Spare respiratory capacity, oxidative stress and excitotoxicity

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Abstract

Chronic exposure to glutamate (glutamate excitotoxicity) exacerbates neuronal damage in the aftermath of stroke and is implicated in a variety of neurodegenerative disorders. Mitochondria play a central role in the survival or death of the exposed neuron. Calcium, oxidative stress and ATP insufficiency play closely interlocked roles that may be investigated with primary neuronal cultures.

Glutamate as an excitotoxin

The ability of the ubiquitous amino acid glutamate to act as a specific neurotransmitter at synapses relies on the strict control of its compartmentation. In the absence of synaptic stimulation, glutamate in the synaptic cleft is maintained at approx. 1 μM [1], a concentration insufficient to activate glutamate receptors. The concentration in the neuronal cytoplasm may be close to 10 mM, whereas further concentration within synaptic vesicles may raise the concentration to approx. 100 mM. The gradients across the plasma and vesicle membranes are directly or indirectly dependent upon ATP generated by the *in situ* mitochondria, and the bioenergetic disruption induced by brain ischaemia and consequent abolition of oxidative phosphorylation leads to a rapid energy failure, inhibition of the Na^+/K^+-ATPase at the plasma membrane and collapse of the Na^+ electrochemical gradient responsible for maintaining the 10000-fold glutamate gradient across the plasma membrane. As a consequence, extracellular glutamate concentrations can rise up to 100-fold within a few minutes of the initiation of ischaemia [2]. Cells in the ischaemic core die rapidly, but neural damage is exacerbated by the diffusion of glutamate into the partially oxygenated penumbra surrounding the core. Excitotoxicity is the process by which neurons undergo predominantly necrotic cell death in response to this pathological exposure to glutamate [3]. Whereas artificial excitotoxicity can be induced by kainate-induced non-desensitizing activation of AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors (e.g. [4]), *in vivo* excitoxicity is ascribed predominantly to NMDA (*N*-methyl-D-aspartate) receptors. NMDA receptors are central to learning and memory, and two features necessary for these processes underlie the toxicity of chronic NMDA receptor activation. First, the receptor only partially desensitizes in the continued presence of glutamate; secondly, in addition to Na^+, the receptor also conducts Ca^{2+}.

The role of oxidative stress

Aspects of glutamate excitotoxicity can be studied *in vitro* using primary neuronal cultures. Early work by Choi et al. [5] established the importance of Ca^{2+} entry, whereas Tymianski et al. [6] and Randall and Thayer [7] each described the *in vitro* phenomenon of DCD (delayed Ca^{2+} deregulation) whereby individual neurons in culture maintaining a stable elevated $[Ca^{2+}]_c$ (cytoplasmic free Ca^{2+} concentration) in the presence of glutamate underwent a stochastic failure of Ca^{2+} homoeostasis, followed shortly after by necrotic cell death. An extensive subsequent literature has focused on the mechanisms underlying excitotoxicity. An early influential paper [8] reported that superoxide anion, O_2^-, could be detected by EPR upon NMDA receptor activation of cerebellar granule neurons, and this led to a widely accepted concept that NMDA-receptor-mediated oxidative stress played a key role in DCD. In its simplest form, the hypothesis states that free radicals produced as a consequence of receptor activation and Ca^{2+} entry into the cell-induced damage to the mitochondria and the cell precipitating DCD and cell death. The nature of the EPR technique meant that DCD could not be measured during exposure of the cells to glutamate in the EPR tube. Some years later we investigated the relationship between NMDA receptor activation, $[Ca^{2+}]_c$ elevation, O_2^- levels and DCD at a single-cell level using multiple fluorescent indicators and additionally monitoring $\Delta\psi_m$ (mitochondrial membrane potential) [9]. It was found that activated NMDA receptor caused no change in the levels of O_2^- detected by dihydroethidine oxidation to fluorescent ethidium, but that, in individual cells, the initiation of DCD was associated with a massive increase in superoxide. This experiment did not in itself distinguish cause and effect, but parallel experiments in the presence of a potent superoxide dismutase mimetic trapped virtually all the superoxide, but was totally without effect on the timing or extent of DCD. We

Key words: ATP, excitotoxicity, glutamate, ischaemia, mitochondrion, oxidative stress.

Abbreviations used: $\Delta\psi_m$, mitochondrial membrane potential; $[Ca^{2+}]_c$, cytoplasmic free Ca^{2+} concentration; DCD, delayed Ca^{2+} deregulation; MPT, mitochondrial permeability transition; NMDA, *N*-methyl-D-aspartate; ROS, reactive oxygen species.

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concluded that the increase in O_2^- reported previously was a downstream consequence of DCD with the consequent failure of antioxidant defences.

The role of mitochondria

Although mitochondrial oxidative stress appeared not to be the primary mechanism underlying DCD, there was increasing evidence for a central role of mitochondria. In neuronal cultures, where glycolysis was sufficiently active to supply the cell's ATP demands even in the presence of glutamate, it was found that mitochondrial depolarization by the combination of respiratory inhibition by rotenone and ATP synthase inhibition by oligomycin protected the cell against DCD [10,11]. No such protection was afforded by oligomycin alone, suggesting that mitochondrial depolarization and the consequent inhibition of mitochondrial Ca^{2+} uptake were responsible for the protection. Isolated mitochondrial show a net accumulation of Ca^{2+} when the cation's concentration rises above the 'set-point', which for brain mitochondria is at approx. 0.5 μM [12]. Measurements of $[Ca^{2+}]_c$ during NMDA receptor activation report maintained values in excess of this [13], suggesting that the mitochondria should be continually loaded with the cation. Massive Ca^{2+} accumulation by the *in situ* mitochondria in glutamate-exposed neurons has been shown by several groups [10,14,15]. Although the ability of isolated mitochondria to accumulate Ca^{2+} with no apparent bioenergetic damage is massive, it is not infinite, and ultimately the MPT (mitochondrial permeability transition) is induced, with consequent inner membrane permeabilization, Ca^{2+} dumping back to the cytoplasm, depolarization and energetic collapse [16].

Evidence for the MPT in DCD has been controversial [11,17–19]. Using a semi-quantitative method for monitoring $\Delta\psi_m$ in cultured cerebellar granule neurons, Ward et al. [20] showed that DCD in individual neurons was accompanied by a total mitochondrial depolarization in the affected cell. Since mitochondria must depolarize when exposed to the very high $[Ca^{2+}]_c$ that occurs during DCD, this does not show that the MPT was responsible. The conventional technique for establishing a role for the MPT is to see whether cyclophilin D inhibitors such as cyclosporin A delay the MPT. However, most experimenters failed to see any protection. Since it was later reported that mitochondria isolated from cerebellar granule neurons were not sensitive to cyclosporin A [21], these experiments were inconclusive. Individual mitochondria visualized in neuronal processes swell and cease movement after glutamate exposure [22], but this reflects Ca^{2+} accumulation and can generally be reversed by glutamate removal. Some of the best evidence for the MPT has come from structural studies by Pivovarova et al. [18], who observed excitotoxic Ca^{2+} overload in a subpopulation of mitochondria in cultured hippocampal neurons using conventional and electron microscopy coupled with electron probe X-ray microanalysis [18].

Spare respiratory capacity

Is DCD then simply a matter of mitochondrial Ca^{2+} overload and induction of the MPT or do additional factors come into play? Although the NMDA receptor has a typical 10:1 selectivity for Ca^{2+} over Na^+ [23], the cell is exposed to 100-fold higher concentration of Na^+ over Ca^{2+}, with the result that Na^+ fluxes greatly exceed those for Ca^{2+}. Indeed $[Na^+]_c$ (cytoplasmic free Na^+ concentration) rises dramatically on NMDA receptor activation [24]. It would be predicted that the consequent Na^+/K^+-ATPase activation would impose a high ATP demand on the cell, which would be reflected in an increased rate of *in situ* mitochondrial respiration. Until recently there has been no suitable technique for monitoring the respiration of neurons and other cells attached to glass coverslips. However, in 2004, we published a technique in which oxygen uptake by cultured neurons in a closed confocal perfusion chamber could be detected by quantifying the downstream oxygen tension as medium was slowly superfused over the cells [25]. The prediction of an increased respiration rate by the *in situ* mitochondria was immediately confirmed, indeed maximal NMDA receptor activation (100 μM glutamate plus 10 μM glycine in a Mg^{2+} free medium) utilized 100% of the respiratory capacity of the *in situ* mitochondria, since an identical respiratory stimulation was attained with an optimal concentration of protonophore [26]. The consequence of this finding was that any reduction in respiratory capacity might lead to an energy deficit that would facilitate DCD, and this was demonstrated with low concentrations of the complex I inhibitor rotenone [26]. Several years earlier, Novelli et al. [27] had proposed the 'energy-linked excitotoxicity, hypothesis based on a rather different hypothesis, namely that an energy deficit would result in plasma membrane depolarization, which would remove the voltage-dependent block from the NMDA receptors.

The current hypothesis is in apparent conflict with the proposed benefits of 'mild uncoupling' [28], which states that a decrease in ROS (reactive oxygen species) generation can be achieved by a modest increase in mitochondrial proton conductance and consequent slight mitochondrial depolarization. The hypothesis is based on observations with isolated mitochondria, particularly utilizing succinate, where the generation of ROS could be decreased by protonophore [29]. Sustained electron transport in intact cells cannot occur in the absence of NAD^+-linked respiration and so it is superoxide generation in the presence of such substrates that is more relevant in the present context. The generation of ROS with NAD^+-linked substrates is much lower than that of succinate, and reports are ambiguous as to whether ROS production still responds to mild uncoupling [30]. In cultured neurons metabolizing glucose, we found a negligible decrease in mitochondrial O_2^- production in the presence of low-nanomolar concentrations of protonophore [31], and, of course, since uncouplers uncouple, i.e. divert the proton current away from the ATP synthase, the capacity for ATP generation decreases. In response to glutamate this decrease in ATP generating

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capacity outweighed any effect on ROS production and increased DCD at all protonophore concentrations [31].

Although our focus has been on ATP generation, it is not our intention to diminish the importance of oxidative damage. We investigated this by depleting endogenous glutathione stores in cultured neurons by complexation to monochlorobimane [32]. The advantage of this technique is that the formation of the complex can be monitored by its fluorescence. Theoretical calculation predicts that glutathione depletion will lead to an oxidative shift in the thiol redox potential of the residual glutathione [33], and this was confirmed in parallel experiments with cells transfected with the redox-sensitive roGFP (reduction/oxidation-sensitive green fluorescent protein) [32]. The bioenergetic consequences of glutathione depletion were unexpected. In parallel with the formation of the fluorescent adduct, the respiration of the cells linearly declined. No initial change in cell viability was found, nor was there any effect on oligomycin-insensitive respiration (monitoring proton leak) or maximal respiration in the presence of FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone). The decline was focused on a decrease in oligomycin-sensitive respiration, reflecting mitochondrial ATP synthesis. To distinguish between a decrease due to lowered cellular ATP demand and one due to difficulty in mitochondrial ATP generation or export, extra-mitochondrial ATP demand was increased by activation of voltage-sensitive Na^+-channel using brevetoxin. Control cells coped with this increased demand, but monochlorobimane-treated cells showed little capacity to enhance their respiration and showed enhanced DCD. The conclusion was that the oxidative shift in thiol redox potentially damaged ATP generation or its export to the cytoplasm, with the most likely locus being the adenine nucleotide translocator [32].

Most of this research was performed at the Buck Institute for Age Research, where the central aim was to understand why aging is the primary risk factor in neurodegenerative diseases. It is therefore relevant to try to relate the acute cell death experiments similar to those reported in this review to the slow neuronal loss that may occur over decades in the human disease. Our 'spare respiratory capacity hypothesis' focuses on the stochastic changes in ATP demand in individual neurons in response to patterns of input and synaptic activity. As long as the spare capacity of an individual neuron exceeds that maximal ATP demand that the neuron will ever be called upon to supply then and ATP crisis is avoided. Since mitochondrial ATP-generating capacity declines with age, the statistical chances of an ATP crisis in individual neurons will increase with age. The role of oxidative damage in this view is primarily to decrease maximal ATP-generating capacity and thus to increase the statistical chances of an ATP deficit. This is consistent with the Ca^{2+}-overload hypothesis discussed above, since one of the first manifestations of an ATP deficit will be a failure of plasma membrane Ca^{2+} pumps and exacerbated Ca^{2+} accumulation by the cell, whereas a lowered $\Delta\psi_m$ is known to enhance the MPT [16].

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