D (WILLIAMS 1995). Presumably reflecting this subtype specificity – and thus regional specificity, as NR2B-containing NMDA receptors are expressed preferentially in the adult forebrain, in a nonuniform distribution between various forebrain structures and neuronal populations (WATANABE et al. 1993; MONYER et al. 1994) – ifenprodil and related compounds appear to exhibit less side effects than broad spectrum NMDA antagonists (KEMP et al. 1999).

Even while efforts are underway to improve the molecular profile of NMDA antagonist drugs, it is worth noting that the simple physiological channel blocker, Mg^{2+}, responsible for conferring voltage sensitivity to NMDA receptors (Nowak et al. 1984), has shown promise as a therapeutic agent in animal models of stroke, as well as traumatic brain injury (VINK and CERNAK 2000). It also has been used extensively in humans for the prevention of seizures associated with preeclampsia and eclampsia (MASON et al. 1994; ANTHONY et al. 1996) and has been suggested to reduce the risk of cerebral palsy in human infants born to preeclamptic mothers (NELSON and GRETHER 1995). Of course, the beneficial effects of Mg^{2+} may not be limited to NMDA receptor antagonism. To the extent that it penetrates into the CNS in a given disease setting, it would likely reduce glutamate release, and inhibit voltage-gated Ca^{2+} channel-mediated Ca^{2+} entry into neurons and vascular smooth muscle (the latter effect leading to enhancements of cerebral blood flow).

Finally, there may be some settings where NMDA antagonists, regardless of molecular mechanism of action, may not be beneficial. As noted above, NMDA receptor overactivation may already be limited by endogenous tissue factors such as lowered extracellular pH, Zn^{2+}, and oxygen free radicals. In addition, there has been recent recognition that the ability of NMDA antagonists to reduce Ca^{2+} influx may concurrently increase the likelihood of apoptosis for neurons that are in a state of relative Ca^{2+} starvation versus Ca^{2+} overload (LEE et al. 1999). In the developing rat brain, brief administration of NMDA antagonists has been shown to induce widespread apoptotic neuronal death (IKONOMIDOU et al. 1999); in agreement with that observation, the effect of ethanol to promote massive programmed cell death of central neurons in immature rat brains may reflect its ability to inhibit NMDA receptors (IKONOMIDOU et al. 2000).

2. AMPA/Kainate Antagonists

As discussed already, AMPA/kainate receptors can directly mediate excito-
toxic cell death, albeit less powerfully than NMDA receptors. The competitive
AMPA/kainate receptor antagonist NBQX is effective in reducing neuronal
loss following both global (SHEARDOWN et al. 1990) and focal (BUCHAN et al.
1991) cerebral ischemia, spinal cord ischemia (XU et al. 1993), and brain
trauma (WRATHALL et al. 1992), although the possibility of a contribution from
cerebral hypothermia has been raised (COLBOURNE et al. 1997). The noncom-
petitive AMPA receptor antagonist GYKI-52466 has also exhibited neuro-
protective effects in studies of global (LE PEILLET et al. 1992) or focal (SMITH
and MELDRUM 1992; XUE et al. 1994) ischemia.

In addition, AMPA/kainate receptor antagonists may be of special value
in certain settings. Although the death of most cortical neurons induced by
brief glutamate exposure at neutral pH is AMPA/kainate receptor antagonist-
insensitive (KOH and CHOI 1991), lowering pH selectively enhanced AMPA/
kainate receptor-mediated neurotoxicity, perhaps by delaying recovery of
intracellular Ca^{2+} homeostasis (MCDONALD et al. 1998b). In addition, a
small subpopulation of neurons, largely GABAergic, that express Ca^{2+}-
permeable AMPA receptors exhibits prominent vulnerability to AMPA
receptor-mediated excitotoxicity (KOH and CHOI 1988; JONAS et al. 1994;
TURETSKY et al. 1994). Brief glutamate exposure raises intracellular Ca^{2+} and
destroys these cells even when NMDA receptors are blocked. Ca^{2+}-permeable
AMPA receptors are likely also permeable to Zn^{2+} and hence confer vulner-
ability to Zn^{2+} neurotoxicity (SENSI et al. 1997; WEISS and SENSI 2000). Besides
protecting neuronal subpopulations expressing Ca^{2+}-permeable AMPA recep-
tors, AMPA antagonists could have value in reducing the excitotoxic loss of
oligodendrocytes, which likely also express Ca^{2+}-permeable AMPA receptors
(see above).

The prevalence of Ca^{2+}-permeable AMPA receptors in populations of
selectively vulnerable neurons in certain disease settings highlights a
potentially important therapeutic role for AMPA/kainate antagonists. In
amyotrophic lateral sclerosis, the motor neurons that undergo selective degen-
eration express AMPA receptors that are Ca^{2+}-permeable due to low levels
of GluR2 expression (SHAW and INCE 1997). Indeed, brief kainate exposure
induced a Ca^{2+}-dependent and Ca^{2+}-permeable AMPA receptor antagonist-
sensitive death in spinal motor neurons but not dorsal horn neurons (VAN DEN
BOSCH and ROBBERECHT 2000). In the context of transient global cerebral
ischemia, the prevalence of Ca^{2+}-permeable AMPA receptors may rise among
hippocampal CA1 neurons, a population of cells particularly vulnerable to this
type of insult, due to a downregulation in expression of GluR2 relative to other
AMPA receptor subunits (PELLEGRINI-GIAMPIETRO et al. 1992).

Historically, the roles of AMPA and kainate receptors in neuronal physi-
ology have been difficult to distinguish, due to insufficient pharmacology. With
the development of selective, noncompetitive AMPA receptor antagonists

(PELLETIER et al. 1996), it has become possible to discriminate between the relative contributions of AMPA and kainate receptors to several phenomena. Experiments with cultured cortical neurons have suggested that slowly-triggered excitotoxicity, induced by prolonged exposure to kainate (see above) is mediated predominantly by AMPA receptors, suggesting that activation of cortical neuronal kainate receptors alone may not suffice to induce cell death (TURETSKY et al. 1998). Moreover, there is reason to consider that selective kainate receptor antagonism could potentially be counterproductive, as activation of presynaptic kainate receptors by synaptically released glutamate inhibits excitatory transmission in the hippocampus (SCHMITZ et al. 2000), a phenomenon that may reflect a direct negative-feedback pathway for glutamate release. Of note, the AMPA-selective antagonists developed to date exhibit noncompetitive kinetics, providing effective blockade even in the context of excess extracellular glutamate.

3. Metabotropic Glutamate Receptors

Eight metabotropic glutamate receptors (mGluRs), which are linked to G-proteins rather than ion channels, have been identified and segregated into three groups based on sequence similarity and mechanisms of signal transduction (NAKANISHI and MASU 1994; CONN and PIN 1997). Group I mGluRs (mGluR1 and -5) couple via phospholipase C to phosphoinositide turnover and Ca^{2+} release from intracellular stores, whereas group II (mGluR2 and -3) and III (mGluR4, -6, -7, and -8) receptors couple to the inhibition of adenyl cyclase and reduction in cAMP levels. Although mGluRs do not directly mediate excitotoxicity, they can modify excitotoxicity and thus may be useful targets for therapeutic manipulation. The first clue to neuroprotective actions was the demonstration that the nonselective mGluR agonist, trans-1-amino-cyclopentane-1,3-dicarboxylic acid (tACPD), could attenuate glutamate-induced neuronal death (KOH et al. 1991); nonselective activation of mGluRs also reduced infarct volume in vivo after focal ischemia (CHIAMULERA et al. 1992).

Since mGluR group II and III receptors typically have inhibitory effects on circuit excitation and glutamate release, whereas group I receptors are typically proexcitatory (CONN and PIN 1997; CARTMELL and SCHOEPP 2000), it is plausible that agonists selective for group II or III mGluRs would have more powerful antiexcitotoxic effects than nonselective agonists. The mechanisms by which group II/III mGluRs downregulate transmitter release are not entirely understood but likely involve inhibition of presynaptic voltage-gated Ca^{2+} channels (STEFANI et al. 1996) and activation of presynaptic K^+ conductances (SLADECZEK et al. 1993). Initial studies with group II agonists suggested antiexcitotoxic actions against NMDA-induced degeneration in vitro (BRUNO et al. 1995a; PIZZI et al. 1996), although available drugs had confounding weak agonist/antagonist activity at NMDA receptors (BUISSON et al. 1996; CONTRACTOR et al. 1998). The more selective group II mGluR agonist, (+)-2-

aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740), was subse-
quently found surprisingly to lack antiexcitotoxic effects either in vitro or in
vivo (LAM et al. 1998; BEHRENS et al. 1999). However, the group III mGluR
agonists L-(+)-2-amino-4-phosphonobutyric acid and L-serine-*O*-phosphate
did reduce trauma-induced neuronal death in vitro, adding to the protective
effects of an NMDA antagonist (FADEN et al. 1997). Recently, it was demon-
strated that the selective group III mGluR agonist (+)-4-phosphonophenyl-
glycine attenuated NMDA-induced excitotoxic neuronal death both in cortical
cultures and in vivo; these effects were completely abolished in mice lacking
the mGluR4 gene (BRUNO et al. 2000).

   Agonists at group I mGluRs enhance neuronal excitability through
several mechanisms, including regulation of Ca^{2+} and K^+ channels (CONN and
PIN 1997). In addition, these mGluRs have complex modulatory effects upon
NMDA receptors, inducing both a rapid, membrane-delimited reduction of
NMDA receptor currents (YU et al. 1997a) and a long-term NMDA receptor
upregulation via Src family kinase-mediated phosphorylation of NR2 recep-
tors (BEHRENS et al. 2000); the latter effect may be more important from
the standpoint of excitotoxicity. Consistent with proexcitatory and NMDA
receptor-enhancing actions, activation of group I mGluRs generally potenti-
ates excitotoxicity. In cell culture studies, neuronal death secondary to NMDA
or kainate exposure (BRUNO et al. 1995b; BUISSON and CHOI 1995; STRASSER et
al. 1998) or traumatic injury (MUKHIN et al. 1996) was potentiated by group I
mGluR agonists and attenuated by antagonists.

III. Blocking Downstream Mediators

1. Downstream Effects of Cellular Ca^{2+} Overload

Many enzymes, including proteases, lipases, endonucleases, kinases, and phos-
phatases are activated directly or indirectly by increases in intracellular Ca^{2+}
concentration and may contribute to cellular damage after excitotoxic recep-
tor activation. Calpain inhibition attenuated neuronal death triggered by
exogenous excitotoxins in vitro (BRORSON et al. 1994) and following transient
global ischemia in rodents (LEE et al. 1991). More recently, MDL 28,170, a
potent inhibitor of calpains, decreased infarct volume after focal ischemia
when administered even 6h postocclusion (MARKGRAF et al. 1998). Ca^{2+}-
activated cytoplasmic phospholipase A_2 (cPLA$_2$) can catabolize phospholipids
to liberate arachidonic acid (DUMUIS et al. 1988), which may augment excito-
toxicity by reducing glutamate reuptake (YU et al. 1986), promoting glutamate
release (FREEMAN et al. 1990), and producing free radicals in the process of
downstream metabolism (see next section). cPLA$_2$ gene deletion increased the
resistance of mice to focal ischemia-induced brain injury (BONVENTRE et al.
1997).

   Although Ca^{2+}-activated endonucleases have also been suggested to con-
tribute to excitotoxic death, the poor selectivity of available endonuclease

inhibitors needs to be kept in mind (Kure et al. 1991; Roberts-Lewis et al. 1993; Zeevalk et al. 1993; Posner et al. 1995).

2. Free Radical Formation

Cytosolic Ca^{2+} loading consequent to glutamate receptor stimulation triggers the formation of multiple free radical species, which have deleterious effects on proteins, DNA, and lipids. Antioxidants can reduce neuronal death induced by exogenous excitotoxins in culture or by intrastriatal injection of excitotoxins in vivo (Dykens et al. 1987; Miyamoto and Coyle 1990; Monyer et al. 1990).

At least four pathways may link an excitotoxic increase in intracellular free Ca^{2+} to free radical overproduction: xanthine dehydrogenase, cyclooxygenases, nitric oxide synthases, or mitochondrial electron transport. Elevated intracellular Ca^{2+} indirectly converts xanthine dehydrogenase into xanthine oxidase, which can produce superoxide radicals ($^{\bullet}O_2^-$) (Dykens et al. 1987; Atlante et al. 1997). However, due to the low expression of this enzyme in the human brain, the pathophysiological relevance of this free radical pathway in humans remains uncertain (Sarnesto et al. 1996). Cyclooxygenase (COX)-mediated metabolism of arachidonic acid to a prostaglandin intermediate can also lead to the production of toxic $^{\bullet}O_2^-$ (Chan et al. 1985; Wei et al. 1986). Fenamate derivatives, which inhibit both COX-1 and -2, decreased the cell death induced by either NMDA or kainate in isolated chick retina (Chen et al. 1998). In cortical neuronal cultures, NMDA-induced excitotoxicity was decreased by a specific COX-2 inhibitor, NS-398 (Hewett et al. 2000). Consistent with a role for COX proteins in brain injury subsequent to focal ischemia, prostaglandin production was observed to increase early (15 min) following focal ischemia in rats and was attenuated by pretreatment with the fenamate derivative meclofenamate (Bucci et al. 1990). Significantly, COX-2 inhibition afforded neuroprotection against focal ischemia when administered to rats postocclusion (Nogawa et al. 1997).

Rises in intracellular Ca^{2+} concentration can also activate neuronal nitric oxide synthase (nNOS), which forms the weak oxidant, nitric oxide (Dawson and Snyder 1994). In the presence of $^{\bullet}O_2^-$, however, nitric oxide can be converted to peroxynitrite, a powerfully destructive free radical (Beckman and Koppenol 1996). Thus, nNOS plays a central role in mediating cell death induced by the overactivation of NMDA receptors in neuronal culture (Dawson et al. 1991; Dawson et al. 1996) and in mouse striatum in vivo (Ayata et al. 1997), as well as in rodents following focal ischemia (Huang et al. 1994). An inducible form of nitric oxide synthase, iNOS, which is expressed in inflammatory (Iadecola et al. 1995a), vascular (Iadecola et al. 1996), and glial (Endoh et al. 1994) cells after cytokine exposure in culture (Hewett et al. 1994) or after the onset of ischemia in vivo, can also contribute to excitotoxic damage. In vitro, cytokine-dependent induction of iNOS in astrocytes potentiated NMDA-mediated neuronal death (Hewett et al. 1994), and

aminoguanidine, an inhibitor of iNOS, reduced infarct volume following focal ischemia, even when administered 24 h following occlusion (Iadecola et al. 1995b; Zhang et al. 1996).

While mitochondria have the ability to buffer elevations in intracellular Ca^{2+} (Gunter and Pfeiffer 1990; Wang and Thayer 1996), excessive Ca^{2+} accumulation in mitochondria may lead to the uncoupling of energy production from electron transport and the formation of toxic levels of free radicals (Dugan et al. 1995; Reynolds and Hastings 1995; Schinder et al. 1996; White and Reynolds 1996). Pharmacological blockade of mitochondrial Ca^{2+} uptake substantially decreased glutamate-mediated neuronal death in culture (Stout et al. 1998). Furthermore, additional increases in free radical production may occur if mitochondria release their Ca^{2+} stores into the cytoplasm, amplifying the Ca^{2+}-dependent free radical cascades mentioned above (White and Reynolds 1996).

Beneficial results have been obtained with several free radical scavenger drugs in animal studies of ischemic or traumatic brain injury (Clemens and Panetta 1994), although the magnitude of neuroprotection observed has typically not been very large. Additionally, recent clinical trial experience with the antioxidant tirilazad mesylate in subarachnoid hemorrhage was not especially encouraging (Ranttas Investigators 1996; Kassell et al. 1996; Haley et al. 1997). It is possible that more powerful antioxidant agents may yield greater therapeutic benefits. The spin trapping agent, α-phenyl-N-tert-butyl nitrone (PBN) reduced infarct volume following focal ischemia (Cao and Phillis 1994) when administered up to 3 h after ischemia onset (Zhao et al. 1994), perhaps reflecting an ability of its breakdown product, N-t-butyl hydroxylamine, to inhibit mitochondrial superoxide production (Atamna et al., 2000).

Recent reports have suggested that Zn^{2+}-induced neuronal death may also in part be mediated by an increase in oxidative stress. In neuronal cultures, Zn^{2+} exposure caused an early increase in reactive oxygen species production and lipid peroxidation, and antioxidants attenuated neuronal death triggered by Zn^{2+} (Kim et al. 1999a,b; Sensi et al. 1999); however, in other studies a relatively lower prominence of free radical-mediated injury after Zn^{2+} exposure was observed (L.L. Dugan and D.W. Choi, unpublished results).

3. The Role of PARP

A particularly damaging consequence of reactive oxygen species formation may be single-stranded DNA breakage, leading to activation of the repair enzyme, poly(ADP-ribose) polymerase-1 (PARP-1), and consequent depletion of cellular NAD^+ and energy stores (Szabo and Dawson 1998). Consistent with the idea that PARP-1 activation leads to lethal energy depletion under excitotoxic conditions, pharmacological inhibition or gene deletion of PARP-1 attenuated neuronal death induced by glutamate receptor agonists in vitro (Zhang et al. 1994; Eliasson et al. 1997). PARP-1 knockout mice also exhibited increased resistance to focal ischemia (Zhang et al. 1994; Eliasson

et al. 1997; ENDRES et al. 1997) as well as to damage induced by intrastriatal NMDA injection (MANDIR et al. 2000). Several PARP inhibitors have demonstrated neuroprotective effects in rodent focal ischemia studies (ENDRES et al. 1997; TAKAHASHI et al. 1997).

F. A Cautionary Note for Antiexcitotoxic Strategies: Enhanced Apoptosis?

The basic nature of excitotoxicity – influx of cations into cells through over-activated glutamate receptors, leading to acute cell swelling and subsequent death – is suggestive of necrosis. Indeed, multiple studies support the notion that excitotoxic glutamate receptor overactivation in vitro typically induces necrosis (GOTTRON et al. 1997; GWAG et al. 1997; CHIHAB et al. 1998). However, any insult can probably induce programmed cell death in certain circumstances, and excitotoxicity is no exception, particularly when excitotoxic conditions are mild or when target neurons are immature (BONFOCO et al. 1995; MCDONALD et al. 1997). Apoptosis after excitotoxic insults may also be favored by reductions in extracellular Na^+ and Ca^{2+}, as occur in ischemic tissue; these disturbances alter the ionic driving forces governing NMDA receptor currents, increasing, in particular, the relative contribution of proapoptotic K^+ efflux (YU et al. 1997b, 1999). In vivo, intrastriatal injection of excitotoxins (FERRER et al. 1995; PORTERA-CAILLIAU et al. 1995; QIN et al. 1996) and cerebral ischemia (LINNIK et al. 1993; MACMANUS et al. 1993) lead to neuronal death outcomes that lie on a spectrum of morphological and biochemical phenotypes, ranging from necrosis to classic apoptosis, with many cells exhibiting a mixture of markers. The greater prominence of apoptosis after excitotoxin administration in vivo compared to in vitro, particularly at sites remote from an injection site in vivo, may reflect in part loss of innervation or trophic support originally provided by destroyed injection-site neurons.

The idea that certain insults may drive neurons simultaneously towards excitotoxic necrosis and apoptosis argues for caution in selecting neuroprotective strategies, since maneuvers that attenuate one type of death may have little effect or even a deleterious influence on the other. For instance, upstream antiexcitotoxic approaches reducing glutamate release or glutamate receptor activation may have a general tendency to reduce excitotoxic necrosis but enhance apoptosis triggered by other independent events. The converse may also be true. One might conceivably use mild proexcitotoxic manipulations such as the activation of group I mGluRs to raise intracellular Ca^{2+} and attenuate neuronal apoptosis (ALLEN et al. 2000).

Compared to upstream antiexcitotoxic approaches, downstream strategies aimed at blocking intracellular injury events may afford more opportunity for blocking excitotoxic necrosis without promoting apoptosis. Indeed, some strategies, such as free radical scavengers, may be effective against both excitotoxic necrosis and apoptosis. Besides a reduced risk of enhancing apoptosis,

downstream approaches may generally offer a longer therapeutic window of opportunity than upstream approaches. However, since glutamate receptor overactivation triggers multiple parallel injury cascades, downstream approaches may be unlikely to achieve the neuroprotective efficacy of upstream approaches, unless several pathways are simultaneously targeted.

If the contribution of excitotoxic necrosis to injury is large enough, upstream antiexcitotoxic approaches alone may be of value, but if apoptosis is prominent, it may be necessary to add concurrent blockers of apoptosis. Alternatively, it may be possible to separate a necrotic phase of injury from an apoptotic phase in time and/or space. For example, after ischemic insults, excitotoxic necrosis may be most prominent near the ischemic core and at early time points, whereas apoptosis may be more prominent in penumbral regions and at later time points.

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