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Glutamate excitotoxicity in transient global cerebral ischemia

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Abstract. The glutamate excitoxicity hypothesis of ischemic cell damage holds that cell damage caused by transient cerebral ischemia is triggered by glutamate, released during ischemia from the intracellular compartment into the synaptic cleft: high extracellular glutamate levels activate ionotropic glutamate receptors, thus inducing an overflow of calcium ions into the neurones and a calcium-induced activation of catabolic processes. However, several arguments (and much of the evidence) in favour of this hypothesis do not bear closer examination. On the other hand, evidence is accumulating that, after transient ischemia, calcium fluxes through ionotropic glutamate receptors of the non-NMDA type may play a major role in the manifestation of ischemic cell damage. Calcium fluxes through non-NMDA receptors are determined by mRNA editing of non-NMDA receptor subunits: calcium fluxes are blocked in the presence of an edited subunit. A possible role of mRNA editing in the development of ischemic cell damage is discussed.

Key words: brain, global ischemia, glutamate, non-NMDA receptors, mRNA editing

INTRODUCTION

The mechanisms of ischemic cell damage triggered by transient global cerebral ischemia are still not fully understood. It has not even been established whether it is the disturbances occurring during ischemia or those following the insult that play the most prominent role in the manifestation of cell damage. Knowledge of the irreversible changes leading to ischemic cell damage is of fundamental importance for therapeutic intervention in this pathological process. In developing pharmacological therapies it should be taken into account that clinically meaningful treatment must target post-ischemic changes.

It is widely accepted that glutamate is involved in the development of ischemic cell damage. However, it is not yet clear whether the glutamate released during ischemia triggers ischemic cell damage or whether the action of this excitatory amino acid is more critical during recovery from ischemia. The excitotoxic hypothesis holds that glutamate, released during ischemia from the intracellular into the extracellular space, plays a major role in the development of the ischemic cell damage triggered by transient global cerebral ischemia. According to this view, glutamate released during ischemia accumulates to high levels in the synaptic cleft after the breakdown of uptake systems caused by energy disturbances. Glutamate then binds to ionotropic glutamate receptors, and thus activates sodium and calcium fluxes into the cell. The sharp increase in intracellular calcium activity during ischemia causes neuronal damage by an overactivation of a cascade of calcium-dependent catabolic processes such as lipolysis and proteolysis.

There are several observations supporting this excitotoxic hypothesis of glutamate: (1) it has in fact been shown that glutamate is neurotoxic *in vivo* under certain conditions: (2) the distribution of some ionotropic glutamate receptors closely resembles the regional vulnerability of the brain to transient global cerebral ischemia; (3) glutamate antagonists are neuroprotective in different models of transient global cerebral ischemia; (4) high levels of glutamate are released from the intracellular compartment into the synaptic cleft during ischemia; (5) lesioning of the excitatory input to the vulnerable hippocampal CA1-subfield is neuroprotective; (6) glutamate is neurotoxic *in vitro*.

In the present review, all the above mentioned arguments in favour of the excitotoxic hypothesis of ischemic cell damage will be discussed critically. Emphasis will be placed on a balanced assessment of arguments in favour and against an involvement of intra-ischemically released glutamate in the development of neuronal cell damage after transient global cerebral ischemia. In addition, arguments will be put forward for a post-ischemic role of glutamate receptors in the triggering of ischemic cell damage.

EXCITOTOXICITY OF GLUTAMATE *IN VIVO*

The observation of the excitotoxic activity of glutamate *in vivo* dates back to the experiments of Olney and coworker (Olney 1969, Olney and Sharp 1969) who injected monosodium glutamate into infant animals by various routes. The reason for these experiments was the inclusion of glutamate as a flavoring agent in all baby foods in the USA at that time. A few hours after a subcutaneous dose of glutamate, animals were sacrificed and their brains examined for damage. Acute neuronal necrosis was found in brain areas in which the blood-brain barrier was not fully developed, such as the hypothalamus.

The major concern regarding these experiments, if they are quoted in support of the excitotoxic hypothesis, is the fact that relatively high doses of glutamate were used (whole body glutamate concentrations were in the range of about 20 mM (Olney 1969, Olney and Sharp 1969)). In addition, D- and L-glutamic acid and D- and L-aspartic acid showed the same potency in producing neuronal cell damage (the D-stereoisomers were even slightly more potent than the L-forms; Olney et al. 1971), whereas the affinity to glutamate receptors is much higher for L- as for the D-stereoisomers and for L-glutamic acid as compared to L-aspartic acid (Foster and Roberts 1978).

REGIONAL DISTRIBUTION OF GLUTAMATE RECEPTORS

Ionotropic glutamate receptors are subdivided according to their pharmacological characteristics into the N-methyl-D-aspartate (NMDA), alpha--amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) and kainate receptors (Monaghan et al. 1989). The regional distribution of the NMDA receptor grossly resembles the regional vulnerability of the brain to transient global cerebral ischemia, being higher in the hippocampus than in the cortex and higher in the hippocampal CA1--subfield than in the CA3-subfield and dentate gyrus (Cotman et al. 1987). The AMPA receptor is more evenly distributed throughout the different hippocampal subfields (Martin et al. 1993) whereas the density of the kainate receptor is markedly higher in the CA3- than in the CA1--subfield (Cotman et al. 1987).

Recently, molecular studies have shown that NMDA and non-NMDA receptors are composed of different subunits with divergent regional distributions throughout the brain (Hollmann et al. 1989, Bettler et al. 1990, 1992, Boulter et al. 1990, Keinänen et al. 1990, Nakanishi et al. 1990, Egebjerg et al. 1991, Moriyoshi et al. 1991, Werner et al. 1991, Herb et al. 1992). Five different subunits have been identified for the NMDA receptor (NMDAR1, NMDAR2A-D), none of which is particularly concentrated in the hippocampal CA1-subfield (Monyer et al. 1992), a region most vulnerable to transient global cerebral ischemia (Kirino 1982). For the AMPA and kainate receptor subunits the situation is even more complex, because two different forms exist for each subunit (flip and flop version of GluR1-4 produced by alternative splicing) with different electrophysiological characteristics and different distributions throughout the forebrain (Sommer et al. 1990). In addition, the channel characteristics of AMPA/kainate receptors for ions strongly depend on a post-transcriptional modification termed mRNA editing, a critical reaction for the control of calcium fluxes through non-NMDA receptors (Hollmann et al. 1991, Sommer et al. 1991, Burnashev et al. 1992, Egebjerg et al. 1993, Köhler et al. 1993). Owing to this molecular and functional diversity of ionotropic glutamate receptors, it is difficult to establish any clear relationship between regional receptor density and vulnerability of the respective region to transient ischemia. The relationship between NMDA receptor density and vulnerability is, therefore, not an obvious one, and the observation that antagonists of non-NMDA receptors are neuroprotective even when given after a significant delay following ischemia (see below) argues against an intra-ischemic role of the NMDA receptor in the development of neuronal cell damage.

NEUROPROTECTION BY GLUTAMATE ANTAGONISTS

The observation of a relationship between NMDA receptor density and vulnerability stimulated the use of NMDA antagonists to counteract ischemic cell damage in models of transient global cerebral ischemia (for a recent review see Hossmann 1994). The first experiments performed with the non-competetive NMDA antagonist dizocilpine (MK-801) showed a marked dose-dependent protection of CA1-hippocampal neurones in gerbils subjected to 5 min transient cerebral ischemia, and complete prevention of ischemic cell damage in animals treated with 30 mg/kg of MK-801 (Gill et al. 1987). However, in these experiments, brain temperature was not controlled although this parameter critically affects the outcome after transient cerebral ischemia. Control of brain temperature is a prerequisite in pharmacological studies, because even mild hypothermia significantly reduces neuronal cell damage after ischemia (Busto et al. 1987). In fact, it has since been shown that MK-801 produces hypothermia and that the protective effect of this drug in models of transient global cerebral ischemia is most probably due to hypothermia rather than to any direct function of MK-801 as a non-competetive NMDA receptor antagonist (Buchan and Pulsinelli 1990a). The results obtained with MK-801 as a neuroprotectant in different models of transient global cerebral ischemia are therefore

variable (see Hossmann 1994). On the other hand, AMPA/kainate receptor antagonists such as 2,3-dihydro-6-nitro-7-sulphamoyl-benzo(F)-quinoxaline (NBQX) have proven to be neuroprotective in different models of transient global cerebral ischemia (Buchan et al. 1991, Diemer et al. 1992, Nellgard and Wieloch 1992, Li and Buchan 1993, Sheardown et al. 1993). A neuroprotective effect was achieved even when this drug was applied during the recovery period after cerebral ischemia, thus arguing against any critical involvement of intra-ischemically released glutamate in the triggering of ischemic cell damage. It is noteworthy that NBQX was neuroprotective even when given 24 h after ischemia (Li and Buchan 1993, Sheardown et al. 1993), thus pointing to a major post-ischemic role of the non-NMDA glutamate receptor in the development of ischemic cell damage.

RELEASE OF GLUTAMATE DURING ISCHEMIA

A sharp increase in glutamate levels in the extracellular compartment during ischemia (Benveniste et al. 1984) may be viewed as an argument for a role of glutamate in the induction of ischemic cell damage. However, excitatory neurotransmitters such as glutamate or aspartate are not selectively released during ischemia. Instead, ischemia induces a shift of both inhibitory and excitatory amino acids from the intra- to extracellular compartment (Hagberg et al. 1985). In fact, the relative increase in inhibitory neurotransmitters such as GABA may even by more pronounced than that of glutamate or aspartate (Hagberg et al. 1985). But even if we focus on the release of glutamate, the relationship between extracellular glutamate levels and the extent of cell damage is not a close one: during ischemia, glutamate levels increase to the same extent in vulnerable and non-vulnerable brain structures (Globus et al. 1990). Even in the hippocampus, extracellular glutamate levels are identical in the CA1- and the CA3-subfields (Mitani et al. 1992).

The best evidence in favour of the excitotoxic hypothesis would be obtained by pharmacologically manipulating the extent of glutamate release during ischemia: if this excess glutamate produced during the event itself contributes to the subsequent manifestation of ischemic cell damage, a pharmacological reduction of extracellular glutamate levels should cause an attenuation of cell damage and *vice versa*. In fact, such a relationship could not be established: theophylline treatment of animals significantly enhanced the release of glutamate during ischemia but did not change the extent of cell damage in the hippocampal CA1-subfield (Lekieffre et al. 1991), while cell damage could not be reduced by the broad spectrum glutamatergic receptor antagonist kynurenic acid eventhough this compound sharply reduced the release of glutamate during ischemia (Lekieffre et al. 1992). It was therefore concluded that "the extracellular accumulation of glutamate during forebrain ischemia does not play a major role in the hippocampus" (Lekieffre et al. 1992).

NEUROPROTECTION BY LESIONING OF THE EXCITATORY INPUT TO THE HIPPOCAMPAL CA1-SUBFIELD

Further evidence apparently indicating an involvement of intra-ischemically released glutamate in the induction of subsequent cell damage comes from experiments in which the excitatory input to the hippocampal CA1-subfield was lesioned. Different strategies have been used to study the effect of this treatment on the density of ischemic cell damage (Arvin et al. 1982, Wieloch et al. 1985, Onodera et al. 1986, Globus et al. 1987, Jørgensen et al. 1987, Kaplan et al. 1989, Buchan and Pulsinelli 1990b). In all these studies, ischemic cell damage could be significantly reduced in the hippocampal CA1-subfield, which was taken as evidence of a glutamate-evoked excitotoxic process. However, none of these studies established whether, in treated animals, the response of energy metabolism to transient ischemia was altered by lesioning the excita-

tory input to the CA1-subfield. Thus the beneficial effect could have been produced by a slowing of the rate of ATP depletion during ischemia and, a consequent reduction of the disturbances in energy metabolism below the threshold for induction of irreversible injury.

A major argument for a role of glutamate in ischemic cell damage comes from the observation that, during ischemia, the release of glutamate is blocked in animals in which the excitatory input to the hippocampal CA1-subfield has been lesioned (Benveniste et al. 1989): in the intact hippocampal CA1 tissue, glutamate levels increased six-fold during ischemia but only 1.4-fold in animals in which the CA3-subfield was lesioned by kainate injection. However, in a recent, carefully performed study this effect could not be reproduced (Mitani et al. 1994): in CA3-subfield lesioned animals the ischemia-induced efflux of glutamate was retarded and peak glutamate levels were significantly lower in lesioned than in non-lesioned animals; however, the clearance of glutamate from the extracellular space was markedly slowed down in lesioned animals so that the glutamate integral (glutamate levels over time) was about twice as high in lesioned as in non-lesioned animals (Mitani et al. 1994).

EXCITOTOXIC EFFECTS OF GLUTAMATE *IN VITRO*

Important support for the excitotoxic hypothesis of ischemic cell damage is provided by evidence of the neurotoxicity of glutamate *in vitro* (neuronal cell cultures, Choi 1985, Rothman 1985). When cortical neurones are exposed to glutamate for a short period of time, neuronal cell damage develops in a delayed fashion (Choi 1985), a phenomenon resembling the delayed dying of neurones in the hippocampal CA1-subfield after short periods of transient global cerebral ischemia (Kirino 1982). It was, therefore, suggested that glutamate plays a pivotal role in hypoxic-ischemic cell damage (Rothman and Olney 1986). However, if results from *in vitro* experiments are taken to support a hypothesis for the development of brain pathology *in vivo*, we have to illustrate that the changes occurring *in vitro* are similar to those observed *in vivo*, i.e. that the pattern of damage to neurones in cell culture after transient glutamate exposure is comparable to the pattern of damage found in neurones after transient cerebral ischemia. In fact, the pattern of cell damage produced *in vitro* by a transient glutamate exposure differs considerably in many ways from the pattern of cell damage produced by transient ischemia:

1. *In vitro* cortical neurones exhibit a high vulnerability to glutamate: when cortical neurones are exposed to 0.5 mM glutamate for only 5 min, more than 80% of neurones are damaged 24 h after exposure (Choi et al. 1987). *In vivo*, however, cortical neurones are particularly resistant to transient global cerebral ischemia and most neurones can survive even an ischemic insult of up to one hour's duration providing that post-ischemic hemodynamic disturbances can be prevented (Hossmann et al. 1987).
2. *In vitro* non-NMDA receptor antagonists have no significant effect on the extent of delayed cell damage produced by incubating neurones in the presence of 0.1 or 0.5 mM glutamate for 5 min (Koh and Choi 1991), whereas *in vivo*, non-NMDA receptor antagonists are neuroprotective in models of transient global cerebral ischemia (see above). In contrast, NMDA receptor antagonists such as MK-801 have a strong neuroprotective effect *in vitro* (Koh and Choi 1991) but not *in vivo* (see above).
3. The effects of transient glutamate exposure on cell damage of neurones *in vitro* is usually studied in an extremely artificial system, i.e. the serum is taken away during glutamate exposure and throughout the recovery period of up to 24 h (Choi et al. 1987, Koh and Choi 1991). Withdrawal of neurotrophic factors from neurones in culture is designed to stimulate programmed cell death and we cannot exclude the possibility that incubation of neurones in the absence of serum during and after glutamate exposure may predamage the cells and theirby increase their vulnerability. In fact, when these experiments were repeated in the presence of serum, both cortical and hippocampal neurones were extremely resistant to transient glutamate exposure (Dux et al. 1992).

Recently, the effect of transient glutamate exposure on hippocampal neurones *in vitro* was studied using the hippocampal slice model (Djuricic et al. 1994). Interestingly, the effect of transient glutamate exposure on slice metabolism was markedly different to the effect of transient *in vitro* ischemia (incubation in the absence of glucose and oxygen): exposing slices to 1 mM glutamate for 15 min did not affect energy state or protein synthesis, even when glutamate uptake was blocked by L-trans-pyrollidine-2,4-dicarboxylic acid. In the presence of 10 mM glutamate, energy state and protein synthesis were only transiently disturbed immediately after exposure and this effect was completely reversed after two hours of recovery (Djuricic et al. 1994). In addition, the inhibition of protein synthesis found imediately after 10 mM glutamate exposure could not be reversed by glutamate antagonists, which argues against a glutamate receptor mediated process (Djuricic et al. 1994). Incubating slices in ischemia-like conditions, in contrast, produced a transient disturbance of energy metabolism but a permanent inhibition of protein synthesis (Djuricic et al. 1994) similar to transient cerebral ischemia *in vivo*.

It is well established that, during ischemia, glutamate is released from the intra- into the extracellular compartment. However, in our critical review of the available data we have not been able to find any direct proof of the excitotoxic hypothesis, namely that glutamate released during ischemia is critically involved in processes leading to ischemic cell damage. On the other hand, as indicated above, evidence has been provided implicating glutamate receptors (non-NMDA type) in the manifestation of ischemic cell damage induced by transient global cerebral ischemia (non-NMDA receptor antagonists are neuroprotective).

ROLE OF GLUTAMATE DURING RECOVERY FROM ISCHEMIA

The observation that non-NMDA receptor antagonists are neuroprotective even when applied after a delay of up to 24 h following ischemia (see above) illustrates a post-ischemic involvement of glutamate receptors in ischemic cell damage. Interestingly, it has been shown recently that, after transient global cerebral ischemia, the stimulation-evoked influx of calcium ions into pyramidal cells of the hippocampal CA1-subfield is markedly increased compared to controls, and that this additional calcium uptake correlates closely with the extent of cell damage (Andiné et al. 1992). The sharp increase in calcium uptake can be blocked completely with the non-NMDA receptor antagonist NBQX but only partially with the NMDA receptor antagonist MK-801 (Andiné et al. 1992). It can be concluded, therefore, that calcium fluxes through AMPA/kainate receptors may play a central role in the development of ischemic cell damage. One way in which calcium fluxes through AMPA/kainate receptors could contribute to ischemic cell damage is by changes in the process of mRNA editing of glutamate receptor subunits (GluR2, GluR5 and GluR6), a critical reaction for the control of calcium fluxes through non-NMDA receptor channels (see below).

RNA EDITING OF GLUTAMATE RECEPTOR SUBUNITS

It was previously believed that glutamate receptors of the NMDA type gate both mono- and divalent cations, whereas non-NMDA receptors (AMPA/kainate receptors) gate only monovalent cations. Recently, however, it has been demonstrated that calcium fluxes can also be elicited in certain cell types by activation of glutamate-operated ion channels belonging to the AMPA/kainate family (Müller et al. 1992). Calcium currents through AMPA/kainate receptors are controlled by the mRNA editing reaction (Sommer et al. 1991). The reaction of mRNA editing takes place predominantly in the second transmembrane spanning region of the receptor subunits, a part of the molecule forming the ion channel (Sommer et al. 1991). In this particular region the DNA sequence is CAG, coding for glutamine. At the mRNA level, however, three subunits (GluR2, GluR5 and GluR6) belong-

ing to the AMPA (GluR2) or kainate (GluR5, GluR6) family exhibit both the sequence CAG (unedited) and CIG (edited). The triplet CIG is then translated to arginine, a positively charged amino acid, which blocks calcium fluxes: when the receptor is composed of subunits originating from unedited mRNA the receptor gates calcium ions, but calcium fluxes are blocked in the presence of an edited subunit (Burnashev et al. 1992).

In the physiological state, the GluR2 subunit is completely edited and AMPA sensitive receptors are permeable to calcium only when the GluR2 subunit is absent (Sommer et al. 1991, Burnashev et al. 1992). For kainate receptors, the situation is more complex, because the GluR5 and GluR6 subunits are both edited to varying extents in different brain regions (Sommer et al. 1991, Köhler et al. 1993, Paschen and Djuricic 1994, Paschen and Djuricic 1995). There are two possible routes through which changes in the mRNA editing of GluR2, GluR5 or GluR6 may contribute to ischemia-induced cell damage: (1) by an ischemia-induced selective down-regulation of the expression of the GluR2 subunit, or (2) by an alteration in the extent of editing of one of the partially edited subunits (GluR5 or GluR6).

As regards 1: Evidence has, in fact, been presented by in situ hybridisation techniques that, in the hippocampal CA1-subfield after transient global cerebral ischemia, the mRNA levels of the GluR2 subunit are significantly more reduced than those of the GluR1, GluR3 or GluR4 subunits (Pellegrini-Giampetro et al. 1992). However, the authors did not provide data illustrating that the observed changes in GluR2 mRNA levels are accompanied by a corresponding decrease in GluR2 protein content. A marked decrease in GluR2 mRNA levels after transient cerebral ischemia has also been found by Pollard et al. (1993), but the authors did not look at GluR1, GluR3 or GluR4 mRNA levels so that these experiments failed to establish whether the decrease in GluR2 mRNA levels was selective with respect to the other subunits. Other groups, in contrast, could not find any selective decrease of GluR2 mRNA levels after transient forebrain ischemia (Diemer et al. 1994). It remains, therefore, to be established whether transient cerebral ischemia induces a selective decrease in GluR2 mRNA levels which is accompanied by a similar decrease in the respective protein content.

As regards 2: Ischemia-induced changes in the extent of GluR2, GluR5 and GluR6 mRNA editing have also been studied recently (Paschen et al. 1996). After 30 min transient forebrain ischemia and different periods of recovery the GluR2 subunit remained completely edited, showing this to be a very stable reaction. The extent of editing of the GluR5 subunit was significantly increased in the striatum. This increase may reflect adaptive changes in neurones with a low vulnerability to transient ischemia, or it may be caused by a selective damage of neurones containing a high concentration of unedited GluR5 molecules. If this interpretation is valid, a low level of GluR5 mRNA editing could be viewed as a potential risk factor in different pathological states of the brain. The extent of editing of the GluR6 subunit decreased significantly in all brain structures studied, illustrating a global response to transient cerebral ischemia. Whether this decrease arises from a disturbance of the editing reaction or from glial proliferation (GluR6 mRNA editing is low in glial tissue (Paschen et al. 1994)) is not known at present.

Results presented by Andiné et al. (1992) point to a possible post-ischemic role of stimulation--evoked calcium influxes through non-NMDA glutamate receptors in the manifestation of ischemic cell damage. Calcium fluxes through ionotropic non-NMDA glutamate receptors are controlled by mRNA editing of glutamate receptor subunits. Two different mechanisms have been proposed by which mRNA editing may contribute to ischemic cell damage: (1) by a selective ischemic-induced decrease of the expression of the GluR2 subunit which is completely edited and which, therefore, inhibits calcium fluxes through AMPA receptors, or (2) by an increased sensitivity of neurones exhibiting a low level of editing of the GluR5 subunit. Whether mRNA editing is involved in the development of ischemic cell damage remains to be established in further experiments.

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