Review article

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The role of excitotoxicity in neurodegeneration

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Abstract

A body of evidence suggests that the mechanisms of excitotoxic neuronal damage evoked by excessive or prolonged activation of the excitatory amino acid receptors may be involved in pathogenesis of brain damage in acute insults and in chronic neurodegenerative diseases. In this review we briefly discuss several selected mechanisms of the excitotoxicity, focusing attention on the role of ionotropic glutamate receptors, calcium transients and calcium-mediated cell injury. In the second part of this paper we provide information on elements of excitotoxicity in brain diseases.

Key words: *calcium, excitatory amino acid receptors, neurodegeneration, NO, oxidative stress.*

Introduction

The hypothesis of excitotoxicity which has been initially formulated by Olney [143], explains neurotoxic effects of glutamate by excessive neuronal excitation. According to this hypothesis excessive stimulation of the glutamate receptors by increased concentrations of their agonist, or their prolonged activation by moderately elevated concentrations of the excitatory amino acids, leads to neuronal hyperactivation and damage. The hypothesis was based on early findings that glutamate induces neurotoxicity both *in vitro* and *in vivo* [120]. They were followed by discovery that glutamate and some other amino acids cause neuronal depolarization and excitation [40,41]. Then the role of glutamate has been recognized as the main excitatory neurotransmitter in the mammalian central nervous system, acting on specific receptors [38,63,206]. A significant progress in understanding of the mechanism of excitotoxicity has been made with a discovery that the excitotoxic neuronal injury is mainly mediated by a specific subclass of the ionotropic glutamate receptors selective to the agonist N-methyl-D-aspartate (NMDA), which are highly permeable to calcium [34,163,164], and that calcium and oxidative stress play an important role in excitotoxicity [21,33,35,36,66]. Understanding of the hypothesis of excitotoxicity requires recalling of the basic information about the excitatory neurotransmission and calcium homeostasis and on the conditions leading to excessive activation of the glutamatergic system and calcium imbalance which may result in neurodegeneration. Oxidative stress, which is inherent in excitotoxicity, is the subject of a separate paper of this issue.

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Glutamatergic neurotransmission and its disturbances

Glutamate is involved both in the rapid excitatory transmission and in the slowly developing plastic changes connected with learning, memory and neuronal development [1,51]. Normal functioning of the excitatory neurotransmission depends on maintenance of the homeostasis based on regulation of glutamate release from the vascular pool in the presynaptic nerve endings, its uptake by the surrounding astroglia, and on the receptor sensitivity to glutamate. Therefore the metabolic pool of glutamate should be kept away from the glutamate receptors. However, under pathological conditions glutamate from both, vascular and metabolic pools may be released to the extracellular space as a result of hyperexcitation, cell injury or energy imbalance, by the leakage from the cytoplasm or reversal of the activity of glutamate transporters normally taking glutamate into the cell. Moreover the excitotoxic neuronal injury may result from the toxic effect of the exogenous agonists of glutamate receptors or from dysfunction of the receptors dependent on their aberrant subunit composition or disturbed energy metabolism of neurons.

Excitatory amino acid receptors, ionotropic and metabotropic

Glutamate is a primary excitatory neurotransmitter in the brain, acting through two major classes of receptors: glutamate-gated ion channels (ionotropic glutamate receptors, iGluRs) such as NMDA, AMPA and kainite and G protein-coupled metabotropic receptors (mGluRs) [147,175]. Until now eight mGluRs subtypes (mGluR1-8) have been identified and classified into group I mGluRs (mGluR1 and mGluR5), group II mGluRs (mGluR2 and mGluR3) and group III (mGluR4 and mGluR6-8) [175].

NMDA receptors permeable to Ca^{2+}, Na^{+} and K^{+} consist of tetrameric, heteromeric subunit assemblies with different physiological and pharmacological properties and are differentially distributed throughout the CNS [43,84,147,179]. Voltage dependent blockade by Mg^{2+} ions is a characteristic feature of NMDA receptors. Voltage dependent means that in a course of depolarisation the degree of block decreases. This feature makes NMDA receptor ideally suited for mediating neuronal plasticity (change of quantitative into qualitative signal), but under certain conditions like prolonged depolarisation in ischemia block by Mg^{2+} may be completely removed leading to massive influx of Ca^{2+}. Three major subunit families of NMDA receptor, designated NR1, NR2 and NR3 have been cloned. Functional receptors in the mammalian CNS are most probably formed by combination of NR1 and NR2 subunits containing the glycine and glutamate recognition sites, respectively [83,101]. NR3 subunits seem to inhibit receptor function and are expressed at higher levels during development [46].

Alternative splicing generates eight isoforms for the NR1 subfamily [224] which description is beyond the scope of the current review. NMDA receptor channels formed by various combinations of NR1 and NR2 subunits differ in gating properties, magnesium sensitivity and pharmacological profile (e.g. see [147]). Only the heteromeric assembly of NR1 and NR2B subunits, for instance, are potentiated in a glycine-independent manner by the polyamines spermine and spermidine and are selectively blocked by ifenprodil and related compounds. *in situ* hybridisation indicates overlapping but different expression for NR2 mRNA e.g. NR2A mRNA is distributed ubiquitously like NR1 with highest densities occurring in hippocampal regions and NR2B is expressed predominantly in the forebrain but not in the cerebellum where NR2C predominates.

Glycine is a co-agonist at NMDA receptors' glycineB site meaning that its presence is a prerequisite for channel activation by glutamate or NMDA (for review see [43]). Physiological concentrations of glycine reduce one form of relatively rapid NMDA receptor desensitization. Recently it has been suggested that D-Serine may be more important than glycine as an endogenous co-agonist at NMDA receptors in the telencephalon and developing cerebellum [81].

The endogenous polyamines spermine and spermidine have multiple effects on the activity of NMDA receptors. These include an increase in the magnitude of NMDA-induced whole-cell currents seen in the presence of saturating concentrations of glycine, an increase in glycine affinity, a decrease in glutamate affinity, and voltage-dependent inhibition at higher concentrations [212]. The stimulatory effect is also controlled by NR2 subunits in heteromeric complexes – it is observed at heteromeric NR1A/NR2B receptors but not at heteromeric NR1A/NR2A or NR1A/NR2C receptors [213]. Glycine-

dependent stimulation is mediated *via* an increase in glycine affinity and probably involves a second binding site as it is also seen at NR1A/NR2A receptors [213]. The voltage-dependent inhibitory effect of higher concentrations of spermine is similar for NR1A/NR2A and NR1A/NR2B receptors but is apparently absent at NR1A/NR2C receptors and is mediated at the Mg^{2+} channel site. Ifenprodil and its analogue eliprodil were found to block NMDA receptors in a spermine-sensitive manner and were proposed to be polyamine antagonists [29].

AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid) receptors are involved in mediating most forms of fast glutamatergic neurotransmission. There are four known subunits GluR1 to GluR4 – sometimes termed GluRA to GluRD – which are widely, but differentially, distributed throughout the CNS [84]. The types of subunits forming these receptors determine their biophysical properties and pharmacological sensitivity. Two alternative splice variants of GluR1 to GluR4 subunits designated as 'flip' and 'flop' have been shown to differ in their expression throughout the brain and during development and to impart different pharmacological properties [186]. Further non-NMDA ionotropic receptor subunits are designated GluR5, GluR6, KA1 and KA2 and normally form receptor assemblies previously designated as high affinity kainate receptors. Kainate receptors were previously thought to be largely presynaptic, for example they are expressed in the dorsal root ganglia. More recent evidence indicates that they are also postsynaptically involved in neurotransmission in some pathways [112].

AMPA is selective for GluR1 to GluR5 containing receptors and induces strong desensitization. Cyclothiazide is a selective positive modulator of AMPA receptors whereas concavalin-A is much more effective on kainate preferring receptors [148]. 2,3-benzodiazepines such as GYKI 52466 are non-competitive AMPA receptor antagonists and are much less active at kainate receptors [18]. The GluR2 subunit imparts particular properties to heteromeric AMPA receptors. Receptors lacking this subunit show relatively high Ca^{2+} permeability [28], and are sensitive to block by toxins such as Joro Spider toxin, Philanthotoxin-343 and Argiotoxin-636. The GluR2 subunit may be present in edited and unedited forms and the presence of a positively charged arginine (R) residue in the position 586 (channel wall) of edited receptors renders them Ca^{2+} impermeable [28]. Unedited homomeric GluR2 receptors are most prominent at early stages of development and show a much more limited distribution in the adult brain. There are some indications that Ca^{2+}-permeable AMPA receptors are expressed at higher levels under certain pathological conditions such as global ischemia [151].

A different type of role in synaptic transmission is connected with metabotropic glutamate receptors (mGlu receptors) (for reviews see: [90,153,188]). Since they produce rather slow, enzymatic response through second messenger systems, they do play a rather modulatory role. So far, 8 subtypes have been described which are divided into three families based on the type of second messenger coupling and amino acids homology. Group I mGluRs are positively coupled to PLC through Gs protein and their activation leads to an increase in PI hydrolysis i.e. production of DAG and IP3 which mediate various secondary effects e.g. release of Ca^{2+} from ER stores in case of IP3. There are two subtypes of mGluRs belonging to this group i.e. mGluR 1 and 5 which have different neuronal distribution and show quite different pharmacology. Group II mGluRs are negatively coupled to adenylate cyclase through Gi protein i.e. their activation inhibits cAMP production. Two subtypes belonging to this group i.e. mGluR 2 and 3 are hardly distinguishable by presently available pharmacological tools but show different functions e.g. majority of mGluR3 is located on glial cells while mGluR2 are mainly located presynaptically on neurons regulating release of transmitters. The least explored group III of mGluRs contains 4 subtypes i.e. mGluR4, 6, 7, 8. All of them are as group II coupled negatively to adenylate cyclase and show diverse distribution and function. For example, mGluR6 are mainly located in the retina, mGluR4 are concentrated on the striatum and mGluR7 in limbic structures. Pharmacological tools allowing differentiating these subtypes are just starting to emerge.

Interestingly, the function of mGluRs can be not only affected by agents acting at the primary transmitter site (so called orthosteric ligands), but also by allosteric modulation within the transmembrane domain. Agents acting at this site may produce a wide range of effects ranging from allosteric inhibition to positive modulation and even activation of transduction mechanisms in some cases. This site has been described for most of

mGluRs and is noncompetitive in nature e.g. increasing concentrations of an agonist cannot overcome inhibition by an allosteric antagonist.

Physiological and pathological aspects of glutamatergic transmission

Apart from the receptor desensitization, glutamatergic neurotransmission is terminated by the removal of glutamate from the synaptic cleft by several types of transporters into the astroglia and neurons. This process depends on ATP production, activity of the Na^+, K^+ -ATPase and consequently on the physiological segregation of sodium mainly outside the cells. Glutamate uptake is mediated by specific transporters [42]. Five of them (EAAT1 – EAAT5) expressed in the human CNS have been cloned. Isoforms EAAT1 and EAAT2, which play the main role in glutamate transport in the brain, are expressed in astro- and microglia. EAAT3 isoform was identified in the cerebral neurons, while EAAT4 was found in the cerebellar neurons and EAAT5 in the retina. Disturbances in the activity of these proteins or reversal of glutamate transport evoked by energy deficiency or by disruption of sodium gradient may lead to retention of glutamate in the synaptic cleft and to prolonged neurotoxicity [166].

Sustained stimulation of glutamate receptors leads to neuronal depolarization and ionic fluxes triggering subsequent mechanisms of neuronal damage [45, 142]. Initial processes include influx of Na^+, Cl- and water to neurons mediated by non-NMDA (AMPA/KA) receptors and partially by voltage-dependent sodium channels. This is responsible for swelling of the postsynaptic structures, dendrites, neuronal cell bodies and of the astrocytes, spearing presynaptic axons [92,165]. However, elimination of Na^+ and Cl- from the medium which prevents cellular swelling does not preclude neuronal death [32]. Glutamate-evoked neurodegeneration is mainly mediated by the NMDA receptors and influx of Ca^{2+} to neurons [54]. AMPA receptors are indirectly involved in this effect, as they mediate depolarization which releases a voltage-dependent inhibition of the NMDA channel by Mg^{2+}. This results in neuronal calcium overload, disturbances in signal transduction and neurodegeneration. An additional factor, which prolongs and exacerbates the excitotoxic insult is the secondary increase in the extracellular glutamate concentration [52]. Glutamate released to the extracellular compartment in the primary focus of the excitotoxic insult (mechanical injury, stroke) may diffuse and cause secondary excessive depolarization of neurons. Such a positive feedback known as a glutamatergic loop has been considered the main factor responsible for propagation of the necrotic zone around the ischemic focus in stroke [11].

The model of excitotoxicity evoked by increased levels of glutamate has been challenged with the concept of slow excitotoxicity, in which neurodegeneration develops in spite of normal extracellular concentration of glutamate and in the absence of exogenous excitotoxins [139,166,220]. It was based on findings that conditions leading to reduced ATP production as hypoxia, deficit of supply or utilization of glucose, intoxication with ouabain, cyanate or some pesticides interfering with the oxidative chain, lead to increased sensitivity of neurons to glutamate excitotoxicity and to release of Mg^{2+} block of the NMDA receptors [139,220]. Mitochondria, which are responsible for aerobic ATP production in cells play the key role in that hypothesis. Mitochondrial alterations evoked by inhibition of the oxidative chain or by the oxidative stress may inhibit ATP production and induce neurodegeneration implicating excitotoxic mechanisms [8,127,196,199]. ATP deficiency slows down the activity of Na^+, K^+-ATPase responsible for maintenance of the plasma membrane potential. This increases neuronal excitability and probability of activation of voltage-dependent ionic channels and NMDA receptors [52,161], and inhibits the sodium-dependent transporters [96,162]. The hypothesis of slow excitotoxicity might explain several mechanisms of chronic neurodegenerative disorders [7,200].

The role in neurodegeneration of the additional factors modulating excitatory neurotransmission and consequently excitotoxicity has been suggested. Desensitization, phosphorylation/dephosphorylation and inactivation processes, that are calcium dependent, control neurotransmission through NMDA and AMPA/KA receptors and their disturbances can lead to the increase in glutamate-evoked neurotoxicity [50]. A number of studies have demonstrated that while agonists of group I mGluRs may be neurotoxic, several group II and III mGluR agonists were found to be neuroprotective [6,20]. Endogenous or exogenous ligands of the excitatory amino acid receptors may modulate excitatory neurotransmission. Apart from recognized excitatory

amino acid neurotransmitters glutamate and aspartate, also other endogenous substances which are agonist of glutamate receptors, have been implicated in excitotoxic mechanisms mediated by NMDA receptors or group I mGluRs. Quisqualate and several sulfhydryl amino acids including homocysteine represent these substances. Homocysteine which has been associated with neurodegeneration in Alzheimer's disease [159] was shown to induce *in vitro* neurotoxicity mediated by NMDA receptors [117]. The results of more recent studies demonstrated involvement of both NMDA and group I mGluRs in homocysteine-evoked excitotoxicity [222]. Kynurenic acid, synthesized in astrocytes, is the only known endogenous antagonist of the NMDA and AMPA/KA receptors. It has been suggested that disturbances in its biosynthesis from tryptophan might take part in development of neurodegenerative processes [178]. Hypomagnesemia resulting in a decrease in brain Mg^{2+} concentration may result in the release of the voltage-dependent block of the NMDA receptors and in the induction of excitotoxicity [56]. The other factor which may potentiate excitotoxicity is accumulation in the brain of Alzheimer's patients of the β-amyloid peptide (βAP), the product of altered metabolism of β-amyloid precursor peptide (βAPP). Aggregated βAP contributes to a loss of neuronal calcium homeostasis by disrupting the function of membrane proteins involved in the regulation of intracellular calcium concentration [128]. βAP activates voltage operated calcium channels (VOCCs) type L and N, and also increases activity of NMDA receptors.

The role of Ca^{2+} ions in excitotoxicity

Normal intracellular Ca^{2+} concentration below 10^{-7} M contrasts with its levels in the range of 10^{-3} M and 10^{-4} M in the extracellular space and within ER, respectively [152,156]. Under normal conditions the influx of calcium to the neuronal cytosol from the extracellular space requires activation of the selective voltage-operated calcium channels (VSCCs) of different subtypes (e.g. L, N and P channels) [203] or of the receptor-operated channels (ROCs), mainly glutamatergic (see above), but also subtypes of cholinergic and serotonergic receptors. Calcium release from the ER stores is mediated by two types on intracellular calcium receptors/channels, sensitive either to inositol trisphosphate (IP3) or ryanodine [182,183]. Release of calcium by these receptors is potentiated by low-moderate increases in the intracellular calcium concentration. This phenomenon is called calcium-induced calcium release (CICR). High Ca^{2+} concentrations inhibit this effect. The cellular system of calcium buffering inside neurons is complex. It includes calcium binding proteins from the EF-hand family [154]. Mitochondria and endoplasmic reticulum accumulating Ca^{2+} by uniport powered by the mitochondrial potential and the sarcoplasmic/endoplasmic Ca^{2+}-ATPase (SERCA) transporting calcium ions to the lumen of the ER, respectively, also constitute this system [97,136,137]. The mechanism of Ca^{2+} release from the cells encompasses a plasma membrane transporting Ca^{2+}-ATPase and sodium/calcium exchange [16,133,192].

The efficient system of calcium homeostasis in neurons enables Ca^{2+} ions to play a fundamental role in the control of neuronal excitability, the coupling of depolarization with neurotransmitter release in the presynaptic endings, and signal transduction postsynaptically [13,68]. In addition, calcium is involved in the induction of the retrograde transynaptic signaling mediated by second messengers such as NO and eicosanoids, which may play a role in synaptic plasticity of glutamatergic neurotransmission [45]. Calcium ions bind to intracellular proteins acting as calcium sensors and mediators of calcium signaling [154]. Complex of Ca^{2+} with calmodulin binds to several enzymes like protein kinases, phosphatases and adenylate cyclases [68,156]. The signaling role of calcium is not limited only to short term regulation of the synaptic activity by the posttranslational modification of the existing synaptic proteins. Calcium signal is transmitted to the nuclei and induces changes in gene expression. This function of calcium seems to be basic for long lasting changes in the activity of neurons in cellular mechanisms of memory. Stimulation of different pathways of calcium signaling and resulting changes in gene expression depends on the way of calcium influx to neurons [13,68].

The mechanisms of disturbances in calcium homeostasis in neurotoxicity are not entirely clear. The excessive influx of Ca^{2+} to neurons in the excitotoxicity was shown to be mediated mainly by the slowly desensitizing NMDA receptors/channels which are highly permeable to calcium [34]. Moreover, stimulation of the NMDA receptors may activate additional mechanisms of calcium entry to

neurons [22]. Acute excitotoxicity is accompanied by depolarization and decrease of sodium gradient, which may inhibit or even reverse the transport [17,102]. However, the role of reversed sodium/calcium exchange in excitotoxicty has been still disputed [3,92,93,191,193]. Equally unclear is the role of intracellular Ca^{2+} mobilization in the NMDA receptor-mediated excitotoxicity. Calcium influx throughout the NMDA receptors may activate ryanodine receptors using the mechanism of CICR [103]. Dantrolene, which is an inhibitor of CICR in skeletal muscles, was shown to limit *in vitro* and *in vivo* the release of calcium from neurons stimulated by agonists of the NMDA receptors, and to prevent neurodegeneration [64,110,121,125,134,183]. However, several previous studies demonstrated that neurodegeneration evoked by excitotoxicity is dependent on the presence of calcium in the extracellular compartment [160]. Also the capacity of ryanodine-sensitive calcium stores which are heterogeneously expressed in the brain of various species [170], seems to be rather limited. In addition, there are data pointing to low sensitivity to dantrolene of the isoform of ryanodine receptors mainly expressed in brain, which might suggest that the neuroprotective effect of dantrolene is not related to inhibition of CICR [207,221]. There are conflicting data in the literature concerning the role of disturbances in intracellular calcium buffering in the mechanisms of excitotoxicity e.g. in brain ischemia (reviewed in [168]). It is known that excitotoxicity may lead to inhibition of the calcium transporting ATPases in neurons, plasma membrane Ca^{2+}-ATPase (PMCA) and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) [109,150]. Excitotoxicity-evoked disturbances in the activity of mitochondria and endoplasmic reticulum seem to play a fundamental role in the mechanisms of neurodegeneration, and will be discussed separately.

It has been a matter of discussion whether calcium-mediated neurotoxicity mainly depends on the concentration of calcium ions in the cytosol, or on the total calcium load entering the neurons, and what is the role of cellular localization of disturbances in calcium homeostasis that are critical for neuronal injury. Although excitotoxicity mediated by activation of NMDA receptors may be accompanied by increases in intracellular calcium concentration to even 10 μM [198], it seems that duration of such an imbalance rather than Ca^{2+} level is a critical factor determining neurotoxicity [197]. There is evidence indicating that calcium neurotoxicity depends on the load of calcium that enters neurons upon excitation rather than on the intracellular calcium levels [58,80,119]. It was demonstrated that treatment of neurons with kainate or with high potassium concentrations activating the L type of VOCCs induces much lower neurotoxicity than NMDA applied at concentrations that evoke comparable calcium transients [80,126,172]. These findings support the so called source-specific hypothesis pointing to the role of ways of calcium influx in excitotoxicity [171,197]. These authors point to the role in excitotoxicity of early increases in the intracellular calcium concentration of specific microdomains in the postsynaptic nerve endings in the vicinity of the NMDA receptors, and to the role of PSD-95 protein in NMDA receptor-mediated neurotoxicity [173]. In addition, one should remember that during neuronal development moderate and controlled influx of calcium to neurons *via* VSCCs has trophic protective effects on neurons and prevents apoptosis, while an excessive decrease in the intracellular calcium concentration promotes apoptosis [27,65]. According to the calcium set-point hypothesis neurons at different developmental stages have determined optimal interval of intracellular calcium concentration, while concentrations falling behind these limits induce necrotic or apoptotic neuronal death [104].

In neurons the system of calcium signaling is highly developed and participates in the short- and long-term regulation of the neuronal activity and gene expression [37]. Consequently, almost all the important calcium-mediated intracellular processes were accused of involvement in the mechanisms of calcium-induced neurodegeneration, including excessive activation of lipases, proteases and endonucleases [180], inhibition of protein biosynthesis [150], and disturbances in calcium signaling at specific postsynaptic sites [5]. In fact, phospholipid hydrolysis mediated by both, NMDA receptors and group I mGluRs and also by phospholipases A2 and C has been demonstrated [87,122,123]. A resulting accumulation of arachidonate and its metabolites [124] may significantly influence membrane properties and neuronal functions. Several metabolites of arachidonic acid have neuroactive properties. Excitotoxicity excessively and in an

uncontrolled way activates neuronal calcium-sensitive neutral proteases, calpains [105,169]. This may result in degradation of several important proteins. Cleavage of spectrin leads to damage of the neuronal cytoskeleton. Calpains degrade also the plasma membrane calcium ATPase [205]. Proteolysis should be considered an important interference with the mechanism of intracellular signal transduction.

Several calmodulin-binding proteins, e.g. the calcium and calmodulin-dependent protein kinase II (CaM KII) and MAP2, as well as protein kinase C (PKC) are particularly sensitive to calpain-mediated cleavage evoked by excitotoxicity *in vivo* and *in vitro* [79]. Products of CaM KII degradation reveal a calmodulin- or phospholipids-independent activity, respectively [94,98]. An increase in the intracellular calcium level may inhibit protein biosynthesis by suppressing elongation of the protein chain [214]. However, the mechanism of sustained inhibition by excitotoxicity of protein biosynthesis is not clear. It has been proposed that it may be triggered by an excessive release of calcium from the intracellular stores [150,157]. This may lead to activation of the RNA-dependent protein kinase (PKR) normally involved in antiviral cell protection and in the regulation of cell growth and differentiation. Phosphorylation by PKR of the initiation factor eIF-2α suppresses protein synthesis in cells [150].

The role of oxidative stress and nitric oxide in excitotoxicity

The separate paper of this issue presents the role of oxidative stress in neurodegeneration. Here we only intend to remind investigations focused on the role of oxidative stress in the pathogenesis of excitotoxic neuronal death [57,158]. Oxidative stress refers to the cytotoxic effects of the reactive oxygen species (ROS), particularly superoxide anion (O^{2}-), hydroxy radical (OH), and hydrogen peroxide (H_2O_2) which are generated as byproducts of normal and aberrant metabolic processes utilizing molecular oxygen. In normal conditions ROS are degraded by several enzymes and non-enzymatic factors. However, in pathological condition the balance between production and degradation of ROS is disturbed which leads to accumulation of ROS. Excitotoxicity leading to uncontrolled calcium transients activates several metabolic pathways generating free radicals. Excitotoxicity and activation of calpains leads to proteolytic transformation of xantine dehydrogenase into xantine oxidase. The activity of that enzyme results in accumulation of superoxide anion (O^{2}-) and potentiates oxidative stress [181]. Moreover, production of the free radical is a side effect of oxidative metabolism of arachidonate [31]. Mitochondrial calcium overload induced by excitotoxicity and resulting disturbances in the activity of the respiratory chain leads to enhanced generation of oxygen radicals [138]. Oxygen radicals generated as a result of the excitotoxic insult can attack proteins, nucleic acids, and lipid membranes, thereby disrupting cellular functions and integrity.

Studies of the last decade have implicated nitric oxide (NO) as one of mediators in neurodegenerative disorders and neuronal injures [135]. Nitric oxide, first identified as endothelium-derived relaxing factor (EDRF), is an important neuronal messenger molecule [67]. In the brain three different isoenzymes of NO synthase (NOS) were identified: endothelial (eNOS), inducible (iNOS), and neuronal (nNOS) [185]. nNOS and eNOS each produce low concentrations of NO over a relatively long period of time and are dependent on Ca^{2+}/calmodulin activation, whereas iNO produces high concentration of NO and its activation does not depend on calcium signal. It has been suggested that nNOS and iNOS play an important role in the excitotoxicity. NO produced by nNOS seems to be a main factor in acute excitotoxicity, whereas iNOS, practically absent in healthy brain tissue, is activated in pathological condition and its highest activity was observed 48 hours after brain ischemia. This suggests that iNOs may take part in delayed neuronal death.

The mechanism of NO-mediated neurotoxicity is complex. Conditions of oxidative stress and increased production of superoxide anion result in kinetically favorable side-reaction of low energy (triplet) nitroxyl anion, which is probably produced by iNOS, with O^{2-}. This reaction generates the peroxynitrite radicals ($ONOO^{-}$) that oxidize free thiols in the cytosol and form disulfide linkages, affecting protein functions in the cell [114]. It was suggested that peroxynitrite is one of the main factors in induction of apoptosis in many cells [48]. NO is known as a modulator of neurotransmitters release. In the pathological conditions NO may increase release of glutamate, which leads to overstimulation of NMDA receptors. This may increase the excitotoxic effects of this neurotransmitter, which lead to the neuronal death.

Increased NO concentration and protein nitrosylation may also lead to mitochondrial alterations.

The role of dysfunction of ER and mitochondria in excitotoxicity

Dysfunctions of intracellular calcium stores in the endoplasmic reticulum (ER) and of the mitochondrial calcium buffer are presently considered important neurodegenerative factors. Excessive stimulation of glutamate receptors activates influx of Ca^{2+} throughout NMDA receptors and mobilization of intracellular Ca^{2+} mediated by group I mGluRs and in the mechanisms of CICR. This is an important way of cellular signaling but also a possible mechanism potentiating the pathological calcium signal [182]. Moreover, depletion of ER calcium stores may produce severe ER stress because high intraluminal calcium levels are necessary for folding and processing of newly synthesized proteins [150]. In addition to the inhibition of protein synthesis by ER stress, which has been mentioned above, the role of ER stress in the induction of neuronal apoptosis was suggested [149].

In the conditions of increased intracellular calcium concentration mitochondria accumulate Ca^{2+} in the energy dependent process. On the one hand, this mechanism prevents pathological increases in calcium concentrations in the cytosol, but on the other hand excessive accumulation of Ca^{2+} causes disruption of the mitochondrial electron transport chain, inhibition of oxidative phosphorylation and ATP formation [57,138]. Mitochondrial calcium overload, excessive production of free radicals and deenergization of the mitochondria induce opening in the inner membrane of a large conductance channels known as the mitochondrial permeability transition (MPT) pores. This causes matrix space to expand and can lead to outer membrane rupture and release of caspase-activating proteins like cytochrome c, located within the intermembrane space into the cytosol. The alternative mechanism opens channels in the outer membrane without concomitant swelling of the organelle [75]. Release of the caspase-activating proteins into the cytosol induce cellular death, mostly in the apoptotic way.

Initially it was generally accepted that the excitotoxic neuronal injury is consistent with a necrotic type of cell death. Necrosis is a passive process, characterized by cell and organelle swelling with release of the intracellular contents into the extracellular space. This usually results in inflammatory reactions, vascular damage, edema and injury to the surrounding tissues. Necrosis and apoptosis are two distinct forms of cell death and have different implications for the surrounding tissue. Apoptosis is connected with cell shrinkage, organelle relocalization and compaction, chromatin condensation, and DNA cleavage into large fragments. The whole nucleus may fragment into rounded bodies containing chromatin pieces [4,111]. Recent studies have provided evidence that some subpopulations of neurons submitted to the excitotoxic insult may die *via* apoptosis [4]. It has been shown that although an acute exposure to excitotoxic glutamate concentrations kills neurons rapidly by necrosis, following withdrawal of glutamate, the surviving neurons eventually undergo delayed apoptosis. Similar effects have been observed with other excitotoxic stimuli. Although apoptosis and necrosis are usually regarded as distinct types of cell death, there is increasing evidence that they may represent only the extreme ends of the continuum of biochemical and morphological changes. It was suggested that at least some early stages of the excitotoxicity like an increase in the intracellular Ca^{2+} concentration or synthesis of stress dependent factors like c-Fos may be mutual for both types of cell death [111].

Elements of excitotoxicity in acute insult and in neurodegenerative diseases

Acute CNS insults

Stroke. Microdialysis studies in animals show an increase in extracellular glutamate concentration during experimental ischemia [11, 73]. In man, there is also an increase in the CSF and plasma content of glutamate and glycine in patients with progressive, but not with stable stroke [30]. Because of energy deficit during ischemia, an increase in extracellular glutamate concentration *per se*, is not necessary to evoke damage through activation of glutamate receptors. Simply other factors may increase neuronal vulnerability to physiological levels of glutamate by e.g. a decrease of resting membrane potential or intracellular buffering of Ca^{2+} ions. Apart from glutamate, oxidative stress, inflammatory reactions and break-down of the blood-brain barrier may also play a role [70,184]. Interestingly, in the

four vessel occlusion (4-VO) global 10 min ischemia model in rats there is a post-ischemic decrease in expression of GluR2 AMPA receptor subunit, which makes AMPA receptors more permeable to calcium [151]. In general, AMPA receptor antagonists seem to be more active in global ischemia models [144] while both NMDA and AMPA receptor antagonists show a moderate activity in focal ischemia [107,155]. In post ischemia treatment regimes NMDA receptor antagonists in general show better efficacy in permanent ischemia models [70,71].

Several recent clinical trials with glutamate antagonists have consistently failed to show beneficial effects in stroke. In these studies some CNS related side effects such as agitation, hallucinations, confusion and dizziness were reported for competitive and high affinity uncompetitive NMDA receptor antagonists [47]. This fits well with the fact that neuroprotective effects of most agents in animal models of stroke can only be expected at high doses of NMDA receptor antagonists producing clear side effects, which has often been a severe dose-limiting factor in clinical studies. $Glycine_B$ antagonists initially seemed to be promising since, apart from myorelaxation/ataxia, they are less prone to produce other side effects. However, a recent clinical trial with GV-150526 also failed to show any benefit [108].

The first generation NR2B selective agents such as ifenprodil have been reported to provide neuroprotection without producing side effects [53] but it is not certain that this is due to actions at NMDA receptors. Ro 25-6981 is more selective as a high-affinity antagonist of NMDA receptors containing the NR2B subunit [61]. Ro 25-6981 administered in a 2 mg/kg i.v. bolus followed by 4.4 mg/kg/hr for 6 hrs provided 37% protection in the middle cerebral artery occlusion (MCAo) model in rats without effects on the rotarod [62]. Other NR2B selective compounds such as CP-101,606 and CP-283,097 show structural similarity to ifenprodil however, recently it has been reported that most if not all of these agents produce a prolongation of the QT interval in the cardiac action potential due to blockade of human ether-a-go-go-related gene (HERG) potassium channels [69]. The Na^+ channel blocker and glutamate release inhibitor BW 1003C87 given 10 mg/kg i.v. 15 min post-injury significantly reduced fluid percussion injury induced focal brain edema in the rat [140]. Also other glutamate release inhibitor BW 619C89 administered at 50 mg/kg 30 and 60 min after the onset of 2 hours of transient MCAo ischemia in rats decreased cortical infarction volume assessed 72 hours later, but the effect vanished when the drug was given 5 min after reperfusion [89].

To sum up, it seems that there is no evidence for efficacy of NMDA receptor antagonists in stroke as evidenced by clinical trials. This is somewhat surprising considering their good efficacy in animal models. The failure of clinical trials could be related to heterogeneity of patients, too long a time window for initiation of treatment, too low a dose used or other factors (see [118]). In contrast to NMDA receptor antagonist, some hope remains for AMPA receptor antagonists since it has been hypothesized that AMPA receptors play a more important role in acute excitotoxicity due to increased sensitivity by lowered pH [130]. Likewise, glutamate release inhibitors could be useful. In the authors opinion, it is more likely that rather an agent with several mechanism of action or combination of several agents will find therapeutic use than a single mechanism based treatment.

CNS trauma. Following traumatic brain injury in rats there are clearly perturbations of energy homeostasis and a significant increase of extracellular glutamate, as was shown in the microdialysis experiments [146,219]. There is also evidence for a role of oxidative stress, since transgenic mice over-expressing superoxide dismutase show relative resistance to traumatic brain injury [132] and free radicals are known to cause a secondary increase in glutamate levels by inhibition of uptake [219]. An increase in glutamate content has also been observed in patients using brain microdialysis [88] or CSF sampling [145]. Interestingly, a delayed rise in extracellular levels of glutamate that persisted for several days after traumatic insult has been observed in some patients, and was often connected with a bad prognosis (death) [26].

Pretreatment with either NMDA or AMPA receptor antagonist provides neuroprotection in animal models of brain trauma, however in case of post-treatment only AMPA receptor antagonists seem to be active [12,60]. In contusive spinal trauma model NBQX showed neuroprotective effects both in histological and functional parameters [215,216,217], while NMDA receptor antagonists showed no

persistent beneficial effect on recovery [2,86]. In contrast, in infant rats NMDA receptor antagonists such as (+)MK-801 or CPP are neuroprotective while AMPA antagonists like NBQX are not. The glutamate release inhibitor, riluzole attenuated fluid percussion cortical injury when applied shortly after the insult [202]. In infants subdural hematoma, common causes of mortality associated with child abuse [55] and in a rat model of this insult dextromethorphan and memantine combined with flunarizine were neuroprotective [95]. The NR2B selective agents may offer a more promising profile as indicated by positive effects of first generation NR2B selective agents such as ifenprodil [49]. The NR2B selective agent CP-101,606 was also protective in traumatic brain injury [141] and in subdural haematoma [195] and well tolerated in TBI patients [25]. Clinical trials with the NMDA channel blocker Cerestat were suspended due to lack of effect and safety concerns.

Chronic neurodegenerative diseases

Huntington's disease. The pattern of neuronal loss in the striatum in Huntington's disease is similar to that obtained after excitotoxic lesions in animals [177]. Also striatal neurodegeneration produced by mitochondrial toxins 3-nitropropionic acid (3-NP) and malonate – inhibitors of complex II-III, represents a similar type of damage and is attenuated by lesions of the glutamatergic inputs, the glutamate release inhibitor lamotrigine and/or NMDA receptor antagonists such as MK-801 and memantine [78,106,176]. Hence, it is likely that mitochondrial dysfunction evoked by these toxins triggers a chain of reactions including excitotoxicity. In fact, there are data indicating a deficit of mitochondrial complex II-III activity in the brains of Huntington's patients [24]. However, results of clinical trials of Rochester Huntington's Study Group with Remacemide (NMDA channel blocker +/-Co Q10) in several hundred patients failed to confirm this assumption [91].

Amyotrophic lateral sclerosis (ALS). The pattern of neuronal loss in the spinal cord in ALS patients is similar to that obtained after excitotoxic lesions induced with kainate in animals, which suggests involvement of AMPA receptor [85]. Moreover, the CSF from ALS patients contains an excitotoxic factor that activates AMPA receptors [39]. It has also been reported that there is loss of glutamate uptake (EAAT-2) protein (not mRNA) in the spinal cord and motor cortex [167]. Editing of mRNA for the GluR2 subunit of AMPA receptors is reduced in the ventral grey matter of patients with ALS, which favors higher Ca^{2+} permeability of these channels [190]. However, the majority of clinical trials with glutamate antagonists completed to date have not been encouraging. Dextromethorphan (NMDA channel blocker) showed no benefit [19,74]. Similar negative results were obtained with glutamate release inhibitor Lamotrigine [59]. In contrast, riluzole (glutamate release inhibitor) has been found to increase survival for several months in a clinical trial involving c.a. 1000 patients [10,99]. This agent (Rilutek) has been registered as a neuroprotective treatment for ALS in several countries.

Alzheimer's disease. Over 15 years ago Greenamyre suggested that glutamate might be involved in the pathomechanism of neurodegenerative diseases, like Alzheimer's disease [76]. There are many indirect indications that this might be the case. E.g. in samples from brains of Alzheimer's patients there is a decrease in astroglial glutamate carrier EAA2 in the frontal cortex [113]. It was shown in the *in vitro* study that constituents of senile plaques stimulate microglia to produce an unknown neurotoxin having agonistic properties at NMDA receptors [72]. Moreover, *in vitro* β-amyloid peptide enhances the toxicity of glutamate [23,129] and augments NMDA receptor mediated transmission [218]. For compilation of evidence see: [44].

So far, the moderate affinity, uncompetitive NMDA receptor antagonist memantine is the only substance profiled for neurodegenerative dementia that has been used clinically. Low doses of memantine very effectively attenuate lesions of cholinergic neurons in the nucleus basalis of Meynert (NBM) in rats produced by direct injection of NMDA [208,209]. The effective dose produced quite low peak plasma levels < 1 μM demonstrating the good neuroprotective potential of memantine even at such low, therapeutically relevant doses. Similarly, lesions of the NBM produced by a direct injection of mitochondrial 3-NP are also inhibited by memantine [210]. This indicates that NMDA receptors might be a likely link between different factors contributing to the neuronal insult. It has also been suggested that inflammation might play an

important role in neurodegeneration in Alzheimer's disease. Wenk's group [211] using a ChAT assay observed that memantine infused s.c. by Alzet minipumps prevented the loss of cholinergic neurons in the NBM produced by chronic inflammation through infusion of lipopolysaccharide (bacterial wall component, LPS). Memantine also attenuates hippocampal neuronal damage produced by a direct injection of β-amyloid [131]. Thus, memantine is an NMDA receptor antagonist that shows symptomatological improvement in animal models and clinical trials, pivotal clinical trials proving evidence for neuroprotective activity in Alzheimer's disease are pending.

HIV dementia. There are a number of indications that glutamate might be involved in some aspects of AIDS related neurological deficits [116]. Gp 120 (HIV coat protein) *in vitro* produces toxicity that is attenuated by NMDA receptor antagonists such as MK-801 and memantine [115]. This toxicity is probably secondary to glutamate release from glial cells rather than a direct agonistic effect of Gp 120. In line with this *in vitro* evidence, neurodegeneration in transgenic mice over-expressing gp120 is attenuated by memantine [194].

Parkinson's disease. There is some evidence that neurodegeneration of dopaminergic pathways of the substantia nigra pars compacta (SNc) in Parkinson's disease involves excitotoxicity [15,77,174]. Application *in vitro* of MPTP (its metabolite MPP^+ is a mitochondrial complex I inhibitor) inhibits the astroglial glutamate transporter [82], likely through free radicals. In rats, NMDA receptor antagonists protect against damage of dopaminergic neurons induced by the dopaminomimetic methamphetamine [187], however possibly due to hypothermia. MPTP-induced toxicity in monkeys, is prevented either by NMDA receptor antagonists or by lesion of the descending cortico-striatal glutamatergic pathway [9,14,189,223]. Similarly, in rats, damage to the SNc produced by a direct injection of MPP^+ into this structure is attenuated by NMDA receptor antagonists [196]. Based on the above data one could speculate that NMDA receptor antagonists should provide some degree of neuroprotection in Parkinson's patients.

Glaucoma. Mild chronic intravitreal elevation in glutamate concentration by intravitreal glutamate injections over 3 months in rats results in 42% death of retinal ganglion cells after 5 months. Concurrent daily injections of memantine 10 mg/kg/day completely prevents this cell death [201]. Also retinal ischemia induced in rats by elevating intraocular pressure also causes an elevation in the concentration of glutamate and glycine causing loss of retinal ganglion cells [100]. Memantine 10 mg/kg was protective even when given up to 4.5 hours post ischemia [100]. Memantine 20 mg/kg/day administered by osmotic minipumps starting 2 days before ischemia also increased retinal ganglion cell survival [100]. Memantine is presently in phase II clinical trials for the indication in glaucoma.

Multiple sclerosis (MS). In a mouse model of allergic encephalomyelitis there is a deficit of astroglial enzymes (glutamate dehydrogenase and glutamine synthase) responsible for degradation of glutamate taken up from the extracellular space. This may lead to an increase in extracellular glutamate and neurotoxicity seen in this disease. Memantine (10-20 mg/kg) dose-dependently ameliorated neurological deficits in experimental autoimmune encephalomyelitis (EAE) in Lewis rats. This therapeutic effect was not *via* interactions with the immune system *per se* and implies that effector mechanisms responsible for reversible neurological deficits in EAE may involve NMDA receptors [204].

Concluding remarks

A great progress was made in understanding the excitotoxic neuronal damage, however, further studies on the molecular level are required for learning the exact mechanism of this process. Stroke along with head and spinal cord injury are the CNS diseases in which the role of excitotoxic mechanisms in neuronal tissue degeneration has been well documented in animal models. While the results of experimental therapeutic strategies in both these acute diseases, directed to inhibition of excitatory amino acid receptors are promising, the results of clinical tests are disappointing. Although the specific etiology and exact pathogenesis of neurodegenerative disorders remain unknown, there are indications that the mechanisms of excitotoxicity participate in neurodegeneration in such chronic diseases like Parkinson's disease, Huntington's disease, Alzheimer's disease, ALS and glaucoma. Consequently, it seems that in these

diseases therapeutic strategies directed against excitotoxicity might delay the progress of neurodegeneration.

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