Minireview

Glutamate neurotoxicity, oxidative stress and mitochondria

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Abstract The excitatory neurotransmitter glutamate plays a major role in determining certain neurological disorders. This situation, referred to as 'glutamate neurotoxicity' (GNT), is characterized by an increasing damage of cell components, including mitochondria, leading to cell death. In the death process, reactive oxygen species (ROS) are generated. The present study describes the state of art in the field of GNT with a special emphasis on the oxidative stress and mitochondria. In particular, we report how ROS are generated and how they affect mitochondrial function in GNT. The relationship between ROS generation and cytochrome *c* release is described in detail, with the released cytochrome *c* playing a role in the cell defense mechanism against neurotoxicity.

Key words: Glutamate neurotoxicity; Reactive oxygen species; Mitochondrion; Oxidative stress; Cytochrome *c*

1. Introduction

In the last three decades, the excitatory neurotransmitter glutamate has been shown to cause certain neurological diseases [1–3]. This situation, referred to as 'glutamate neurotoxicity' (GNT), is characterized by time-dependent damage of many cell components leading to cell death. In the biochemical processes leading to cell death, reactive oxygen species (ROS), i.e. superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2), are generated in different cell compartments due to several reactions. However, cells can prevent ROS damage in virtue of the presence of a variety of molecules that work as antioxidants. Moreover, significant processes occur due to the presence of ion channels [4] and other reactions including *S*-nitrosation, thiolation and catecholamine oxidation (for references see [5]).

At present, exactly how cell death due to neurotoxicity occurs is rather obscure. In particular, ROS sources, scavengers and mechanisms of action remain to be fully established. In the present study, these issues are considered with a special emphasis on oxidative stress and mitochondria. These organelles are responsible for biological oxidations: they can produce ROS and are subjected to oxidative stress with a severe impairment of their functions including cell energy supply and metabolite transport [6–8].

2. ROS production in brain

A consensus has been achieved on the conclusion that GNT takes place as a result of glutamate binding to the *N*-methyl-D-aspartic acid (NMDA) receptor and to a minor extent to other receptor subtypes and that it depends on neuronal Ca^{2+} overloading (for references see [1,9]). Briefly, GNT is caused by glutamate binding to its receptor/s. This involves essentially two components. The first component marked by acute neuronal swelling, depends on the uptake of extracellular Na^+ and Cl^- uptake by the cell that causes plasma membrane depolarization; this causes Ca^{2+} channel opening that triggers the second component that is marked by delayed neuronal degeneration. In this case, the massive influx of extracellular Ca^{2+}, together with any Ca^{2+} release triggered from intracellular stores, increases cytosolic free Ca^{2+} and initiates a cascade-like effect leading to cell death. It should be noted that, in a Na^+–Ca^{2+}-free medium, NMDA receptor-mediated K^+ efflux may contribute to neuronal apoptosis [10].

The elucidation of the variety of events occurring downstream of neuronal Ca^{2+} overloading is still a matter for further research. As far as mitochondria are concerned, it has been reported that they accumulate Ca^{2+} (for references see [9]). On the other hand, ROS generation undoubtedly takes place in GNT [11–15] and is likely due to Ca^{2+} influx in the cytosol; consistently both (+)-5-methyl-10,1-dihydro-5H-dibenzo(*a*,*d*)cyclohepten-5,10-imine hydrogen maleate (MK801), a selective NMDA receptor antagonist, and EGTA, which can remove calcium ion, were shown to prevent ROS production as well as cell death [13]. Thus, in spite of the occurrence of many cell defense mechanisms against ROS, widely diffused in the liver but relatively deficient in the brain [16], in GNT a condition of 'oxidative stress' can develop (for references see [17]).

One of the most outstanding problems to be considered when studying the role of ROS production in neurotoxicity is the dependence of ROS formation on the time after glutamate exposure; indeed, ROS can be produced according to a variety of mechanisms and manifest different effects in a time-dependent manner.

The first suggestion of a causal relationship between GNT

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and ROS production was proposed by Dykens et al. [18] who observed that neuron damage caused by the excitotoxin kainate is prevented by both superoxide dismutase that can remove $O_2^{-\bullet}$ and by mannitol, used as an $OH^{\bullet}$ scavenger. In other studies, cortical and/or cerebellar neuron damage prevention was also found when using other antioxidants including α-tocopherol, ascorbic acid, ubiquinone, or 21-amino steroids [19,20]; conversely, the depletion of the antioxidant glutathione was found to exacerbate toxicity [21]. In a different experimental approach, cultured cortical neurons from mice over-expressing the free radical scavenging enzyme superoxide dismutase were found to be resistant to glutamate toxicity and showed the involvement of glutathione in neurodegeneration (for references see [22]). Finally, electron paramagnetic resonance spectroscopy provided direct evidence that NMDA receptor activation leads to the generation of superoxide radicals [11]. Shulz et al. provided the first in vivo evidence that excitotoxic neuronal injury is linked to free radical generation and showed the involvement of glutathione in neurodegeneration (for references see [23]).

Interestingly, Alzheimer's disease also involves free radical accumulation [24].

Some aspects of the processes leading to ROS production have been investigated in detail. Xanthine oxidase (XOD) was proposed as ROS producer [25] as allopurinol, an inhibitor of this enzyme, was found to prevent ROS production. It was suggested that Ca^{2+}-induced activation of one member of the cysteine proteases converts xanthine dehydrogenase (XDH) into XOD thus allowing for H_2O_2 and $O_2^{-\bullet}$ production [1,18]. Surprisingly, no XDH/XOD activity has been detected in neurons [18,26], until Atlante et al. [13], taking advantage of a modified XOD assay [27], showed that XOD activity, derived from XDH via an enzymatic cleavage [18,28,29], can produce $O_2^{-\bullet}$ at the beginning of GNT.

Beside the XOD-dependent ROS production, in the brain the presence of ROS is due to mitochondrial electron flow via the respiratory chain [6,7,30,31] with a different individual contribution depending on the different types of cells investigated and different experimental conditions used. The ROS production by mitochondria could derive from electron leaking from the transport chain to oxygen at high potential sites [6,30–32]. Moreover, defects in electron transport complexes or other perturbations of mitochondria can be responsible for excess production of ROS leading to glutamate toxicity [31,33–36]. A strong indication of the mitochondrial ROS generation in neurotoxicity derives from the evidence that the enhanced cellular $O_2^{-\bullet}$ production in the presence of glutamate is abolished by rotenone/oligomycin [14]. It should be noted that, in this case, ROS production was measured over a relatively long period of time, i.e. about 1 h after beginning glutamate treatment.

According to Choi [1], arachidonic acid metabolism by oxidases could lead to the production of oxygen free radicals that can trigger peroxidative degradation of lipid membranes and other destructive events [37]. Interestingly arachidonic acid and ROS were found to inhibit glutamate uptake in astrocytes via two distinct and additive mechanisms [38].

Ciani et al. [39] demonstrated that phospholipase A_2 and nitric oxide synthase activated by Ca^{2+} influx in the cell give rise to ROS whose deleterious action can be counteracted either by inhibiting these enzymes or by scavenging the excess of free radicals produced by them.

3. Mechanism of ROS action

ROS reactivity with DNA, proteins and lipids makes it easy to blame them for a variety of pathologies including GNT. Since mitochondria per se are vulnerable targets of ROS [7,40,41], one could conclude that initial ROS production could reduce mitochondrial respiration, with mitochondrial damage becoming acute due to ROS-induced alterations in mitochondrial DNA, proteins or lipids. However, these events are considered to occur over a relatively long time. The understanding of the mechanism of ROS action could take advantage of experimental work aimed at establishing the time course of the investigated process. In this respect, we have investigated the relationship between both mitochondrial ROS production and release of cytochrome *c* (cyt *c*) in the early phase (up to 30 min) of GNT [42]: cyt *c* release takes place owing to the ROS generation both in glutamate-treated cerebellar granule cells and in sister control cultures incubated in the presence of a ROS generating system. The released cyt *c* can work as a ROS scavenger and electron donor to the respiratory chain. Interestingly, cyt *c* release was found to occur in cerebellar granule cells from mitochondria that were essentially coupled and intact and that had a negligible production of oxygen free radicals. In agreement with [14,35], mitochondria from cells treated with glutamate for 3 h were mostly uncoupled and produced ROS at a high rate. The cytosolic fraction containing the released cyt *c* was able to transfer electrons from superoxide anion to molecular oxygen via the respiratory chain and was found to partially prevent glutamate toxicity when added externally to cerebellar neurons undergoing necrosis. These findings confirm Skulachev's proposal that cyt *c* can work as an antioxidant, derived from experiments carried out in a heterologous system [43,44], and has the added dimension that cyt *c* release is shown in vitro to be part of a cellular and mitochondrial defense mechanism against oxidative stress in the early phase of neurotoxicity.

A comparison made between mitochondrial function, as investigated in cell homogenate, and cell viability in 3 h GNT [42] shows that in this time during ROS production, the cells appear to be essentially intact, whereas mitochondria are already damaged, as shown by the decrease in both respiration and ATP synthesis [35,42]. Glucose oxidation was investigated with respect to both the electron flow via respiratory chain and the mitochondrial shuttles. In both cases a significant impairment was found [35,45]. However, as a result of cyt *c* release, an increase of the oxidation of externally added NADH was found, probably occurring via the mitochondrial NADH-b_5-oxidoreductase [45,46]. Consistently, activation in lactate production and plasma membrane NADH-oxidoreductase activity was found in glutamate-treated cells. It should be noted that no significant release of both intermembrane space and matrix enzymes was found under the same conditions [45], showing that in this process no membrane permeability transition (MPT) takes place (see [47]). The picture emerging from these results and from [14,34, 48–50] (Fig. 1) is that there are two phases of ROS formation after exposure to glutamate: an early ROS production coupled to XOD activation, and perhaps to other as yet unidentified processes, and a later ROS production mostly due to mitochondria. Long glutamate treatment results in permanent damage of mitochondria and large uncoupling, which occurs simultaneously with high mitochondrial ROS produc-

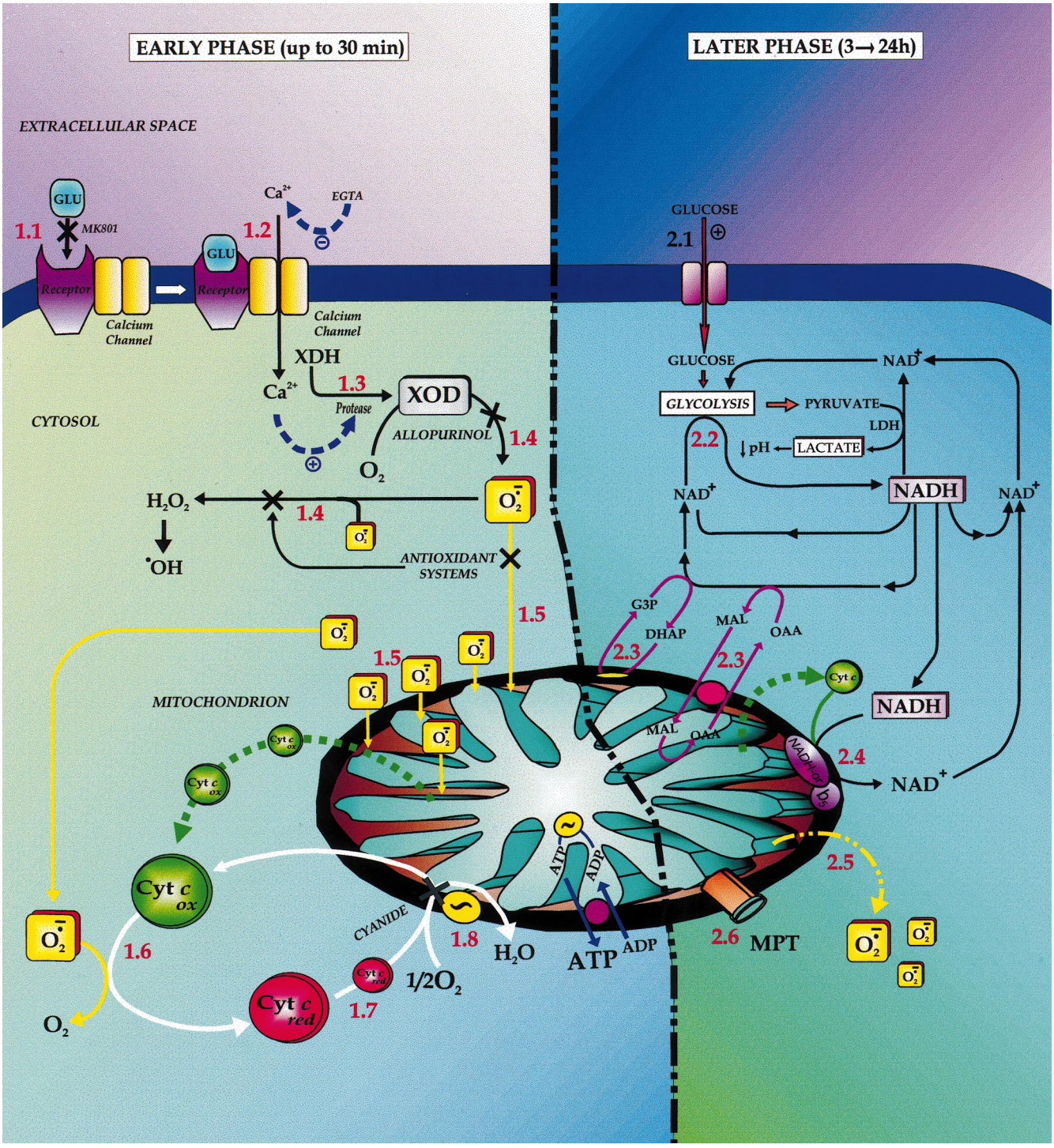

Fig. 1. The GNT time course. The time course of GNT is described as occurring in two phases. (1) Early phase (up to 30 min): 1.1, glutamate binding to receptor; 1.2, calcium influx in cytosol; 1.3, XDH→XOD conversion; 1.4, ROS production; 1.5, ROS-induced cyt *c* release; 1.6, Cyt *c* as ROS scavenger; 1.7, Cyt *c* as electron donor; 1.8, Cyt *c*-dependent energy generation. (2) Later phase (3–24 h): 2.1, glucose uptake increase; 2.2, increase in lactate production via glycolysis; 2.3, mitochondrial shuttle impairment; 2.4, NADH oxidation via mitochondrial NADH-b_5-oxidoreductase; 2.5, massive ROS production by mitochondria; 2.6, mitochondrial permeability transition. Abbreviations used: Cyt *c*, cytochrome *c*; DHAP, dihydroxyaceton-phosphate; G3P, glycerol-3-phosphate; GLU, glutamate; GNT, glutamate neurotoxicity; MAL, malate; MK801, (+)-5-methyl-10,1-dihydro-5H-dibenzo(*a,d*)cyclohepten-5,10-imine hydrogen maleate; MPT, mitochondrial permeability transition; H_2O_2, hydrogen peroxide; $O_2^{-\bullet}$, superoxide anion; •OH, hydroxyl anion; OAA, oxaloacetate; PFK, phosphofructokinase; ROS, reactive oxygen species; XDH, xanthine dehydrogenase; XOD, xanthine oxidase.

tion. In this case, cytosolic Ca^{2+} deregulation is followed by MPT [9,51]. However, although this may provide an attractive unifying hypothesis, particularly since oxidative stress facilitates the opening of the transition pore, it would caution against an apparent consensus [52,53] that the MPT, observed with isolated mitochondria, occurs in intact cells as a component of necrotic neuronal cell death. Interestingly the induction of a non-specific permeability transition pore in the inner

mitochondrial membrane was suggested as a first step in mitochondrial destruction [54,55]. Such a suggestion was based on the failure of mitochondria to sequestrate rhodamine 123. Since this process is accompanied by ultrastructural alterations in a granule cell, including clumping of the chromatin, swelling of the endoplasmic reticulum and mitochondria and disruption of the mitochondrial cristae, we could consider these events to occur in the later phase of neurotoxicity.

In agreement with [13,42], Luetjens et al. [50] demonstrated that the delayed superoxide production in excitotoxicity as well as in apoptosis, occurs secondary to a defect in mitochondrial electron transport and that mitochondrial cyt *c* release occurs upstream of this defect.

We conclude that in the early phase of GNT the non-mitochondrial ROS production triggers a cell defense mechanism in which a protective role is exhibited by cyt *c* released owing to ROS production. In the later phases of neurotoxicity ROS production is a result of cell dysfunction leading to cell death in a process in which mitochondrial damage, that occurs as a self-propagating process [41], could be considered one of the most relevant causes both in ROS production and in cell impairment [56]. In particular, local damage could impair mitochondrial energy production [15,40,42,52], enhancing depletion of cellular energy stores, and leading to the impairment of certain homeostatic or protective mechanisms [24].

There are other ROS cell targets including phosphofructokinase (PFK), creatine phosphokinase, and lactate dehydrogenase that are susceptible to oxidative inactivation. In this context it should be noted that the activities of several enzymes susceptible to oxidative inactivation, including PFK, are reportedly reduced in post-mortem brain from Alzheimer's disease patients and in gerbil brain after ischemia–reperfusion [57].

In addition, the increasing ROS production is expected to deplete cellular antioxidant defenses, leading to a general enhancement of oxidative stress and radical-mediated injury throughout the cell [39], this being the final factor in the cascade of events leading to neuronal injury after excitotoxic NMDA receptor activation [11,34].

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References

[1] Choi, D.W. (1988) Neuron 1, 623–634.
[2] Coyle, J.T. and Puttfarcken, P. (1993) Science 262, 689–695.
[3] Choi, D.W. (1994) Prog. Brain Res. 100, 47–51.
[4] Duprat, F., Guillemare, E., Romey, G., Fink, M., Lesage, F. and Lazdunski, M. (1995) Proc. Natl. Acad. Sci. USA 92, 11796–11800.
[5] Wolin, M.S. and Mohazzab-H., K.M. (1997) in: Oxidative Stress and the Molecular Biology of Antioxidant Defenses (Scandalios, J.G., Ed.), pp. 21–48, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
[6] Papa, S. and Skulachev, V.P. (1997) Mol. Cell. Biochem. 174, 305–319.
[7] Lenaz, G., Cavazioni, M., Genova, M.L., D'Aurelio, M., Merlo Pich, M., Pallotti, F., Formiggini, G., Marchetti, M., Parenti Castelli, G.P. and Bovina, C. (1998) Biofactors 8, 195–204.
[8] Atlante, A., Passarella, S., Quagliariello, E., Moreno, G. and Salet, C. (1990) J. Photochem. Photobiol. 7, 21–32.
[9] Nicholls, D.G. and Budd, S.L. (2000) Physiol. Rev. 80, 315–360.
[10] Yu, S.P., Yeh, C.-H., Strasser, U., Tian, M. and Choi, D.W. (1999) Science 284, 336–339.
[11] Lafon-Cazal, M., Pietri, S., Culcasi, M. and Boeckaert, J. (1993) Nature 364, 535–537.
[12] Budd, S.L. and Nicholls, D.G. (1996) J. Neurochem. 67, 2282–2291.
[13] Atlante, A., Gagliardi, S., Minervini, G.M., Ciotti, M.T., Marra, E. and Calissano, P. (1997) J. Neurochem. 68, 2038–2045.
[14] Castilho, R.F. and Nicholls, D.G. (1999) J. Neurochem. 72, 1394–1401.
[15] Pereira, C.F. and Oliveira, C.R. (2000) Neurosci. Res. 37, 227–236.
[16] Siesjo, B.K., Agardh, C.D. and Bengtsson, F. (1989) Cerebrovasc. Brain Metab. Rev. 1, 165–211.
[17] Cadenas, E. and Davies, K.J. (2000) Free Radic. Biol. Med. 29, 222–230.
[18] Dykens, J.A., Stern, A. and Trenkner, E. (1987) J. Neurochem. 49, 1222–1228.
[19] Majewska, M.D. and Bell, J.A. (1990) NeuroReport 1, 194–196.
[20] Puttfarcken, P.S., Getz, R.L. and Coyle, J.T. (1993) Brain Res. 624, 223–232.
[21] Bridges, R.J., Stanley, M.S., Anderson, M.W., Cotman, C.W. and Chamberlin, A.R. (1991) J. Med. Chem. 34, 717–725.
[22] Chan, P.H. (2001) J. Cereb. Blood Flow Metab. 21, 2–14.
[23] Schulz, J.B., Lindenau, J., Seyfried, J. and Dichgans, J. (2000) Eur. J. Biochem. 267, 4904.
[24] Mattson, M.P. and Goodman, Y. (1995) Brain Res. 676, 219–224.
[25] Flower, R.J., Moncada, S. and Vanet, J.R. (1985) in: The Pharmacological Bases of Therapeutics (Goodman, A.G., Gilman, L.S., Rall, T.W. and Murad, F., Eds.), pp. 674–715, Macmillian, New York.
[26] Markley, H.G., Faillace, L.A. and Mezey, E. (1973) Biochim. Biophys. Acta 309, 23–31.
[27] Atlante, A., Valenti, D., Gagliardi, S. and Passarella, S. (2000) Brain Res. Protoc. 6, 1–5.
[28] McCord, R.C. (1985) Int. Ophthalmol. Clin. 25, 15–19.
[29] Dykens, J.A. (1994) J. Neurochem. 63, 584–591.
[30] Chance, B., Sies, H. and Boveris, A. (1979) Physiol. Rev. 59, 527–603.
[31] Halliwell, B. (1992) J. Neurochem. 59, 1609–1623.
[32] Kwong, L.K. and Sohal, R.S. (1998) Arch. Biochem. Biophys. 350, 118–126.
[33] Ankarcrona, M., Dypbukt, J.M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S.A. and Nicotera, P. (1995) Neuron 15, 961–973.
[34] Dugan, L.L., Sensi, S.L., Canzoniero, L.M.T., Handran, S.D., Rothman, S.M., Lin, T.-S., Goldberg, M.P. and Choi, D.W. (1995) J. Neurosci. 15, 6377–6388.
[35] Atlante, A., Gagliardi, S., Ciotti, M.T., Marra, E., Passarella, S. and Calissano, P. (1996) NeuroReport 7, 2519–2523.
[36] Carriedo, S.G., Yin, H.Z., Sensi, S.L. and Weiss, J.H. (1998) J. Neurosci. 18, 7727–7738.
[37] Chan, P.H. and Fishman, R.A. (1980) J. Neurochem. 35, 1004–1007.
[38] Volterra, A., Trotti, D. and Racagni, G. (1994) Mol. Pharmacol. 46, 986–992.
[39] Ciani, E., Groneng, L., Voltattorni, M., Rolseth, V., Contestabile, A. and Paulsen, R.E. (1996) Brain Res. 728, 1–6.
[40] Ernster, L. (1993) in: Active Oxygen, Lipid Peroxides and Antioxidants (Yagi, K., Ed.), pp. 1–38, CRC Press, Boca Raton, FL.
[41] Poderoso, J.J., Boveris, A. and Cadenas, E. (2000) Biofactors 11, 43–45.
[42] Atlante, A., Calissano, P., Bobba, A., Azzariti, A., Marra, E. and Passerella, S. (2000) J. Biol. Chem. 275, 37159–37166.
[43] Skulachev, V.P. (1998) FEBS Lett. 423, 275–280.
[44] Korshunov, S.S., Krasnikov, B.F., Pereverzev, M.O. and Skulachev, V.P. (1999) FEBS Lett. 462, 192–198.
[45] Atlante, A., Gagliardi, S., Marra, E., Calissano, P. and Passarella, S. (1999) J. Neurochem. 73, 237–246.
[46] La Piana, G., Fransvea, E., Marzulli, D. and Lofrumento, N.E. (1998) Biochem. Biophys. Res. Commun. 246, 556–561.

[47] Single, B., Leist, M. and Nicotera, P. (1998) Cell Death Differ. 5, 1001–1003.
[48] Reynolds, I.J. and Hastings, T.G. (1995) J. Neurosci. 15, 3318–3327.
[49] Bindokas, V.P., Jordan, J., Lee, C.C. and Miller, R.J. (1996) J. Neurosci. 16, 1324–1336.
[50] Luetjens, C.M., Bui, N.T., Sengpiel, B., Munstermann, G., Poppe, M., Krohn, A.J., Bauerbach, E., Krieglstein, J. and Prehn, J.H.M. (2000) J. Neurosci. 20, 5715–5723.
[51] Beal, M.F., Howell, N. and Bodis-Wollner, I. (1997) Mitochondria and Free Radicals in Neurodegenerative Disease, Wiley-Liss, New York.
[52] Kroemer, G., Dallaporta, B. and Resche-Rigon, M. (1998) Annu. Rev. Physiol. 60, 619–642.
[53] Bernardi, P., Basso, E., Colonna, R., Costantini, P., Di Lisa, F., Eriksson, O., Fontane, E., Forte, M., Ichas, F. and Massari, S. (1998) Biochim. Biophys. Acta 1365, 200–206.
[54] Isaev, N.K., Zorov, D.B., Stelmashook, E.V., Uzbekov, R.E., Kozhemyakin, M.B. and Victorov, I.V. (1996) FEBS Lett. 392, 143–147.
[55] Schinder, A.F., Olson, E.C., Spitzer, N.C. and Montal, M. (1996) J. Neurosci. 16, 6125–6133.
[56] Lemasters, J.J., Qian, T., Bradham, C.A., Brenner, D.A., Cascio, W.E., Trost, L.C., Nishimura, Y., Nieminen, Y. and Herman, B. (1999) J. Bioenerg. Biomembr. 31, 305–319.
[57] Beal, M.F., Hyman, B.T. and Koroshetz, W. (1993) Trends Neurosci. 16, 125–131.