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APOPTOSIS IN THE AGING BRAIN

Molecular and cellular mechanisms of excitotoxic neuronal death

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Abstract Glutamate receptor-mediated excitatory neurotransmission plays a key role in neural development, differentiation and synaptic plasticity. However, excessive stimulation of glutamate receptors induces neurotoxicity, a process that has been defined as excitotoxicity. Excitotoxicity is considered to be a major mechanism of cell death in a number of central nervous system diseases including stroke, brain trauma, epilepsy and chronic neurodegenerative disorders. Unfortunately clinical trials with glutamate receptor antagonists, that would logically prevent the effects of excessive receptor activation, have been associated with untoward side effects or little clinical benefit. Therefore, uncovering molecular pathways involved in excitotoxic neuronal death is of critical importance to future development of clinical treatment of many neurodegenerative disorders where excitotoxicity has been implicated. This review discusses the current understanding of the molecular and cellular mechanisms of excitotoxicity and their roles in the pathogenesis of diseases of the central nervous system.

Keywords Excitotoxicity · Glutamate receptor · Mitochondria · Protease · Neurological disorder

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Introduction

Glutamate is a major excitatory neurotransmitter in the mammalian central nervous system (CNS), and is an important neurotransmitter for neural development, synaptic plasticity, and learning and memory under physiological conditions [1]. Regulation of glutamatergic neurotransmission is critical because improper management of glutamate levels and glutamate receptor activity may impair not only its signaling properties, but can lead to cell death via excitotoxicity. The concept of excitotoxicity was first proposed by Dr Olney in 1969 as a toxic effect of excessive or prolonged activation of receptors by excitatory amino acids (EAAs) [2]. Excitotoxicity might mediate neuronal damage in various neurological disorders including ischemia and neurodegenerative diseases [3], and thus has been an important subject of neuroscience research for decades.

Although the molecular pathways involved in excitotoxicity are still not fully understood at the present, there is a wealth of evidence suggesting that over-stimulation of glutamate receptors produces multiple adverse effects including impairment of intracellular calcium homeostasis, compromise of organelle functions, increase in nitric oxide (NO) production and free radicals, persistent activation of proteases and kinases, increases in expression of pro-death transcription factors and immediate early genes (IEGs). Many studies provide support for each as reviewed in the following sections, although the role of these biochemical events is still incompletely defined. This review will focus on diverse cellular and molecular responses to excitotoxic insult, because the identification of a key post-receptor molecule as a cell death promoter will provide not only insight into the molecular basis of how neuronal cells execute excitotoxic death commitment, but also potential approaches for therapeutic intervention targeting excitotoxic signaling pathways in neurological disorders.

Different roles of glutamate receptors in excitotoxicity

Excitatory amino acids (EAAs) refer principally to glutamate (glutamic acid), but also include various metabolites that act via glutamate receptors including endogenous molecules such as aspartic acid, quinolinic acid (QA), homocysteic acid, and exogenous molecules such as *N*-methyl-D-aspartate (NMDA) and kainate (reviewed in [4]). EAAs produce their actions via a family of receptors generally called glutamate receptors. Glutamate receptor types are classified into three ionotropic classes: NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, and kainate; and three metabotropic classes [5, 6]. Ionotropic receptors are ligand-gated ion channels that open upon the binding of glutamate, leading to the influx of sodium and/or calcium and the efflux of potassium. Metabotropic receptors belong to the G-protein coupled receptor superfamily. Activation of metabotropic receptors leads to changes in cAMP levels and release of Ca^{2+} from intracellular stores [7].

NMDA receptors are tetra-heteromeric structures permeable to sodium, potassium, zinc and calcium. At normal physiological resting membrane potential, magnesium blocks the channel pore. When magnesium is removed, the ligand activated NMDA receptor allows an influx of calcium, leading to postsynaptic depolarization and action potential in the postsynaptic neuron. NMDA receptors play an important role in excitotoxicity as calcium entering through over-activated NMDA receptors results in more cell death as opposed to calcium entering through non-NMDA glutamate receptors or voltage-gated calcium channels [8]. As an additional support, NMDA receptor antagonists can block most excitotoxic effects of glutamate. NMDA receptor-mediated neurotoxicity occurs through distinct calcium signaling pathways that may involve the NMDA receptor specific interaction with postsynaptic density (PSD) proteins, a family of postsynaptic scaffold proteins [7]. Besides changes in calcium levels, over-activation of NMDA receptors could result in mitochondrial membrane depolarization, increases in free radicals and caspase activation [9–11].

AMPA receptors are permeable to sodium, potassium, zinc and occasionally calcium. The efficiency of calcium permeability through AMPA receptors is highly dependent upon the combination of subunits making up the heteromeric receptor [12]. The pre-mRNA editing of one subunit, GluR2, causes the replacement of a neutral glutamine with a positively charged arginine residue in the channel-forming membrane loop segment [13]. Presence of an edited GluR2, as is the case in an overwhelming majority of cells expressing AMPA, renders the heteromeric receptor mostly impermeable to calcium. Calcium-impermeable AMPA receptors can still cause excitotoxicity by allowing sodium influx to slightly depolarize the cell membrane, leading to the subsequent activation of NMDA receptors, as has been demonstrated by several investigators [4, 14]. Some studies suggest that changes in expression of glutamate receptor subunits under certain conditions may affect receptor activity. However, many studies show that changes in glutamate receptor subunits after neurological insults may not be specific, but instead generally affect expression of several glutamate receptor subunits [15, 16]. Under pathological conditions, such as seizures or hypoxia–ischemia, many principal cells may increase their Ca^{2+} influx regardless of the existing stoichiometry of AMPA or NMDA receptor assemblies [17]. It is important to note that the specific AMPA or NMDA receptor patterns expressed after an insult depend upon the age of the animal and history of early-life seizures [18].

Kainate receptors are heteromeric receptors permeable to sodium, potassium, and sometimes calcium [19]. Excitotoxicity enhanced by kainate receptor activation may be due to release of glutamate and sodium influx to depolarize the membrane and release the magnesium blockade of NMDA, leading to the subsequent activation of NMDA receptors [20, 21]. Excitotoxicity resulting from kainate receptor stimulation may be proceeded by apoptotic pathways rather than the necrotic pathway sometimes observed with NMDA receptor-mediated cell death. In addition, there are several studies suggesting that excessive stimulation of non-NMDA glutamate receptors with kainic acid (KA) can induce autophagy and activates lysosomal enzymes, which play an important role in excitotoxic neuronal injury [22, 23].

Metabotropic glutamate (mGlu) receptors have been grouped into three categories (Group I–III) based on pharmacological properties, signal transduction mechanisms, and sequence similarities. Group I mGlu receptors play a role in regulating multiple calcium, potassium, and non-selective cationic channels as well as NMDA and AMPA receptors, which may influence the firing patterns of neurons [24]. Group I mGlu receptors potentiate NMDA receptor activation, thus affecting excitatory neurotransmission, synaptic plasticity, and the generation of long-term potentiation [25, 26]. Group II and III mGlu receptors inhibit various calcium channels and may inhibit presynaptic release of neurotransmitters [27]. A growing number of studies have shown an important and complex role for mGluR in neuronal cell death [28, 29]. Metabotropic receptor stimulation leads to the death of striatal neurons by a mechanism having the biochemical stigmata of apoptosis. Moreover, metabotropic receptor stimulation evidently exerts opposite effects on pre- or postsynaptic mechanisms contributing to the NMDA and KA-induced apoptotic-like death of these neurons [30]. Activation of Group I and Group II/III mGlu receptors may have

opposite effects on neuronal cell survival. Stimulation of group I receptors potentiates neuronal excitation, as well as NMDA receptor activity, and thus, exacerbates excitotoxicity. In contrast, Group II or Group III receptors activation may provide neuroprotection through presynaptic inhibition of glutamate release [31, 32], downregulation of adenylate cyclase activity [33], or modulation of mitogen-activated protein kinase (MAPK)/phosphatidylinositol 3-kinase (PI3K) activity [34]. Opposite effects of group I and Group II/III mGluR activation on EAA release have been shown in rat spinal cord injury model. In this model, treatment with selective group I mGluR antagonists decreased EAA release, whereas the Group II antagonist LY 341495 increased EAA levels. Administration of the Group III agonist L-AP4 also significantly downregulates extracellular EAA levels after spinal cord injury [35].

Mixed forms of cell death in excitotoxicity

Excitotoxic neuronal death in brain is not a uniform event but, rather, a continuum of necrotic, apoptotic, and autophagic morphologies. The characteristics of morphological features of excitotoxic neuronal death might prove useful for analyzing the mechanisms that govern cell death under pathological conditions. Early studies found that excitotoxin-induced cell death was characterized by cell swelling, cytoplasm vacuolization and disruption of cell membranes. These features imply that cells die of necrosis (reviewed in [36]). Later studies found that DNA internucleosomal degradation, chromosome condensation and fragmentation, activation of caspases were observed, indicating dying neurons also exhibit apoptotic features [37, 38]. Thus, it is generally agreed that excitotoxic neuronal death is a mixed form of necrosis and apoptosis [39].

In recent years, it has been suggested that autophagy may be a possible mechanism for non-apoptotic cell death induced by excitotoxins, despite evidence from many species that autophagy represents a survival strategy in times of stress. Increases in glutamate receptor activity could induce expression of proapoptotic proteins such as p53, leading to neuronal injury and death by inducing apoptosis and autophagy [40–42]. Autophagy is reportedly activated in response to acute excitotoxic insults in cultured hippocampus slices and mouse hippocampus [43, 44]. We have found that KA- or the NMDA receptor agonist QA-induced neuronal death was accompanied by increases in the formation of autophagosomes and secondary lysosomes. KA and QA also increase levels of autophagy biomarkers including the autophagosomal membrane-associated form of microtubule associated protein light chain 3 (LC3-II) and beclin-1 [23, 45]. Our recent studies have demonstrated that KA activates the lysosomal enzyme cathepsin B, and that the cathepsin B inhibitor Z-FA-fmk and the autophagy inhibitor 3-methyadenine (3-MA) potently attenuates apoptosis of striatal neurons induced by KA [22], suggesting autophagy contributes to excitotoxic cell death through lysosomes [22, 23].

Mobilization of ions and excitotoxicity

Prolonged overstimulation of the glutamate receptors leads to Ca^{2+} and Na^{+} overload in postsynaptic neurons [46]. Ca^{2+} inflow through voltage-dependent or independent channels can enhance neuronal Ca^{2+} overload under excitotoxic [47] or ischemic [48] conditions. In addition, mitochondrial Ca^{2+} accumulation and its subsequent release may play an important role in maintaining a persistent Ca^{2+} overload. Nevertheless, the combination of increased Ca^{2+} influx into neurons and mitochondrial Ca^{2+} release may not fully account for the irreversible buildup of intracellular Ca^{2+} after excitotoxic stimulation. Conceivably, the delayed increase in cellular Ca^{2+} should be rectified by the mechanisms governing cellular Ca^{2+} extrusion.

In neurons, Ca^{2+} extrusion is enabled by the plasma membrane Ca^{2+} pump (PMCA) and by Na^{+}/Ca^{2+} exchangers (NCX). PMCA has high Ca^{2+} affinity but low transport capacity, whereas NCX has a low affinity, but a higher capacity to transport Ca^{2+} [49]. Inhibition of Ca^{2+} efflux from cells is sufficient to cause a sustained intracellular Ca^{2+} elevation and the demise of non-neuronal cells by activating Ca^{2+}-dependent hydrolytic enzymes including members of the calpain protease family. It has been found that the plasma membrane NCX [50] is cleaved in the ischemic brain and in cultured cerebellar granule neurons (CGNs) exposed to glutamate. In particular, it has been shown that proteolysis of NCX isoform 3 [51] by calpains play prominent roles in the delayed, irreversible excitotoxic Ca^{2+} elevation leading to neuronal demise.

Cl^{-} movement has also been shown to be a central component of the acute excitotoxic response in neurons. The acute excitotoxicity is thought to be mediated by excessive depolarization of the postsynaptic membrane. This results in an osmotic imbalance, which is countered by an influx of Cl^{-}, Na^{+}, and water, and eventually cell lysis. A significant increase in intracellular Cl^{-} concentration ($[Cl^{-}]_i$) is observed in hippocampal neurons during oxygen–glucose deprivation (OGD) [52]. Removal or reduction of Cl^{-} from extracellular medium during EAA exposure completely eliminates the acute excitotoxic response in hippocampal [53] and retinal neurons [54]. Blockage of Cl^{-} entry through the Cl^{-}/HCO_3^{-} exchanger or GABA receptor effectively protects cells against the

acute excitotoxicity. The Cl^- influx is mediated by multiple pathways. $GABA_A$ receptor-coupled anion channels appear to serve as one of the Cl^- influx pathways in neurons exposed to excitotoxic insults, because $GABA_A$ receptor blockers partially blocked excitotoxic injury [55]. Also, an involvement of some other anion channel in the excitotoxic Cl^- influx have been suggested [56]. It is reported that, in cultured cortical neurons, volume-sensitive outwardly rectifying (VSOR) Cl^- channels, but not $GABA_A$ receptors or Cl^- transporters, serve as the pathway for volume-regulatory anion efflux and play a requisite role in varicosity resolution after a sublethal excitotoxic insult [57].

Na–K–Cl cotransporter isoform 1 (NKCC1) also contributes to the Cl^- movement during excitotoxicity. NKCC1 belongs to the cation-dependent Cl^- transporter family and transports Na^+, K^+, and Cl^- into cells under physiological conditions [58]. NKCC1 was involved in ischemic cell death through an association with excitotoxicity. NKCC1 may be involved in K^+ uptake from the paranodal region of myelinated axons and thereby may regulate extracellular ionic environment and the excitability of axons [59]. Development-dependent expression of NKCC1 occurs in rat spinal cords, which regulates intracellular Cl^- in spinal oligodendrocytes [60]. Oligodendrocyte damage in white matter causes axonal demyelination and determines subsequent neurological function deficit [61]. Oligodendrocytes express glutamate receptors including NMDA, AMPA, and KA receptors [62, 63]. Cerebral hypoxia/ischemia, intracerebral injection of AMPA, or spinal cord ischemia causes white matter damage and loss of oligodendrocytes, which is significantly attenuated by AMPA receptor antagonist NBQX [64–66]. Exposure of cultured oligodendrocytes to AMPA or glutamate induces immediate Ca^{2+} influx and leads to cell death after prolonged treatment [67, 68]. Intracellular Ca^{2+} overload, mitochondrial dysfunction, and apoptosis have been implicated as cellular mechanisms in excitotoxic oligodendrocyte damage [69].

Connections of cellular organelles and excitotoxicity

Dysfunction of cell organelles occurs in many human neurological diseases. Cell death in neurons is controlled by the activity of signaling pathways and proteins with a cross-talk between various organelles [70]. In neurons that are dying of excitotoxicity, morphologic changes include swelling of endoplasmic reticulum (ER), Golgi vesiculation, mitochondrial disruption and increases in the number of secondary lysosomes. Recently, mitochondrial and other organelles including ER, lysosomes and peroxisome have been linked to cell stress responses in human diseases such as Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) [71, 72].

Mitochondria

Mitochondria are not only ATP producers through oxidative phosphorylation but also are regulators of intracellular Ca^{2+} homeostasis and endogenous producers of reactive oxygen species (ROS). Mitochondrial injury is understood to have a critical impact on cellular energetics and excitotoxic neuronal death [73]. The mitochondria have been implicated as a central executioner of cell death. Increased mitochondrial Ca^{2+} overload as a result of glutamate receptor over-activation has been associated with the generation of superoxide and the release of proapoptotic mitochondrial proteins, leading to DNA fragmentation/condensation and culminating in cell demise by apoptosis and/or necrosis. On the other hand, it has also been well-established that mitochondrial dysfunction contributes to excitotoxic demise by changing membrane potential and increasing generation of ROS [73]. Dysregulated mitochondrial functions accompanied by disturbed calcium homeostasis have been considered to underlie excitotoxic and other brain injuries [74].

Mitochondria have the ability to sequester large amounts of Ca^{2+}, however this carries a risk of mitochondrial dysfunction [75]. Exposure of neurons to glutamate was previously demonstrated to result in mitochondrial depolarization associated with increased Ca^{2+} uptake into the mitochondria [11]. Activation of NMDA receptors was reported to induce faster mitochondrial Ca^{2+} uptake, and in a more tightly coupled way, compared to kainate or KCl. This observation suggested a privileged access of Ca^{2+} to mitochondria, entering through NMDA receptors, which could be accounted for by the possibility that mitochondria are in closer proximity to NMDA receptors than other routes of Ca^{2+} entry [76]. Excessive influx of Ca^{2+} via NMDA receptors attenuates the mitochondrial membrane potential ($\Delta\psi$), and leads to the opening of the permeability transition pore (PTP). Through the disruption of mitochondrial potential, excess Ca^{2+} can reduce ATP synthesis, rendering the cell more vulnerable to death insults. Mitochondria appear to be the primary mediators of cell death caused by abnormal levels of intracellular Ca^{2+} during excitotoxicity [77, 78].

Mitochondrial Ca^{2+} loading is the critical step in acute glutamate excitotoxicity. NMDA receptor-dependent transient mitochondrial Ca^{2+} loading could initiate oxidative damage and/or inhibit mitochondrial respiration; two factors suggested to precipitate delayed Ca^{2+} deregulation (DCD), a failure of the cell to maintain a low cytoplasmic free calcium concentration [11]. Under the conditions of continued Ca^{2+} entry, plasma membrane Ca^{2+} extrusion

may be the first to fail, whereas after inhibition of the receptor, mitochondrial dysfunction may precipitate DCD. It has been found that even a brief exposure to glutamate initiates DCD and it is apparent that mitochondrial dysfunction is initiated in this period. The damage inflicted by transient Ca^{2+} loading may include cytochrome c release, altering the redox poise of complex III and enhancement of superoxide generation. The critical parameter that becomes rate-limiting to initiate DCD depends on the experimental design. In cultured retinal neurons, a decrease in oxygen consumption upon glutamate exposure has been observed, coincident with the inhibition in the activity of mitochondrial complexes I, II/III, and IV [79]. Curiously, during continuous activation of the NMDA receptors, mitochondria depolarization occurred concomitantly with the DCD, which seems to precede the subsequent necrotic death of the cell. In addition, in cells stimulated with glutamate and glycine, the mitochondria continued to generate ATP, but once started, the NMDA receptor-induced DCD was shown to be irreversible in cultured cerebellar granule cells, as determined upon exposure to antagonists of NMDA or non-NMDA glutamate receptors, Ca^{2+} channel blockers, or even in the presence of inhibitors of the PTP [80].

It is well-known that cytochrome *c* release from the mitochondria to the cytosol is a key indicator of classical apoptotic program development [81, 82]. Two different signal transduction pathways could be involved in apoptosis initiation under the condition of oxygen depletion: one of them is accompanied with cytochrome *c* release [83], while the other is accomplished without this process [84]. The occurrence of apoptosis upon NMDA-mediated excitotoxicity has been controversial. During excitotoxicity, the release of mitochondrial cytochrome *c* associated with a delayed mitochondrial depolarization and production of ROS were documented [85, 86]. Previous reports have also shown that caspase-3, in particular, plays a major role in NMDA excitotoxicity [87]. Moreover, apoptosis-inducing factor (AIF) translocation was observed upon the stimulation of the NMDA receptors in a process requiring the activation of poly (ADP-ribose) polymerase (PARP) and the consequent depletion of NAD^+ [88], although the mechanisms involved are still not completely clear.

In recent years, mitochondria have assumed a great importance by clarifying the link between different signaling molecules (e.g., cytosolic Ca^{2+}) and the commitment to cell death. Mitochondria regulate the death execution phase, marking the point of no return in necrosis and apoptosis. Furthermore, because damaged mitochondria can accumulate in aging as a result of deficient autophagy [89], it will also be important to identify the mechanisms involved in autophagy in neurons committed to die in various neurodegenerative disorders.

Endoplasmic reticulum

Endoplasmic reticulum (ER) is an important cell organelle that is responsible among others for correct folding and sorting of proteins [90]. ER functions can be disturbed by different insults such as accumulation of unfolded proteins and changes in calcium homeostasis [91]. ER disturbance induces expression of chaperones, attenuate protein translation, and activate ER-associated degradation [92]. These occur by the activation of ER sensor proteins controlled by the chaperone Bip/Grp78, which is localized in the ER [93]. ER stress leads to activation of the RNA-dependent protein kinase (PKR)-like endoplasmic reticulum kinase (PERK)/pancreatic eukaryotic translation initiation factor 2 subunit α (eIF2α) kinase, activating transcription factor-6 (ATF6), and the inositol-requiring enzyme 1 (IRE1), which in turn activates distinct signaling cascades mediating the ER stress response [94, 95]. ER stress has been widely studied for its role in unfolded protein response (UPR), in cellular homeostasis and in calcium regulation [90, 91]. Apart from the UPR that is mainly adaptive and restorative in function, prolonged ER stress can trigger mitochondria-dependent and -independent forms of cell death [96–98].

Although little is known about the precise mechanisms responsible for activation of ER stress after NMDA or ischemia–reperfusion, both stimuli cause intracellular Ca^{2+} overload and increased NO production, resulting in apoptotic cell death. Several lines of studies suggest that intracellular Ca^{2+} overload and excessive production of NO deplete Ca^{2+} in the ER, thereby resulting in ER stress [99]. Uehara et al. [100] reported that NO induces *S*-nitrosylation of protein-disulphide isomerase (PDI), an enzyme that assists in the maturation and transport of unfolded secretory proteins and thereby helps to prevent the neurotoxicity associated with ER stress. *S*-nitrosylated-PDI exhibits reduced enzymatic activity and induces cell death through the ER stress pathway. These mechanisms may contribute to the activation of ER stress in NMDA receptor stimulation.

Activation of KA receptors is known to induce different signaling pathways and ion fluxes including elevation of calcium in neurons [101]. Previously, caspase-12 cleavage has been observed in hippocampal neurons lacking the calcium-binding protein hippocalcin [102]. Blocking calcium by 2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM, an intracellular calcium chelator) inhibited caspase-12 cleavage, demonstrating that increased calcium can trigger ER-mediated caspase activation. Neurotoxicity induced by NMDA is influenced, in part, by a mechanism dependent on BiP and CHOP protein induction through excessive ER stress [103]. It has also been shown that Grp78/Bip suppresses ER stress and protects neurons against glutamate-induced excitotoxicity [104]. Data

obtained with salubrinal showed that the inhibition of ER stress is also neuroprotective in vivo. Salubrinal and other compounds preventing ER stress may therefore be of value in novel therapies for excitotoxic and other brain disorders [105].

Lysosomes

Lysosomes contain hydrolytic enzymes necessary for intracellular digestion. Uncontrolled release of lysosomal contents into the cytoplasm causes necrotic cell death. It has been reported that certain cathepsins can directly or indirectly activate caspases. The latter is meditated through cleavage of BID by cathepsin B. The BID cleavage results in BID mitochondria translocation and cytochrome c release. When cells are subjected to limited oxidative stress, some lysosomes rupture or leak their contents leading to a non-necrotic cell death. Activation of the FAS/APO-1 receptors results in a decline of the cytosolic pH and, perhaps as a result, lysosomal labilization. Lysosomal labilization might be an initial and general event in apoptosis [106].

Cysteine proteases from the caspase family play a crucial role in the process. However, there is increasing evidence that lysosomal proteases are also involved in apoptosis. Various lysosomal proteases and their potential contribution to propagation of apoptosis are discussed (reviewed in [107]). Kazuyoshi Tominaga's data [108] indicated that excitotoxin-induced neuronal death was associated with a response of lysosome enzyme: cathepsin E. Our studies showed that lysosomal enzyme cathepsin B was involved in KA-induced excitotoxicity in rat striatum [22].

Several lines of evidence support a positive role of lysosomal and autophagic mechanisms in programmed cell death [109–111]. Apoptotic and autophagic cell death have been implicated, on the basis of morphological and biochemical criteria, in neuronal loss occurring in excitotoxic animal models and neurodegenerative diseases [23, 112]. Cross-talk between apoptosis and autophagy has been reported. Apoptosis is accompanied by an early and marked proliferation of autophagosomal–lysosomal compartments [113, 114]. Autophagy is blocked by inhibitors of apoptosis, as well as by adenovirus-mediated overexpression of Bcl-2. 3-Methyladenine (3-MA), an inhibitor of autophagy, not only arrests autophagic cell death, but it also blocks apoptosis. The neuroprotective effect of 3-MA is accompanied by blocking cytochrome c release from mitochondria and by inhibition of caspase-3 activation, which appears to be mediated by cathepsin B as CA074-Me, a selective inhibitor of this enzyme, fully blocks the processing of pro-caspase-3 [115]. As the lysosome is an important component of autophagy activity and autophagy has now been found to play a role in excitotoxicity, it is believed that the lysosome is a contributor of excitotoxicity.

A lysosomal–mitochondrial axis theory of cell death has been proposed [116]. A few studies have indicated that lysosomal activation was involved in pathogenesis of certain neurodegenerative diseases [117, 118]. Cathepsin inhibitors can be protective in some models of neurodegeneration, and could be therapeutic in ischemic injury, Alzheimer's disease (AD), and other protein deposition diseases in which compensatory responses by lysosomal enzymes may contribute to brain pathology [119, 120].

Contributions of intracellular signaling molecules to excitotoxicity

Accompanying the increase in intracellular calcium is the activation of transcription factors and IEGs, calcium-dependent enzymes, protein kinases and production of ROS. These intracellular signaling molecules make significant contributions to the excitotoxic death of neurons [121, 122].

Free radicals

The CNS is notable for its level of oxygen utilization and ATP synthesis, resulting in a distinct susceptibility to oxidative stress. There is ample evidence to suggest that increased production of ROS may play an important role in excitotoxicity. Generation of ROS can occur with mitochondrial respiration as well as during other aspects of cellular homeostasis maintained through a balance between biosynthesis and catabolism. Oxidative stress is now recognized as being accountable for redox regulation involving ROS and reactive nitrogen species (RNS). Its role is pivotal for the modulation of critical cellular functions such as apoptosis program activation, ion transport and calcium mobilization, notably for neurons, astrocytes and microglia. Mitochondrial dysfunction, cell energy impairment, overproduction of ROS and apoptosis, is a final common pathogenic mechanism in aging and in neurodegenerative disease such as AD, PD and ALS.

Excitotoxicity is associated with marked increases in free intracellular calcium levels [123]. Glutamate-induced excitotoxicity induces cytoskeletal alterations, EAA release, impaired EAA uptake, and the production of ROS. Glutamate excitotoxicity is associated with higher cellular levels of ROS [124, 125]. Glutamate also increases DNA binding of the redox-regulated transcription factors, nuclear factor-κB (NF-κB) and activating protein 1 (AP-1), in human neuroblastoma cells, and increases the expression of the IEGs, c-fos, in murine neuronal cells. These events occur before glutamate-induced apoptosis or necrosis in

several neuronal cell types, suggesting a possible causal role in excitotoxic cell death [126].

Nitric oxide (NO), an RNS which can be produced by three isoforms of NO-synthase in brain, plays a prominent role in excitotoxic neuronal death. Massive generation of the pleiotropic messenger molecule NO has been implicated in many neuropathological conditions and may have similar biochemical consequences as ischemia. At NMDA receptors, glutamate triggers the opening of cation-permeable channels. The entry of Ca^{2+} through these channels into cells stimulates nitric oxide synthetase (NOS) activity by binding to calmodulin, which is a cofactor for NOS. Activation of NOS causes NO production, and NO reacts with superoxide anion (O_2^-) to form peroxynitrite ($OONO^-$), which results in neuronal damage [127, 128].

It has been established that NO triggers a vicious loop strictly dependent on endogenous glutamate release and NMDA receptor activation, which forms the basis of neuronal apoptosis in cerebellar granule cells [129]. NO inhibits the mitochondrial respiratory chain in vitro, stimulates neurotransmitter release from synaptosomes and can cause autocrine excitotoxicity in neuronal cultures. A cyclic process of self-enhancing loops has been suggested to account for NO-mediated neuronal death. NO triggers conditions which lead to an impairment of mitochondrial function and energy failure, followed by impairment of ion pumps and partial hypopolarization. This in turn sensitizes neurons towards glutamate stimulation by releasing the magnesium blockade of NMDA receptors. NMDA receptor-mediated calcium increase enhances depolarization, triggers further calcium increase and favors release of endogenous glutamate [130]. This putatively self-propagating process results in loss of intracellular calcium homeostasis and excitotoxicity. Recently, it has been found that NO switches on the over-expression of metalloproteinase, which, in turn, destroys the environment that surrounds nerve cells. The extracellular proteolytic cascades that are triggered by metalloproteinase can disrupt the extracellular matrix, contribute to cell detachment and lead to anoikis (apoptosis due to cell detachment from the substrate) [131].

Proteases

The mechanism of excitotoxin-induced apoptosis requires activation of cysteine proteases such as calpains and caspases, which work independently [132, 133] and also co-operatively [134, 135] to cause neuronal apoptosis. Studies imply that calpain and caspase-3 inhibitors may provide neuroprotective effects in the animal models of traumatic brain injury and neurodegenerative diseases [136].

Calpains are calcium-dependent proteases. They modulate a variety of physiological processes [137] and can also become important mediators of cell death [134]. Ample evidence documents the activation of calpains in brain ischemia and excitotoxic neuronal degeneration [132]. Calpain activation has been associated with excitotoxicity [138–140]. Activation of calpain has been reported to identify those neurons that are vulnerable to excitotoxic cell death in hippocampal slices exposed to NMDA [141].

Reports indicate that in excitotoxic injury induced by NMDA in vitro [132] or by 3-nitropropionic acid (3-NP) in vivo [142], calpains negatively regulate caspase-3/9 activation and lead to caspase-independent neuronal death. In addition to direct cleavage of caspases, calpains have been shown to cleave several apoptosis regulatory proteins including apoptotic protease-activating factor-1 (Apaf-1) [143], Bax [144, 145], Bid [146–148] and p53 [149]. Calpain-mediated degradation of p53 is correlated with anti-apoptotic effects and degradation of Apaf-1 correlated with a reduced ability of cytochrome c to activate caspase-3-like proteases, whereas the cleavage products of Bcl-2 family proteins exerts proapoptotic function. Therefore, calpains can influence apoptotic pathways at different steps by blocking activation of the caspase cascade and activating other caspase-independent cell death pathways.

The caspase-dependent death pathway is initiated by release of cytochrome *c*, which associates with Apaf-1 to activate caspases [150]. Activated caspases cause neuronal apoptosis via the extrinsic and intrinsic pathways with the final activation of caspase-3 [151]. Caspase-3 was reportedly activated in glutamate-induced apoptosis of cultured cerebellar granule cells [152]. Activation of other caspases such as caspase-6 and caspase-9 in glutamate-induced apoptotic cascade was also reported [153]. Ha and Park [154] reported an increase in caspase-1 and caspases-3/-7 activity following L-glutamate treatment [155].

As seen with other caspases, post-translational activation of caspase-3 requires a proteolytic cleavage of the precursor protein into two subunits (p17 and p12), of which the larger subunit contains the functional catalytic site. Increase in caspase-3 family activity after glutamate stimulation may be attributed to an increase in cleavage of the proenzyme into functional protease, or by an up-regulation of caspase-3 gene expression. A number of studies suggest that caspase-3 can be either auto-activated or activated by members in the same or other caspase families [156]. Caspase-3 may mediate glutamate induced cell death via several mechanisms. Mature caspase-3 cleaves specific cellular proteins, which include the death substrate PARP [157]. Other potential targets for caspase-3 include DNA-dependent protein kinase (DNA-PK) [158], protein kinase C [159], the transcription factors, sterol regulatory element binding proteins (SREBPs) [160] and actin [161].

Protein kinases

While the immediate events in excitotoxic injury, such as NMDA receptor activation and consequent Ca^{2+} influx, are well-established, the subsequent downstream events that result in neuronal death remain less clear. Signal transduction pathways that relay extracellular signals to the nucleus via a series of phosphorylation events are strong candidates for mediating the downstream effects of excitotoxic injury. Several protein kinases including cAMP-dependent protein kinase (PKA), Calmodulin-dependent protein kinase-II (CaMK-II) and some members of protein tyrosine kinases (PTK), such as c-Src, have been shown to transduce Ca^{2+} signaling to ERK1/ERK2 (extracellular signal-regulated kinases) cascade and excitotocixity [162, 163].

ERK1/ERK2, with molecular masses of 44 and 42 kDa, respectively, are classical members of the MAPK superfamily. Both require specific diphosphorylation of both threonine and tyrosine residues at the regulatory sites by MEK1/MEK2 (ERK1/ERK2 kinase) for activation. ERK1/ERK2 cascades play important roles in signal transduction from cell surface to nucleus. The well-documented neurotropic growth factor receptor-mediated activation cascade (Ras/Raf/MEK/ERK) has been thought to play important roles in cell growth, proliferation and survival [164]. ERK1/ERK2 have been found to be activated after relatively mild stimulation of glutamate receptors and to be involved in some activity-dependent functions [165]. Furthermore, ERK1/ERK2 have also been found to be activated in some excitotoxicity-associated events, such as stroke, seizure and AD [166, 167]. ERK1/ERK2 were transiently activated in glutamate-induced apoptotic-like death in cultured rat cortical neurons, and PD98059, a specific inhibitor for MEK1/MEK2, completely inhibited such activation and partially prevented the glutamate-induced apoptotic-like death [168]. Therefore, ERK1/ERK2 might be excessively activated transiently and involved in the glutamate-induced cortical neurotoxicity.

Phosphorylation of the transcription factor cAMP response element binding protein (CREB) represents a potential downstream target of MAPK/ERK activation in models of neuronal death. CREB phosphorylation is observed in hippocampal neurons following both transient [169] and permanent [170] focal cerebral ischemia in the rat, suggesting this event may be important to the injury process. Whilst several CREB kinases are involved in neuronal injury, including PKA, MAPK-activated protein kinase-1 (MAPKAP-K1) and mitogen and stress activated protein kinase-1 (MSK1). Calcium induces ERK via Ras independent PKA-dependent stimulation of the small G-protein, Rap1, and the downstream kinase, B-Raf. PKA signaling pathway has been implicated in NMDA receptor-induced neuronal death [171] and in epidermal cell autophagy in Drosophila [172].

Calmodulin-dependent protein kinase-II (CaMK-II) has been shown to play a key role in mediating some of the biochemical events leading to cell death following an acute excitotoxic insult of cortical neurons. Treatment with DY-9760e, a calmodulin antagonist, resulted in a dose-dependent prevention of neuronal cell death elicited by excitotoxicity, voltage-gated channel opening, and inhibition of ER Ca^{2+} ATPase [173]. Although the mode of cell death and underlying mechanisms are not yet clear, CaMK-II that is regulated by calcium has been shown to induce ERK activation in neurons and vascular smooth muscle cells [174, 175]. Therefore, it is plausible that ERK might be a downstream player of CaMK-II-mediated excitotoxic cell death.

Src constitute a family of tyrosine kinases, which can act as upstream activators of ERK and have been implicated in neuronal cell death mediated by zinc, glutamate and ischemia. Zinc-induced neuronal death can be apoptotic or necrotic depending upon the intensity of Zn^{2+} exposure [176]. Zn^{2+} has been shown to produce oxidative neuronal necrosis in cortical cell cultures via Src family kinase [177]. Glutamate triggers neuronal degeneration after ischemia–reperfusion in brain. It has been suggested that tyrosine phosphorylation, including Src kinase activation, might propagate delayed neuronal death in the mature hippocampus following glutamate overload, after ischemia reperfusion. Similarly, increased activation of Src was seen in microglia of the post-ischemic hippocampus, indicating that Src signaling may be involved in the microglial response to an ischemic insult [178]. Although these studies do not provide evidence for an involvement of ERK in their models, it has been suggested that Src family tyrosine kinases are critical for ERK activation [179]. One study in a hippocampal cell line showed that glutamate-induced neuronal death is accompanied by an activation of Src kinase and ERK. Numerous other studies in non-neuronal cells suggested ERK as a downstream target of Src [180]. In addition, calcium and its regulation by NMDA receptors have been shown to be modulated by Src [181, 182]. Together, these reports suggest that glutamate, zinc and ischemia induce neuronal degeneration via Src-tyrosine kinase. However, the precise role of ERK in mediating Src induced neuronal degeneration still needs to be further investigated.

Transcription factors and immediate early genes

Increased DNA binding of redox-regulated transcription factors, nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1), are associated with the mechanisms of excitotoxicity. Kaltschmidt et al. [183] reported that KA

activates NF-κB. Later studies defined the nuclear translocation and a pro-apoptotic role of NF-κB activation mediated by AMPA/KA receptors [184, 185]. Similarly, the stimulation of glutamate NMDA receptors robustly activates NF-κB through the degradation of NF-κB inhibitor-α (IκB-α) [186, 187]. In other studies, pharmacological upregulation of NF-κB increased glutamate-induced excitotoxicity, while the upregulation of CREB decreased excitotoxicity [188]. Grilli et al. [189] reported a neuroprotective role of aspirin on the glutamate-induced death of hippocampal neurons, opening a new avenue for the study of excitotoxicity. Since then, several studies have reported that the inhibition of NF-κB has neuroprotective effects [187, 190–192]. In studies conducted by Casper et al. [193], neuroprotection against glutamate-mediated excitotoxicity was also found with ibuprofen. The inhibition of NF-κB with a herbal active component glycyrrhiza acid, free radical scavenger OCT14117 [194], and glutamate metabotropic receptor agonists (2S,1′S,2′S)-(carboxycyclopropyl) glycine and L(+)-2-amino-4-phosphonobutyric acid [195, 196] was associated with a neuroprotective effect. Pretreatment with a cell-permeable recombinant peptide inhibitor of NF-κB, selectively blocked quinolinate-induced NF-κB nuclear translocation as well as apoptosis [187]. Studies have suggested that neurotoxicity through glutamate-NMDA receptors or oxidative stress is dependent upon CREB and NF-κB DNA transcription that regulates vitality of neurons [188], suggesting that NF-κB inhibitors could be suitable drugs for blocking excitotoxicity (reviewed in [41]).

In the NMDA-triggered apoptotic process involving NF-κB activation, NF-κB regulated the expression of many proteins including c-Myc and p53, which in turn regulate a broad range of physiological and pathological responses. Levels of both proteins increase upon NF-κB nuclear translocation, and play a prominent role in the control of the cell cycle and apoptosis in dividing cells. AP-1 is a transcription factor sensitive to stress conditions, and induced by diverse stimuli, including glutamatergic stimulation [187]. AP-1 consists of a variety of dimers constituted by proteins of the Jun and Fos families. The Jun proteins (c-Jun, JunB and JunD) can both homodimerize and heterodimerize with Jun or Fos proteins, while the Fos proteins (c-Fos, FosB, Fra-1 and Fra-2) can only heterodimerize with the Jun family members to form transcriptionally active complexes [197]. AP-1 activation is mediated, in part, by the phosphorylation of c-Jun by the c-Jun N-terminal kinases (JNKs). In the Jun family, c-Jun is the most potent activator of transcription. There are direct [198] and indirect lines of evidence [199, 200] that the JNK/c-Jun signaling pathway is important for neuronal death induced by excitotoxicity. Studies show that activation of the AP-1 transcription factor, mediated through Ca^{2+}-permeable GluR4-containing AMPA receptors, is involved in excitotoxicity-induced cell death. Thus, neuronal cells preferentially expressing the GluR4 subunit of AMPA receptors are particularly vulnerable to AMPA-induced excitotoxicity.

Within minutes of neurotransmitter release, the expression of a family of genes termed IEGs is induced in the postsynaptic neuron. IEGs are genes that are responsive to transsynaptic stimulation and membrane electrical activity in neuronal cells. Transcription of these genes occurs rapidly and transiently within minutes of stimulation. Many IEGs encode transcription factors that then induce subsequent waves of delayed-response gene expression. These delayed-response genes encode proteins that are likely to be determinants of neuronal plasticity. These proteins may include neurotransmitter-synthesizing enzymes and neurotransmitter receptors, as well as structural components of the synapse. The prototypic IEG, c-fos, has been reported to be both rapidly and transiently transcribed in response to a variety of neurotransmitters that trigger Ca^{2+} influx in in vitro cell culture systems [201, 202]. The c-fos gene encodes the transcription factor c-Fos, which forms a heterodimer with members of the Jun family of transcription factors via a leucine zipper, forming the transcription factor complex AP-1. Griffiths et al. [203, 204] proposed that the assessment of c-fos mRNA expression levels could be used as a specific indicator of excitotoxicity.

Multiple mechanisms lead to excitotoxicity in neurological disorders

Glutamate receptor-mediated excitotoxicity is closely associated with neurochemical and neuropathological changes occurring in acute neural damage (stroke, spinal cord trauma, and head injury) and neurodegenerative diseases such as AD, PD, Huntington's disease (HD), ALS, Creutzfeldt-Jakob disease, Guam-type ALS/Parkinson dementia (ALS/PDC), and multiple sclerosis (MS). In the past decades, our understanding of the biochemistry, molecular biology, and neurophysiology of the glutamate receptors has exploded. It is becoming increasingly evident that excitotoxicity is involved in pathogenesis of many neurological disorders. The underlying mechanisms by which disease conditions contribute to excitotoxicity appear to be due to dysregulation of glutamate levels and glutamate receptor activity.

Disease conditions associated with hyperactivity of glutamate receptors

Some investigators find that overstimulation of NMDA or AMPA-type glutamate receptors can induce apoptosis in

striatal projection neurons in vitro and in vivo [205, 206]. Intrastriatal infusion of KA can induce excitotoxic lesions in striatum, and has become a well-established chemical model of HD [207]. In HD model, changes in kynurenine metabolism [208] and hyperactivity of glutamate receptors [209] were reported. Schiefer et al. [210] suggested that inhibition of glutamate neurotransmission via specific interaction with mGluRs might be important for both inhibition of disease progression as well as early symptomatic treatment in HD.

Altered NMDAR function has been reported in corticostriatal synapses, and NMDAR mediated current and/or toxicity have been found to be potentiated in striatal neurons from several HD mouse models as well as heterologous cells expressing the mutant huntingtin (mhtt) protein [211]. Several possible mechanisms may allow mhtt to modulate NMDAR function at the receptor level and may contribute to excitotoxicity in HD [212]. Mhtt may modulate NMDARs via intermediate interacting proteins. In a heterologous system, mhtt expression increased Src-mediated tyrosine phosphorylation of NMDARs, an effect enhanced by expression of postsynaptic density 95 (PSD-95) [213]. Other mhtt-mediated alterations of NMDAR phosphorylation were documented in N171-82Q mouse models of HD: down-regulation of PSD-95 expression and of the dopamine D1 receptor pathway that normally acts via protein kinase A activation to phosphorylate Ser897 of NR1 and increase NMDAR activity [214]. Finally, while htt indirectly interacts with NMDARs via PSD-95, mhtt has a reduced ability to interact with PSD-95, increasing the vulnerability of neurons to glutamate-mediated excitotoxicity [213].

Disease conditions associated with secondary excitotoxicity

Mitochondrial dysfunction has been found in HD and other types of neurodegenerative diseases [215–217]. Glutamate-mediated neuronal death is highly influenced by the energy state of the cells, and even physiological concentrations of glutamate become toxic during energy failure [218], causing cell death through a process known as secondary excitotoxicity. The secondary excitotoxicity is refered to cellular injury by glutamate is triggered by disturbances in neuronal energy status, which causes substantial decreases in membrane potential. The NMDA receptor channel is normally blocked by Mg^{2+} ion in a voltage-dependent manner, and this ion is extruded to the extracellular medium when the plasma membrane is depolarized. The resting membrane potential, sustained through the activity of the Na^+/K^+ ATPases, will collapse during ATP-limiting conditions such as impaired glycolytic or mitochondrial metabolism, depolarizing the plasma membrane and causing the extrusion of Mg^{2+} ions and the activation of NMDA receptors by ambient glutamate. Previous studies have shown that neuronal death induced by the accumulation of glutamate and aspartate after inhibition of glutamate transporters is facilitated in the striatum of animals previously treated with the mitochondrial toxin 3-NP, an irreversible inhibitor of complex II of the mitochondrial electron transport chain [219]. Similarly, inhibition of the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), by iodoacetate (IOA) facilitates neuronal damage induced by glutamate transport inhibition or by the intrastriatal administration of glutamate [220]. Mechanisms associated with the increased vulnerability to glutamate toxicity have not been completely elucidated, but recent studies indicate that IOA treatment induces a decrease in the content of glutamate transporter GLT-1 and an increase in the protein and phosphorylation levels of the NR2B subunit of NMDA receptors [221]. Studies have shown that energy substrates such as pyruvate, acetoacetate (AcAc), and beta-hydroxybutyrate (BHB) reduce excitotoxic lesions induced by the intracerebral administration of glutamate or glutamate uptake inhibitors [220, 222]. Energy substrates potentially have the ability to treat excitotoxic neuronal death.

In other conditions, synaptic glutamate concentration can reach toxic levels. Glutamate transporters remove the excitatory neurotransmitter glutamate from the extracellular space after neurotransmission is complete, by taking glutamate up into neurons and glia cells. As thermodynamic machines, these transporters can also run in reverse, releasing glutamate into the extracellular space [223]. Glutamate transport by the plasma membrane transporters is reversible. While the conventional transport direction is inward under physiological conditions, glutamate can also be transported in the outward direction when extracellular $[Na^+]$/intracellular $[K^+]$ decrease and/or intracellular $[Na^+]$/extracellular $[K^+]$ increase [224, 225]. Due to the electrogenicity of glutamate transport, membrane depolarization will also result in a reversal of the transport direction because the driving force for uptake decreases under depolarized conditions. Glutamate transport in the outward direction was also termed reversed transport, to indicate the reversal from the conventional transport direction. Since glutamate is excitotoxic, this transporter-mediated release is detrimental to the health of neurons and axons, and thus, may contribute to CNS disorders [226, 227].

Disease conditions associated with alterations in excitatory amino acid transporters

A family of sodium-dependent excitatory amino acid transporters (EAATs) is of prominent importance for glutamate uptake and for regulating glutamate homeostasis in

the CNS [228, 229]. Thus, EAATs are considered to be a critical buffer against excitotoxicity in CNS disorders. To date, five high-affinity EAATs have been cloned from human and animal tissues, and they are identified as excitatory amino acid transporters 1–5 (EAAT1–5). According to previous studies, EAAT1 and EAAT2 are predominantly expressed in glial cells, EAAT3 and EAAT4 are typically present only in neurons [230, 231], and EAAT5 is located in retinal ganglion cells [232]. A study has shown that astrocytes also express EAAT4 [233]. EAATs are considered to contribute to prevention of excitotoxicity by promoting glutamate uptake.

Many of the original inhibitors of glutamate transporters are substrates of the transporters, and are often referred to as 'substrate-inhibitors' [234, 235]. As the name implies, these compounds are translocated by the transporters, much like glutamate or aspartate. In a cell culture model, one of the more selective substrate-inhibitor trans-pyrrolidine-2,4-dicarboxylate (t-PDC) potentiates glutamate toxicity and increases the sensitivity of neurons to OGD [236]. Similarly, the substrate inhibitor t-PDC causes both NMDA-dependent neurotoxicity and NMDA-independent gliotoxicity in hippocampal mixed cultures [237]. The mechanism of t-PDC-induced gliotoxicity remains unclear, but it is also independent of oxidative stress and glutathione deficiency. As mentioned above, transportable inhibitors have the potential to affect intracellular targets, which is one possible explanation accounting for gliotoxicity. On the other hand, blockade of glutamate transporters by the non-transportable glutamate uptake inhibitor threo-b-benzyloxyaspartate (TBOA) is neurotoxic through activation of NMDA receptors but is not toxic to glia [237]. In a series of experiments, Bonde and colleagues have shown that blocking glutamate transporters with TBOA under normal conditions in rat hippocampal slice cultures results in marked necrotic neurodegeneration, presumably due to increased glutamate in the synaptic cleft, as the effect is blocked by glutamate receptor antagonists [238]. In addition, TBOA exacerbates ischemia in rat hippocampus [239].

In cultures, glutamate uptake into glia has a dramatic effect on the sensitivity of neurons to excitotoxic insults [236]. With the cloning of the transporters, several additional tools became available to manipulate glutamate transporter activity. Using anti-sense knockdown, Rothstein and his colleagues demonstrated that impaired glutamate transporter expression was associated with neurodegeneration in normal animals [240]. In addition, mice deleted of GLT-1 (also called EAAT2), display markedly diminished transport activity, seizures, and increased sensitivity to neurotoxicity [241], convincingly demonstrating that impaired glutamate transport can cause neurodegeneration in an otherwise normal setting.

Studies have demonstrated that activated microglia are a significant source of redundant extracellular glutamate that induces excitotoxic neuronal death [242–244], and thus the regulation of such microglial glutamate may be a key therapeutic strategy against excitotoxicity-driven neurological diseases. Glial activation is a neuropathological hallmark in various neurological disorders [242, 245]. It remains undetermined whether glial activation is neuroprotective or neurotoxic. Activated microglia produce glutamate via the upregulation of glutaminase, then release this glutamate from the connexin32 gap junction hemichannel, and thereby induce excitotoxic neuronal death [246]. A study has shown that activated microglia act on glutamate transporters in oligodendrocytes, leading to a net increase in extracellular glutamate and subsequent oligodendrocyte death [247]. Neurons express fewer EAATs compared to glial cells [248]. Activated microglia may also downregulate or dysregulate astrocytic EAATs, which may also contribute to neurodegeneration in various neurological diseases.

Over the last decade, it has become clear that many neurodegenerative disorders are associated with a change in localization and/or expression of some of the subtypes of these transporters. Alterations in glutamate transporters have been reported for several neurodegenerative disorders including ALS [249], HD [250, 251], PD [252], and AD [253, 254]. This would suggest that therapies directed toward enhancing transporter expression might be beneficial. However, there is also evidence that glutamate transporters might increase the susceptibility of neuronal tissue to insults causing collapse of the normal cellular electrochemical gradients, such as ischemic/hypoxic insult in acute cerebral stroke. It is not clear in most cases whether glutamate dysfunction contributes to pathogenesis, or results from the disease pathology. It is important to determine whether the onset of neurodegeneration precedes or follows glutamate transporter alterations. In any case, decreases in transporter expression could contribute to ongoing pathology by making the tissue more vulnerable to excitotoxicity.

Enhanced excitotoxicity by other signaling molecules

Endogenous compounds able to modulate glutamatergic transmission may interfere with glutamate-induced cell death. Neurotensin (NT) is a 13 amino acid neuropeptide that is implicated in the regulation of luteinizing hormone and prolactin release and has significant interactions with the dopaminergic system. In view of the enhancing effects of NT on glutamate transmission and glutamate-induced neurotoxicity, this peptide may play a relevant role in reinforcing the effects exerted by glutamate on a variety of CNS functions and pathologies, in particular on

glutamate-mediated excitotoxicity. NT immunoreactive cell bodies and terminal systems and their receptors are found in many parts of the brain and interact, preferentially, with the mesolimbic, mesocortical and nigrostriatal dopamine (DA) neurons [255–257]. However, the important role of central NT receptor mechanisms played in modulation of glutamate transmission has not been fully understood [258]. The probable reason is that it has only recently become clear that NT enhances glutamate excitotoxicity in DA neurons and that NT receptors are involved in NMDA induced excitotoxicity through the work of the Tanganelli et al. [259, 260]. NT may be involved in the degeneration of dopaminergic mesencephalic neurons and cortical neurons by enhancing glutamate signaling, leading to excitotoxicity, most likely via a rise of intracellular calcium and/or to an amplification of the NMDA-mediated glutamate signalling. Morphological and biochemical findings obtained in primary cultures of rat cortical neurons and rat mesencephalic dopaminergic neurons [259, 260] strengthen the evidence of an involvement of NT in neurodegenerative processes.

Cyclooxygenase (COX) has two well-studied isoforms, called COX-1 and COX-2. Studies demonstrate that systemic treatment of a commercially available and clinically useful nonselective COX inhibitor, naproxen, ameliorates hippocampal and parenchymal cell death and edema formation mediated by excessive activation of neuronal NMDA receptors in vivo [261]. Studies also demonstrate similar and significant neuroprotection by the COX-2 selective inhibitor rofecoxib (p.o.) in the same in vivo excitotoxic model, supporting the notion that the cell death occurs predominantly via a COX-2-dependent mechanism. Animals overexpressing COX-2, as well as cells derived from said animals, are more susceptible to injury induced by kainate and glutamate [262]. Selective inhibition of COX-2 effectively ameliorates cortical brain damage caused via direct intracortical injection of NMDA [263] and hippocampal oxidative damage following intraperitoneal injection of kainate [264]. Glutamate receptor-mediated injury to cortical, hippocampal, and cerebellar granule cell neurons in vitro is also reduced when COX-2 is pharmacologically inhibited [265, 266]. In addition, COX-2 expression is increased in brains from animals subjected to experimental manipulations mimicking neurological diseases known to have an excitotoxic component. Importantly, up-regulation of COX-2 is reported to occur in neurons and non-neuronal cells in human brains following a lethal cerebral ischemic insult [267], in AD brains [268], in postmortem PD specimens [269], and in spinal cord [270], cortex, and hippocampus of ALS patients [271], indicating that these experimental observations may have direct relevance to human pathology. Pharmacological inhibition of COX-2 or use of COX-2 null mutant animals in these same models has, in most cases, proven beneficial [263, 272, 273].

Conclusions

The signaling pathways and the roles of excitotoxicity have been studied for about half of century. However, we still have limited knowledge on the role of excitotoxicity in CNS neurons and the molecular mechanisms underlying its actions. In particular, its critical roles in neuronal death and underlying molecular mechanisms need to be carefully evaluated in relation to human neurological diseases (Fig. 1).

The involvement of excitotoxicity in human diseases certainly establishes it as a potential target for therapy. However, with the exception of memantine [274], human clinical trials using NMDA receptor antagonists have proven to be disappointing. The reasons for these failures may be diverse and could depend on whether the initial injury develops slowly over time or is rapidly initiated. In the latter case, it is likely that compounds that prevent excitotoxic neuronal injury, after initial receptor binding of glutamate has occurred, may actually be more clinically practical. For example, studies have demonstrated significant neuroprotection by COX inhibitor naproxen and COX-2 inhibitor rofecoxib in in vivo excitotoxic models [261, 275]. The nother potential advantage is that targeting post-receptor signal pathways may avoid excessive blocade of excitotary neurotransmission. The benzothiazole drug riluzole has a number of pharmacological effects that contribute to neuroprotection in experimental paradigms of neurodegenerative diseases including anti-excitotoxic activity, blocking of voltage dependent sodium-channels, free-radical scavenging, anti-apoptotic and neurotrophic effects and inhibition of protein aggregation [276, 277]. Riluzole (up to 200 mg daily) is well tolerated and prolongs survival in ALS [278]. The combined treatment of riluzole and the histone deacetylase inhibitor, sodium phenylbutyrate, significantly extended survival and improved both the clinical and neuropathological phenotypes in G93A transgenic ALS mice beyond either agent alone [279]. Thus far, some potential agents, i.e., anti-glutamatergic drugs, anti-oxidants, enhancers of mitochondrial functions, anti-COX-2 drugs, anti-inflammatory agents, and therapeutic implications of deep brain stimulation, cell transplantation, stem cells therapy, gene therapy, were shown to modify disease progression in human neurodegenerative disorders. Further investigation of upstream signals controlling excitotoxicity, and which cause hyperactivity of glutamate receptors under disease conditions may provide new insights on the mechanisms contributing to neurodegenerative diseases in humans, thereby unveiling new strategies for therapy.

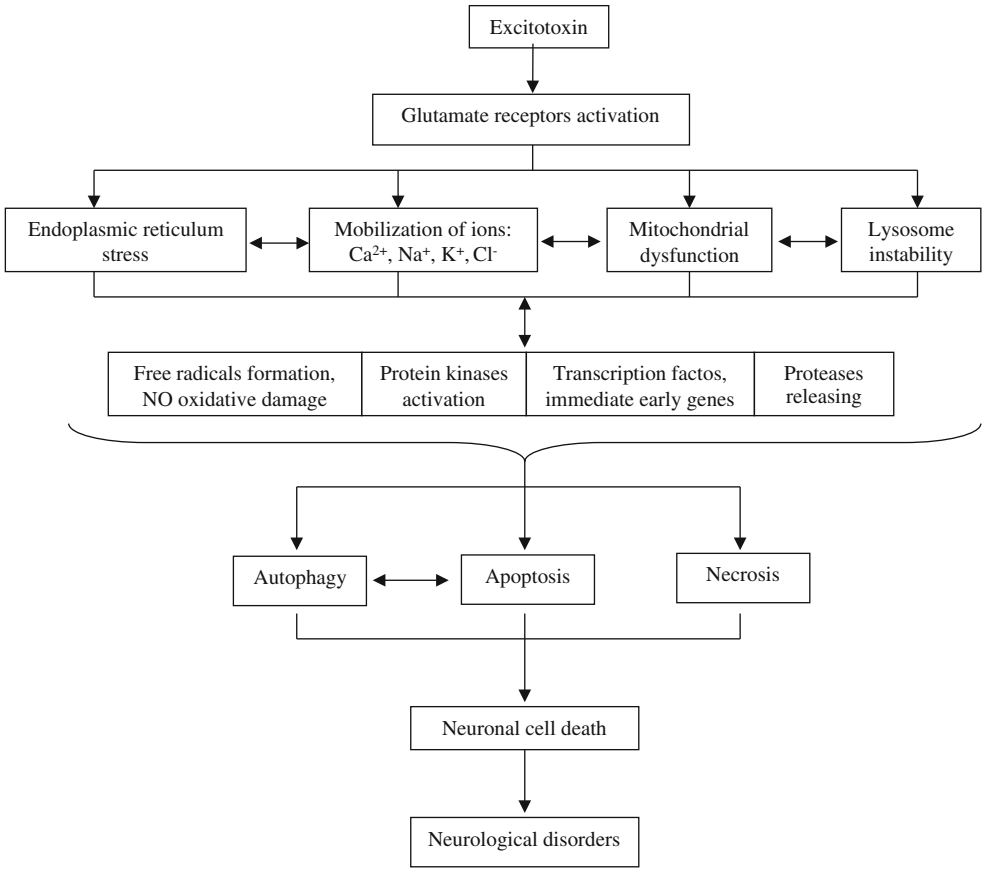

Fig. 1 Mechanisms of excitotoxicity in neurological disorders

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