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Mitochondria, Ca^{2+} and neurodegenerative disease

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Abstract

Mitochondria play a central role in cell biology not only as producers of ATP, but also in the sequestration of Ca^{2+} and the generation of free radicals. They are also repositories of several proteins which regulate apoptosis. Perturbations in the normal functions of mitochondria will inevitably disturb cell function, may sensitise cells to neurotoxic insults and may initiate cell death. Neuronal Ca^{2+} overload, such as follows excessive stimulation of Ca^{2+} permeant excitatory amino acid receptors, can cause cell death. Recent evidence suggests that the accumulation of Ca^{2+} into mitochondria during episodes of cellular Ca^{2+} overload initiates a cascade of events that culminate in cell death. Cell death appears to require not only mitochondrial Ca^{2+} overload, but rather a combination of raised intramitochondrial Ca^{2+} concentration with increased production of nitric oxide and possibly other oxyradical species. Cell death may proceed through either necrotic or apoptotic mechanisms, depending on the rate of consumption and depletion of ATP. Evidence is also accumulating to suggest that more subtle alterations in mitochondrial function may serve as predisposing factors in the pathogenesis of a number of neurodegenerative disorders.

Keywords: Glutamate; Ca^{2+}; Mitochondrion; Apoptosis; Necrosis; Motor neuron disease; Alzheimer's disease

1. Introduction

Mitochondria are central to the maintenance of cell function and viability. Maintained generation of ATP is an absolute requirement for normal cellular homeostasis, and, in the central nervous system (CNS), the bulk of cellular ATP is produced by mitochondrial oxidative phosphorylation. Mitochondria also play important roles in normal cell physiology by virtue of their subtle but significant influence on the spatiotemporal patterning of cellular Ca^{2+} signals (Boitier et al., 1999; Jouaville et al., 1999) which are absolutely central to normal cellular communication, especially in the CNS.

The spectre of neurodegenerative disease terrifies most of us. These diseases are profoundly debilitating, and include a large number of disorders, some with a high incidence in the population. Examples include Alzheimer's disease, Parkinson's disease, motoneuron disease (amyotrophic lateral sclerosis, or ALS, also known as Lou Gehrig's Disease in the USA), multiple sclerosis, the rarer but crippling Huntington's and Wilson's disease, Friedreich's ataxia, and a number of inherited disorders of the mitochondrial genome causing the mitochondrial encephalomyopathies. Our understanding of the pathophysiological mechanisms involved in these diseases remains incomplete. Although the specific causes of neuronal dysfunction and cell death vary in these disorders and follow a number of distinct pathways, disorders of mitochondrial function have been implicated at some level of the pathogenic process in all of these diseases. A major challenge of contemporary neuroscience is to understand the extent to which these changes in mitochondrial function represent primary or secondary components of the pathophysiological process, and to elucidate the basic pathways that lead to the development of disease.

Perhaps it is evident that mitochondrial dysfunction will inevitably manifest itself as cellular dysfunction or death. If the generation of ATP is impaired, a failure of cellular homeostasis must follow, with attendant changes in the ionic balance for Na^+, K^+, Cl^- and Ca^{2+} that will disturb the patterning of electrical signals and of the changes in the concentration intracellular free calcium ($[Ca^{2+}]_c$) that together underpin the transmission of information in the

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CNS. Ultimately, ATP depletion will lead to necrotic cell death. Other forms of mitochondrial injury may lead to the release of pro-apoptotic factors, particularly of cytochrome *c* from the mitochondria and the initiation of the cascade to apoptotic cell death (for a review, see Crompton, 2000). Below, we will address the extent to which these processes operate in acute glutamate neurotoxicity and will also examine briefly their role in the more insidious neurodegenerative diseases. While the importance of these processes in neuronal signalling may be self-evident, our understanding of the function of glial elements and their contribution both to normal signalling in the CNS and to the development of pathology has been growing rapidly in recent years (for a recent review see Haydon, 2001). The contribution of mitochondrial function to glial physiology and of mitochondrial dysfunction to glial pathophysiology is an emerging field of which we still understand relatively little.

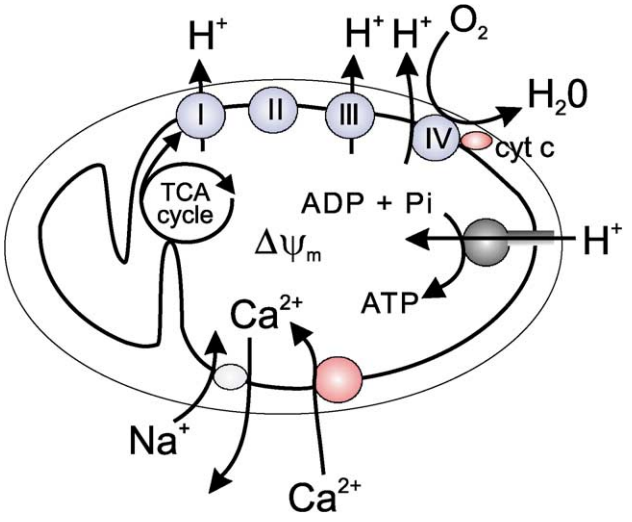

Fig. 1. Cartoon of a mitochondrion to illustrate the basic principles of the chemiosmotic coupling of respiration and phosphorylation. Please see the text for explanation. Solid line represents inner mitochondrial membrane, fainter solid line outer mitochondrial membrane. Roman numerals represent enzyme complexes I–IV of electron transport chain.

2. The basic principles of mitochondrial function

Mitochondria are always described in the textbooks as the 'power house of the cell', providing ATP through the action of the F_1F_0-ATP synthase in the inner membrane. However, mitochondria have other functions that are also important for cell viability. They accumulate Ca^{2+} from the cytosol. They contain proteins such as cytochrome *c* (and others) that are released as a major trigger to caspase activation and the initiation of apoptotic cell death. They are the only organelle outside the nucleus (in animal cells) that contains DNA (mitochondrial DNA or mtDNA), encoding genes responsible for the essential enzymes involved in oxidative phosphorylation, and yet they maintain a complex relationship with the host cell, depending heavily on the import of nuclear encoded proteins to maintain normal function. As they have mtDNA, they can also acquire, carry and transmit mutations that underlie disease, although the genetic principles of such diseases are very different from the Mendelian genetics of nuclear encoded mutations.

The primary function of mitochondria is ultimately to produce ATP. This is achieved through a chemiosmotic mechanism that couples the generation of ATP by the mitochondrial F_1F_0-ATP synthase and the oxidation of carbohydrate derivatives (pyruvate) and the β-oxidation of fatty acids through the tricarboxylic acid cycle (TCA cycle, also known as the Krebs cycle). The basic principles of the chemiosmotic circuit are illustrated in Fig. 1.

The TCA cycle maintains the coenzymes NADH and flavoproteins in a reduced state and so supplies reducing equivalents to the electron transport chain, situated in the inner membrane of the mitochondrion. This comprises five enzyme complexes including: NADH CoQ reductase (complex I), succinate CoQ reductase (complex II), ubiquinol cytochrome *c* reductase (complex III), cytochrome *c* oxidase (COX; complex IV) and ATP synthase (complex V; represented by rod and ball-shaped symbol in Fig. 1). These complexes are large oligomers composed of more than 80 polypeptides, 13 of which are encoded by mtDNA. While the respiratory enzyme complexes transfer electrons to each other and ultimately to molecular oxygen (with the production of water), they translocate protons across the inner mitochondrial membrane. The proton gradient set up in this way provides the energy that drives the motor of the ATP synthase (Mitchell and Moyle, 1967; Stock et al., 2000). This proton gradient is largely expressed as a potential across the mitochondrial inner membrane ($\Delta\Psi_m$), estimated at −150 to −180 mV negative with respect to the cytosol, although quantification of this variable within cells is difficult. It is this potential which lies at the heart of most aspects of mitochondrial function.

One consequence of oxidative phosphorylation is the generation of unpaired electrons. The interaction of these electrons with O_2 results in the generation of molecules with unpaired electrons (O_2^-), known as superoxide ions, highly reactive free radical species (or reactive oxygen species, ROS). Furthermore, other radical species, such as hydroxyl ions (OH^-) and H_2O_2 may also be present in reasonably high concentrations, posing a risk of lipid peroxidation and damage to cell membranes and to DNA. It is worth remembering that this includes mtDNA, which has no associated histones and is less protected from radical damage than nuclear DNA. Mitochondria represent the major source of free radical species in all cells except those specialised to generate free radicals such as macrophages and neutrophils.

3. Studying mitochondrial function in situ

Long established biochemical assays of mitochondrial enzyme activity, histology and electron microscopy provide the foundation for the evidence for the contribution of mitochondrial dysfunction in disease. However, newer approaches to study mitochondrial function in living cells have begun to make a major impact on our understanding of the underlying sequence of events that lead to cellular dysfunction and death. In particular, digital imaging fluorescence microscopy has been applied extensively to estimate a range of variables that inform us about mitochondrial status: $-\Delta\Psi_m$, intramitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$), redox state, the rate of free radical generation within living cells. These studies have revealed the complexity of mitochondrial signalling, but also have shown the important role played by mitochondria in the local control of $[Ca^{2+}]_c$.

3.1. The measurement of $\Delta\Psi_m$

One of the most powerful features of imaging technology is the ability to measure multiple signals simultaneously on a cell by cell basis. Thus, changes in $[Ca^{2+}]_c$ or $[Ca^{2+}]_m$ can be directly correlated, for example, with changes in $\Delta\Psi_m$. It is not appropriate to discuss the measurement of $\Delta\Psi_m$ in too great detail here, and the methodology has been discussed extensively elsewhere (see Duchen et al., 2002). However, as misinterpretations of these measurements do lead to major misunderstandings and confusion in the literature, we will dwell briefly on the principles involved. $\Delta\Psi_m$ is measured by using one of a number of lipophilic cationic dyes that partition between membrane-bound compartments in response to the distribution of potential gradients according to Nernstian principles. At very low concentrations (typically 10–50 nM), fluorescence intensity is a simple function of dye concentration. As the concentrations of dye used increase, the fluorescence intensity fails to increase as expected due to a phenomenon of 'autoquenching', which involves formation of multimers and the transfer of energy between dye molecules. The dyes are concentrated into the cytosol (~10×) and thence into the mitochondria (~5–800×) in response first to the plasma membrane potential and then in response to the mitochondrial potential. At low dye concentrations, the distribution of dye between compartments can be followed using high resolution confocal microscopy. Mitochondrial depolarisation will cause a redistribution of dye from mitochondria to the cytosol (Fig. 2). One of the major problems with these measurements is that the signal is not independent of the plasma membrane potential, and as many reading this essay will be interested in the effects of glutamate on neuronal mitochondria, it is important to realise that plasmalemmal depolarisation will inevitably cause the loss of cytosolic signal, leaving mitochondria effectively bathed in a lower dye concentration. The overall signal from the cell will therefore be reduced even if the mitochondria remain fully polarized (see Nicholls and Ward, 2000). Careful high resolution imaging is necessary to discriminate between this process and true mitochondrial depolarisation, in which case dye redistribution, rather than signal loss is the key observation (see Fig. 2).

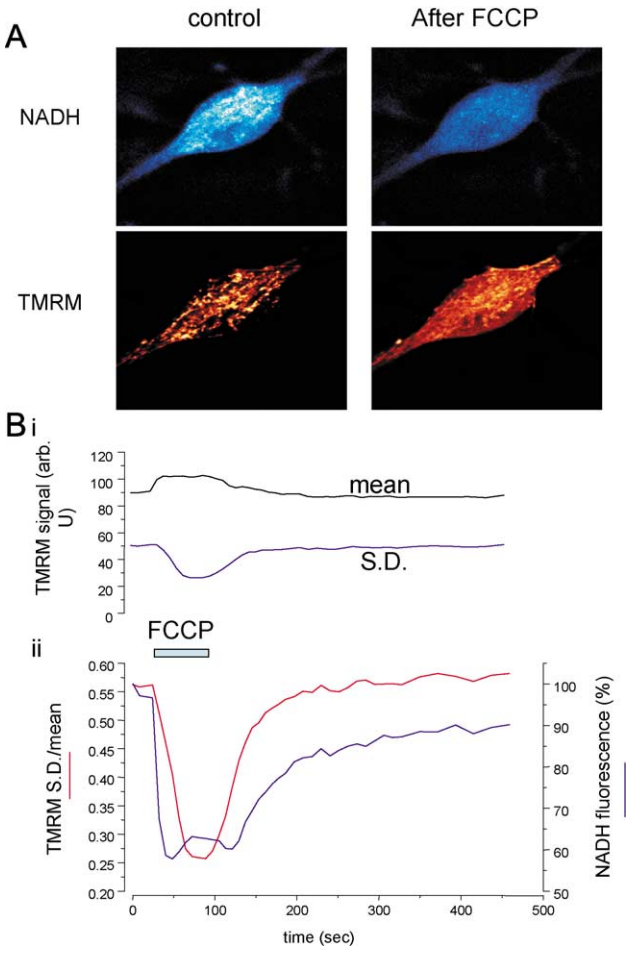

Fig. 2. Basic principles of the measurement of mitochondrial potential ($\Delta\Psi_m$) and NADH autofluorescence. Hippocampal neurons in culture were equilibrated with 30 nM TMRM and images of NADH autofluorescence and TMRM fluorescence (A) were acquired simultaneously on a confocal imaging system (Zeiss 510), illuminating alternately at 543 nm (TMRM—emission at 590 nm) and at 351 nm (NADH—emission measured between 435 and 475 nm). Application of the protonophore uncoupler FCCP (1 μM as indicated) depolarises $\Delta\Psi_m$ and increases the rate of respiration causing oxidation of NADH to NAD^+, seen as a loss of fluorescence signal as indicated in A. The collapse of $\Delta\Psi_m$ after FCCP also causes a redistribution of the TMRM throughout the cell (seen in (A)). However, only a very small increase in the mean cell signal due to slight dequench occurred. This effect is barely apparent when the only mean fluorescence intensity is measured (plotted in panel (Bi)). The redistribution is most effectively quantified through measurement of the standard deviation (S.D.) of pixel intensity in the image series (see panel (Bi)) and then expressed as the ratio of the S.D. to the mean. The change in $\Delta\Psi_m$ (red) and in NADH signal (blue) are plotted with time in panel (Bii) (also see: Duchen et al., 2001; Toescu and Verkhratsky, 2000). The time of application of FCCP is represented by stippled bar.

Alternatively, using higher concentrations of dyes, the high concentration of dye in the mitochondria causes

autoquenching: mitochondrial depolarisation then allows the loss of dye from the mitochondria with little change in the mitochondrial signal but an increase in the cytosolic signal which is readily measured. In practical terms, this is often the more convenient and reliable approach to use. This approach does not cause artefacts due to changes in plasma membrane potential, as the cytosolic signal remains in equilibrium with the mitochondrial dye fraction (for a detailed discussion of solutions to these issues, see Duchen et al., 2001). It is also important to be careful with all these indicators, as almost all of the mitochondrially localised dyes are photosensitizing agents. Prolonged illumination causes phototoxicity and eventually oxidative damage and mitochondrial depolarization (see Duchen, 2000; Zorov et al., 2000; Jacobson and Duchen, 2002).

3.2. Measurement of mitochondrial redox state

In addition to monitoring $\Delta\Psi_m$, it is also possible to measure changes in the redox state of NADH and $FADH_2$. Peak excitation for NADH fluorescence is obtained around 350 nm with an emission maximum around 450 nm. The oxidized form of the pyridine nucleotide (NAD^+) is not fluorescent. Thus, an increase in the emission at 450 nm indicates an increase in the ratio of NADH/NAD^+. These signal changes do not indicate an increase in the total pool of pyridine nucleotides, but rather a conversion between oxidized and reduced forms. Flavoproteins are also fluorescent but in this case, it is the oxidised form, which is fluorescent when excited at around 450 nm (with an emission peak around 550 nm), whereas the reduced form has limited fluorescence. These measurements give an indirect index of the balance between the supply of substrate and oxidation and can be useful as an indicator of changing oxygen consumption—otherwise very difficult to measure from single cells (see Fig. 2).

4. Ca^{2+} and mitochondria

As in all excitable cells, activation of voltage-gated Ca^{2+} channels represents a major routine pathway for Ca^{2+} influx in neurons. Many neurotransmitter receptors routinely expressed by neurons have a significant Ca^{2+} permeability, including excitatory amino receptors such as NMDA, kainate, and some classes of receptors for AMPA (see Carriedo et al., 2000), acetylcholine and serotonin. $[Ca^{2+}]_c$ can also be mobilised from the endoplasmic reticulum through the activation of metabotropic glutamate receptors. We should not forget $[Ca^{2+}]_c$ signalling in astrocytes and microglia, which, as non-excitable cells depend critically on the mobilisation of intracellular Ca^{2+} stores to generate $[Ca^{2+}]_c$ signals. The precise spatiotemporal patterning of $[Ca^{2+}]_c$ signals is absolutely central to the coordination of signalling in the CNS. The functional significance of these signals remains poorly understood when compared to neuronal $[Ca^{2+}]_c$ signalling, but interest has been recently renewed by the discovery that astrocytes can release ATP and glutamate which can in turn modulate neuronal activity (Haydon, 2001; Newman, 2001). Such feedback cycles have recently been implicated in the toxicity of the HIV-AIDS related viral coat protein, gp 120, in relation to AIDS dementia (Bezzi et al., 2001).

Isolated mitochondria have an enormous capacity to accumulate Ca^{2+} driven by an electrochemical potential provided largely by the mitochondrial potential and a low intramitochondrial Ca^{2+} concentration which is in turn maintained by export via a Na^+/Ca^{2+} exchanger. When the cytosolic Ca^{2+} is raised, through any of the mechanisms outlined above, it will accumulate into mitochondria, mediated by a uniporter whose structure remains unknown (but see Beutner et al., 2001). The mitochondrion thus serves as a high capacity, relatively low affinity buffering system removing Ca^{2+} from the cytosol when concentrations rise above 500 nM (for review, see Nicholls and Budd, 2000; Duchen, 1999). The major physiological role of this pathway is the activation of the rate limiting enzymes of the TCA cycle, which will increase the flux through the respiratory chain and increase the rate of ATP production (McCormack et al., 1990; Rizzuto et al., 2000; but see Nicholls and Budd, 2000). Here, we are more concerned with the pathological consequence of maintained pathologically high $[Ca^{2+}]_m$ which can initiate pathology in the mitochondria, on one hand, and with disorders in mitochondrial function that may interfere with the normal participation and integration of mitochondrial Ca^{2+} handling in cell signalling on the other.

5. Ca^{2+} overload, mitochondria and cell death: acute excitotoxicity

5.1. Ca^{2+} overload as an inducer of mitochondrial damage

It has long been clear that elevation of the concentration of free cytosolic Ca^{2+} is critical for many, but not necessarily all types of neuronal cell death (Choi, 1988). The most intensively studied model of cell death induced by Ca^{2+} overload must surely be that of excitotoxic cell death, whereby neurons die in response to prolonged exposure to the excitatory amino acid, glutamate. This is usually studied as a model of the cell death that extends the penumbra of a stroke, as glutamate can accumulate to high concentrations in ischaemic brain spreading to areas outside the focus of acute ischaemia (Szatkowski and Attwell, 1994). It is widely believed that some more subtle form of the same process may contribute to significant pathology in a more insidious chronic process to cause neurodegeneration in other disease models. Early studies assumed that large elevations of $[Ca^{2+}]_c$ per se were toxic, a phenomenon sometimes referred to as 'Ca^{2+} overload'. More recent experiments have suggested that rather than a simple and

inevitable consequence of a high global $[Ca^{2+}]_c$, the route of Ca^{2+} entry may play a crucial role in defining the outcome, although this remains slightly contentious. Such 'source specificity' seems arise as a consequence of microdomains of high $[Ca^{2+}]_c$ established in critical regions of the cell where activation of localised enzyme systems may play an important role in triggering neuronal death (see Tymianski et al., 1993; Sattler et al., 1999).

In many experimental systems, (e.g. in preparations of hippocampal neurons and cerebellar granule cells), glutamate neurototoxicity depends primarily on the activation of NMDA receptors, which are the most highly Ca^{2+} permeant of the subtypes of glutamate receptor. Features of NMDA-mediated excitotoxicity include extracellular Na^+-dependent cell swelling and a Ca^{2+}-dependent process leading to cell death (Choi, 1988). Cell death can be prevented by removal of extracellular Ca^{2+}, firmly establishing raised $[Ca^{2+}]_c$ as a trigger for excitotoxic neuron death, and by blockade of NMDA receptors. Following glutamate application, $[Ca^{2+}]_c$ follows a stereotyped pattern. $[Ca^{2+}]_c$ rises transiently, then staying at a plateau or with an incomplete recovery towards baseline. Following a variable latency, $[Ca^{2+}]_c$ rises again, probably into the μM range, closely accompanied by the collapse of mitochondrial potential (see Fig. 3). Once this has happened, $[Ca^{2+}]_c$ is independent of external Ca^{2+}, and either the plasmalemmal Ca^{2+} ATPase is non-functional, or ATP has been depleted, preventing its operation. The presence of glutamate is only required for the initial $[Ca^{2+}]_c$ transient, although some of the latter phases of the response are also sensitive to NMDA receptor antagonists (e.g. see Vergun et al., 1999) suggesting that glutamate release, either in response to reverberating circuits in the cultures or due to reversed uptake, contributes to sustained Ca^{2+} influx. Nevertheless, it appears that the initial transient $[Ca^{2+}]_c$ rise is pivotal in activating a Ca^{2+}-dependent process responsible for the necrotic cell death.

Under conditions where $[Ca^{2+}]_c$ is elevated due to activation of excitatory amino acid receptors, $[Ca^{2+}]_m$ can rise substantially (Peng and Greenamyre, 1998). Recent evidence has suggested that the rise in $[Ca^{2+}]_m$ plays a central role in initiating the progression to neuronal death under these circumstances. Thus, limiting mitochondrial Ca^{2+} uptake simply by depolarisation of mitochondrial potential protects neurons from delayed cell death following NMDA exposure (Stout et al., 1998; Nicholls and Budd, 2000). These experiments also show that the collapse of $\Delta\Psi_m$ per se cannot be the primary cause for delayed neuronal death following NMDA application, which must be somehow a consequence of the process initiated within mitochondria by the accumulated Ca^{2+} that causes the collapse of $\Delta\Psi_m$.

In the presence of physiological amounts of phosphate, it is estimated that mitochondria can accumulate and retain mM concentrations of total Ca^{2+} (Nicholls and Budd, 2000). However, excessive Ca^{2+} accumulation has at least two deleterious effects, it can lead to the opening of a high conductance pathway referred to as the mitochondrial permeability transition pore (MPTP), and to the deposition of Ca^{2+}-phosphate precipitates. It has also been suggested that high $[Ca^{2+}]_m$ may increase the rate of mitochondrial ROS generation (Dugan et al., 1995) although more recent data suggest that calcium alone may not suffice but may need also to be associated with some damage to the respiratory chain (Votyakova and Reynolds, 2001).

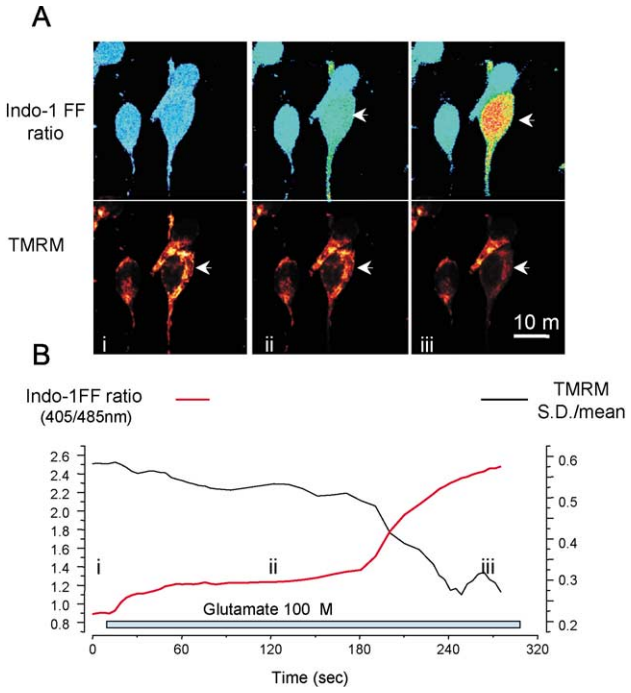

Fig. 3. Changes in $\Delta\Psi_m$ and in $[Ca^{2+}]_c$ in response to the application of glutamate. Hippocampal neurons were loaded with the low affinity $[Ca^{2+}]_c$ indicator, Indo-1 FF (5 μM of the AM ester for 20 min) and then equilibrated with 30 nM TMRM, before exposure to 100 μM glutamate for the period indicated in (B). Indo-1 FF fluorescence was excited at 351 nm and emission measured on two channels at 380–430 and at 470–500 nm, while the TMRM fluorescence was excited at 543 nm and measured at >590 nm. The ratio of Indo-1 emission at 380–430 and at 470–500 is a function of $[Ca^{2+}]_c$ and is shown in (A) at three times (i–iii) as indicated below (red trace) in (B). Glutamate caused a rapid increase in $[Ca^{2+}]_c$ followed after a delay of a few minutes by a secondary massive increase. $\Delta\Psi_m$ was assessed as described in Fig. 2 from the TMRM signal shown at times i–iii in (A) and as the ratio of the S.D./mean of pixel intensities of the TMRM signal (grey trace) in B. These data show a massive redistribution of signal corresponding in time with the secondary $[Ca^{2+}]_c$ increase. These changes were seen only in one cell of four in this field. In the other three cells, $[Ca^{2+}]_c$ rose and then stayed at a plateau level, and no change in TMRM distribution ($\Delta\Psi_m$) was seen.

5.2. Inducers of cell damage: the mitochondrial permeability transition pore (MPTP)

The MPTP is a large conductance pore that forms under pathological conditions in the inner mitochondrial membrane. The exact structure of the MPTP has proven elusive,

although elements of the structure seem to include an adenine nucleotide translocator (ANT), the voltage-dependent anion channel (VDAC), and cyclophilin D, which confers sensitivity of the complex to cyclosporine A, which closes the pore (see: Crompton, 2000). Other proteins—Bcl-2, the peripheral type benzodiazepine receptor—may be associated with the pore. The channel (also called the 'mitochondrial megachannel') is characteristically opened by a combination of high $[Ca^{2+}]_m$, oxidative stress, ATP depletion, high inorganic phosphate and mitochondrial depolarization (for a recent review, see Crompton, 2000). Pore opening is inhibited by Mg^{2+}, ADP, cyclosporin A and modulators of the adenine nucleotide translocase (e.g. bongkrekic acid). It is currently unknown whether the MPTP plays any normal physiological role in mitochondrial homeostasis, or whether it represents only a pathological process—there is some evidence in support of both proposals (see Crompton, 2000; Smaili et al., 2001). It is possible that the pore may manifest in various conductance states and these may define the consequence of its opening. Thus, transient reversible openings of a low conductance state might be expected to have quite different consequences to irreversible opening of a high conductance pathway, which causes mitochondrial swelling, and initiates cytochrome *c* release, caspase activation and apoptotic cell death (see Ichas, this volume, and below), or the collapse of the mitochondrial potential, ATP consumption and depletion and energetic collapse followed by necrotic cell death. As a potential pharmaceutical target, it is of great importance to be clear whether this process contributes significantly to cell death in pathological processes in vivo—there is certainly evidence for this both in the heart (Halestrap, 1999) and in brain (Friberg et al., 1998; Li et al., 2000).

5.3. Inducers of cell damage: the role of free radical species

It seems clear that while Ca^{2+} alone is required, it is not sufficient to cause cell death in these models of cellular Ca^{2+} overload. There are a number of additional factors that have been invoked as contributors to the extent of NMDA-mediated cell death: the generation of reactive oxygen species (ROS), the status of antioxidant defences in the cell or contributions to the calcium signal through the release of Ca^{2+} from endoplasmic reticulum stores. The generation of ROS is closely linked to mitochondrial respiration and mitochondrial function. In addition, nitric oxide (NO) has recently emerged as a central factor in defining the source specificity of NMDA-mediated neurotoxicity. We have found that inhibition of NOS by L-NAME attenuated the glutamate induced loss of $\Delta\Psi_m$ and NO exposure in combination with an otherwise innocuous Ca^{2+} load now provoked mitochondrial depolarization (Keelan et al., 1999). Thus, it appears that elevated $[Ca^{2+}]_c$ acts synergistically with NO to augment mitochondrial depolarization. The 'source specificity' for Ca^{2+} neurotoxicity appears to result from the co-localisation of the NMDA receptors and nNOS through the action of the scaffolding protein PSD-95. Sattler et al. (1999) showed that suppression of PSD-95 expression selectively reduced cell death and NO generation in response to NMDA, without altering the net Ca^{2+} influx. Another interesting speculation involves the possible role of NO generated by an intramitochondrial NOS, although even the existence of such an enzyme remains controversial (see Gharifourifar et al., 1999).

In our hands, in a model of NMDA toxicity in hippocampal neurons, the generation of ROS—superoxide anion, hydroxyl radicals and singlet oxygen—appears to play no part in the collapse of mitochondrial potential (Vergun et al., 2001), although this may not apply to other model systems. Thus, a variety of free radical scavengers had no effect at all on the rate of mitochondrial depolarisation. These studies also revealed that such agents may have a substantial direct effect on the NMDA channels, limiting Ca^{2+} influx and this alone may account for the neuroprotective actions of these agents. Nevertheless, much previous work has shown that ROS are neurotoxic (see: Beal, 1998). While we found no evidence of increased ROS production in the short-term in hippocampal neurons exposed to glutamate (Vergun et al., 2001), others have shown increases in free radical generation following NMDA application to cultured neurons (Lafon-Cazal et al., 1993; Castilho et al., 1999). In the latter study, using electron paramagnetic resonance, increase in ROS generation was delayed in relation to the time course of mitochondrial depolarisation and so may reflect a separate converging pathway that contributes to cell death.

Sub-populations of AMPA and kainate receptors also have high Ca^{2+} permeability and activation of these receptors may cause mitochondrial depolarization and cell death in motoneurons in culture (see Carriedo et al., 2000). In this model, AMPA and kainate appear to increase the rate of ROS formation, and agents that inhibit mitochondrial Ca^{2+} uptake reduce both ROS generation and motoneuron injury (Carriedo et al., 2000). As different neuronal populations differ in the specific numbers and types of glutamate receptors present on the neuronal surface, it is likely that variations in the extent of Ca^{2+} influx will occur in response to activation of glutamate-gated receptors.

Glutamate is able to cause cell damage and death even in cells that do not express ionotropic (or metabotropic) receptors. This toxic process is mediated by the depletion of glutathione (GSH) by competition between glutamate and cysteine for the cysteine transporter, preventing the supply of cysteine that is required for resynthesis of GSH (Murphy et al., 1989; Schubert and Piasecki, 2001), a process which has been referred to as "oxidative" glutamate toxicity. As GSH functions as a major anti-oxidant, removing ROS, this oxidative toxicity involves an increase in net mitochondrial ROS production, following the depletion of antioxidant defence mechanisms (Maher, 2001). The extent to which this mode of cell injury contributes to cell death in vivo in the CNS is not at all clear, but seems likely to be modest,

given the degree of protection conferred by inhibition of NMDA receptors. Oxidative damage may be particularly evident in cultured neurons, or neurons in vivo, at periods in development when NMDA or other excitatory amino acid receptors are not yet fully functional or mature.

5.4. Ca^{2+} buffering and modulation influx pathways

The effect of a given total Ca^{2+} influx on changes in $[Ca^{2+}]_c$ will also depend on neuronal Ca^{2+} buffering power. This varies substantially between populations of neurons, and seems particularly low in motoneurons which are more likely to show degeneration in ALS (Vanselow and Keller, 2000). Although mitochondria contribute substantially to intracellular Ca^{2+} buffering, there are other important intracellular Ca^{2+} buffers such as binding proteins. It has been suggested that low buffering power may play a particularly important role in defining the vulnerability of cells following an excitotoxic stimulus (D'Orlando et al., 2001) and a reduction of Ca^{2+} buffering proteins in motoneurons has been proposed as a cause of selective vulnerability to excitotoxic injury in ALS (Vanselow and Keller, 2000). These arguments are supported by the observation that the cells are protected from injury in transgenic mice overexpressing both a mutant superoxide dismutase (mSOD) gene and parvalbumin have delayed disease onset and longer survival than mSOD overexpressing mice without parvalbumin overexpression (Beers et al., 2001).

Activation of protein kinase C (PKC) prior to glutamate exposure increases neuron death in cultured neurons and in non-neuronal cell lines transfected with NMDA receptor subunits (Felipo et al., 1993; Wagey et al., 2001). Although PKC activation can augment Ca^{2+} influx through NMDA receptors and so increase cell death by increasing Ca^{2+} load, this does not appear to apply in all circumstances (see Wagey et al., 2001). In part, this is because PKC acts on the c-terminus of the NR2A subunit of the NMDA receptor, so promoting cell death in cells expressing NMDA receptors containing this subunit. In NMDA receptors without the NR2A subunit, PKC activation may have other effects. In turn, PKC activation in neurons probably results from Ca^{2+} influx, involving translocation of PKC from the cytosol to the membrane, and its autophosphorylation. PKC activation appears to be dependent on the shape and amplitude of the $[Ca^{2+}]_c$ transient (Hasham et al., 1997). So it seems plausible that PKC is involved in a cycle in which NMDA receptor activation leads to rapid activation of PKC and that activated PKC, will in turn, alter the flux of Ca^{2+} through NMDA receptors and regulate Ca^{2+}-dependent inactivation of these receptors (Lu et al., 2000). Ca^{2+} entry through NMDA receptors and other Ca^{2+}-permeable receptors will of course also activate Ca^{2+}/calmodulin-dependent kinases (CaMKs), calcineurin, nitric oxide synthase, as well as other protein kinases and protein phosphatases. Whether these protein kinases interact with mitochondria and alter their function is not clear.

6. Mitochondrial dysfunction as a cause of neurodegenerative disease

Given the importance of mitochondria to cell function, it seems remarkable that it is only recently that mitochondrial damage has been directly linked to human disease (see: Beal, 1998; Cassarino and Bennett, 1999; Schapira, 2002). A number of diseases have been associated with specific defects in mtDNA leading to defects of the electron transport chain. One difficulty in correlating specific defects in mitochondrial function with a given clinical expression of disease is the delayed development of a disease phenotype, possibly after months or years, which is difficult to model using tissue culture techniques. Furthermore, differences in lifespan or anatomy detract from comparisons between animal models and human disease. The creation of animal models of mitochondrial disease has proven difficult mainly because of the difficulties in introducing site directed mutations into the mtDNA or targeting DNA to mitochondria. Also, because cells generally contain multiple copies of the mtDNA (estimated at ~1–5000 copies per cell—see Jacobs, 2001), establishing a dominant mutant mtDNA population experimentally is difficult.

Specific abnormalities in mtDNA give rise to a group of diseases broadly referred to as the encephalomyopathies, and illuminate the role of mitochondria in disease. Many of these diseases are characterised by recurrent or progressive involvement of vulnerable organs with weakness, hepatic dysfunction, or abnormal visual or cerebral function. It is often stated that these diseases involve tissues with high metabolic rates, such as brain, eye, liver and muscle, but the basis for involvement of one specific tissue rather than another and the basis for an extreme variability in the extent of disease between patients remains obscure (see Nardin and Johns, 2001). As opposed to this group are several newly described mitochondrial disorders, in which nuclear genes encoding proteins targeted to mitochondria are involved (see Schapira, 2002).

One interesting approach to study the consequences of mitochondrial disorders on cell function has been the use of cytoplasmic hybrid cells (cybrids), in which ρ^o cells (where mtDNA has been destroyed) are fused with cells carrying mtDNA defects (Swerdlow et al., 1997; see Cassarino and Bennett, 1999). Further studies have shown that in many neurodegenerative diseases, for example, Alzheimer's disease, Parkinson's disease and ALS, either mitochondrial structure or function is altered (see Beal, 1998). These findings raise the tantalising possibility that some aspects of the neurodegenerative process might be modifiable by agents that modulate mitochondrial function. Potentially therapeutic agents could include anti-oxidants, inhibitors of NOS, or agents that regulate mitochondrial Ca^{2+} flux, all interfering with the mechanisms discussed above.

7. Role of mitochondrial abnormalities in neurodegenerative disease. Is a mitochondrial disorder a predisposing factor in neurodegenerative disease?

Mitochondrial abnormalities have been identified in a large proportion of neurodegenerative diseases. Biochemical analysis of CNS tissue from patients with neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease and ALS has yielded evidence for abnormalities of components of the electron transport chain. However, whether these abnormalities are primary and related to the causation of the disease, or whether they represent a consequence of the disease process remains unresolved. All of these neurodegenerative diseases also share a common feature in that they have abnormal protein aggregations: synuclein in Parkinson's disease, mutant superoxide dismutase (mSOD) in familial ALS (FALS, a heritable subset of patients with ALS), amyloid beta (Aβ) peptide in Alzheimer's disease and Huntingtin and Frataxin in Huntington's disease and Friedreich's ataxia, respectively. It is not known what if any relation exists between mitochondrial dysfunction and the accumulation of these protein aggregates, and, indeed, to the development of the disease phenotype. However, evidence is accumulating to suggest that interplay between abnormal protein aggregates and abnormal mitochondrial function may play a key role in the development of these diseases.

Thus, the most impressive model of Parkinson's disease, that of MPTP (1-methyl-phenyl-4-phenyl-1,2,3,6,-tetrahydropyridine) toxicity experienced by drug users, is associated with damage to complex I of the mitochondrial respiratory chain by the derivative MPP^+ (Langston et al., 1983). Complex I deficiency has also been found in the substantia nigra in patients with sporadic Parkinson's disease (Mizuno et al., 1989; Parker et al., 1989; Schapira et al., 1989). Indeed, it was recently suggested that Parkinson's disease at least in some patients may be associated with the toxicity of insecticides targeted to Complex I (Betarbet et al., 2000). Frataxin is thought to be important in the transport of iron in mitochondria, leading to abnormalities of iron processing which is so important in the normal construction of complexes II and IV of the respiratory chain (Babcock et al., 1997).

In ALS, as in most of these diseases, the long periods between the onset of disease and the analysis of tissue have made the evaluation of mitochondrial abnormalities at post-mortem difficult to interpret. Attempting to circumvent this problem, Vielhaber et al. (2000) evaluated muscle biopsy specimens from patients with ALS and found differences in the activities of cytochrome oxidase (complex IV, COX) between saponin-permeabilized muscle fibres from patients with the sporadic (non-familial) ALS and control subjects. They also found low levels of Mn-SOD in muscle fibres from the ALS patients and multiple mtDNA deletions in 1 of 17 patients. A mutation of a mtDNA-encoded subunit of COX has also been described in a patient with sporadic amyotrophic lateral sclerosis (Comi et al., 1998).

Remarkably, a rare, non-progressive neurological disorder ('konzo') associated with impaired gait seen in rural areas of Africa has been tentatively linked to consumption of cyanogenic compounds present in improperly processed casava plants (Spencer, 1999). The biochemistry of these compounds is poorly understood, but raises the possibility that mitochondrial dysfunction may be involved.

Furthermore, it is suspected, but not proven, that at least some neurodegenerative disorders express abnormal mitochondrial function at a comparatively early stage of the disease. This viewpoint is difficult to determine in human subjects, as mitochondrial function would have to be assessed at pre-symptomatic phases of a disease. However, support for this view has been provided by recent observations that mice overexpressing mSOD have morphological evidence of mitochondrial abnormalities early in the course of disease, prior to the rapid decline in motor function (Kong and Xu, 1998). What remains unclear is the mechanism by which these mitochondrial disorders might predispose specific classes of neurons to degeneration—motoneurons in ALS, basal ganglion cells in Wilson's disease, Huntington's disease and Parkinson's disease.

Evidence is also accumulating for a role of a mitochondrial defect in Alzheimer's disease. Thus, in many patients with Alzheimer's disease, cytochrome *c* oxidase (COX) activity is apparently impaired in the CNS (Kish et al., 1992; Mutisya et al., 1994), and even in other tissues, including platelets (Parker et al., 1994). In ρ^0 cells repopulated with mitochondria from Alzheimer's disease patients, mitochondria showed excessive ROS generation and $[Ca^{2+}]_c$ signalling in the cells was abnormal (Swerdlow et al., 1997). Recent studies have suggested that Aβ may be directly toxic to isolated mitochondria (Casley et al., 2002) and that it also causes a loss of cytochrome *c* oxidase activity in neurons in culture.

The association of these mitochondrial disorders with abnormal Ca^{2+} signalling and/or calcium-induced mitochondrial damage is less clear. In Alzheimer's disease suggested that exposure of neurons to Aβ raises $[Ca^{2+}]_c$ perhaps through the pore forming activity of the peptide (Arispe et al., 1993). In the mSOD over expressing transgenic mouse, motoneurons are protected by the over-expression of parvalbumin, suggesting that improved $[Ca^{2+}]_c$ buffering may be neuro-protective (see above and Beers et al., 2001). Indeed, it has been suggested that motoneuron degeneration may reflect chronic low grade glutamate excitotoxicity in a neuronal pool that has a selective vulnerability to glutamate through an intrinsically low $[Ca^{2+}]_c$ buffering power (Vanselow and Keller, 2000).

There are several additional ways in which sub-lethal mitochondrial disorders may cause subtle alterations in Ca^{2+} signalling that then cause alterations in neural function. These include increased ROS generation, impaired ATP production, or altered mitochondrial Ca^{2+} buffering.

8. Impact of increased mitochondrial ROS generation on cell signalling

Damage to the mitochondrial respiratory chain may cause an increase in the mitochondrial generation of ROS, depending on where in the chain the damage has occurred. Increased generation of ROS may alter cell function in many ways. In the context of this essay, ROS may specifically alter $[Ca^{2+}]_c$ signalling in cells through a variety of processes. Probably the best characterised system is that involving endoplasmic reticulum Ca^{2+} release through ryanodine and/or IP3 receptor-gated channels, both of which show an increase in the probability of opening in response to superoxide, probably through the role of thiol groups that play a critical role in channel gating (Sun et al., 2001). Various plasmalemmal proteins are also influenced directly by radical species—notably the Na^+/Ca^{2+} exchange and the L-type Ca^{2+} channel, which is also very sensitive to changes in metabolic state (see Smith et al., 1989). It is conceivable that sustained increases in the basal rate of mitochondrial ROS formation will lead to a gradual and progressive depletion of cellular antioxidant defences, increased $[Ca^{2+}]_c$, perhaps increased $[Ca^{2+}]_m$ so perhaps promoting a further increase in ROS generation, as high $[Ca^{2+}]_m$ has been shown to increase the rate of radical generation (Dykens, 1994). This sort of scheme contains the basic elements of a positive feedback loop that will ultimately lead to cell damage or even cell death, and may also account in some measure for the slow progression of many of the neurodegenerative diseases.

9. Mitochondria and cell death

Mitochondrial damage can cause cell death. If mitochondrial function is sufficiently damaged, oxidative phosphorylation will fail, and in neurons, glyclolytic ATP production will not suffice to maintain ATP levels. Indeed, the mitochondrial F_1F_0-ATPsynthase may 'reverse', acting as a proton translocating ATPase and consuming ATP at a considerable rate (e.g. see Leyssens et al., 1996). Once ATP is depleted, ionic homeostasis will fail, cells swell and death will follow rapidly. This is an important mechanism of cell death in pathological states and should not be underestimated or belittled.

Mitochondria also hold the key for the initiation of apoptotic or programmed cell death. In most (but not all) pathways of apoptosis, the release of mitochondrial cytochrome *c* is a key event in initiating the cascade of reactions that culminate in cellular autodestruction through the activity of caspases (Budd et al., 2000). The mechanism of cytochrome *c* release remains controversial (for discussion, see Jacobson and Duchen, 2001) and is clearly regulated by the pro- and anti-apoptotic proteins of the BH hand family (bax, bak, bid as pro- and bcl-2 and bclxl as anti-apoptotic proteins). The specific role of the MPTP in regulating cyt *c* release or the release of apoptosis inducing factor (AIF; Ferri and Kroemer, 2001) remains controversial (see Ichas, this issue).

A specific example of apoptotic neuron death occurs when neurons are deprived of growth factors, or when important enzymes in the regulatory pathways of growth factors are inhibited. For instance, a standard model to induce apoptotic cell death involves the application of the PKC inhibitor, staurosporine together with glutamate or alone (Maher, 2001; Yuste et al., 2002). Survival of immortalized hippocampal cells, and primary cortical neurons exposed to glutamate was enhanced activation of PKC by co-incubation with phorbol esters. This suggests that PKC activation in these cells triggers a cell survival mechanism, most likely mediated by the mitogen-activated protein kinase (MAPK) pathway, and more specifically involving extracellular signal-regulated kinase (Erk) and c-Jun NH2 terminal kinase (JNK) pathways (see Maher, 2001).

Recent work has suggested that motoneuron death in mice overexpressing mSOD and in tissue from ALS patients may be produced by apoptotic mechansisms (see Guegan et al., 2001). For instance, in mSOD mice, cytochrome *c* appears to redistribute from mitochondria to cytosol as the disease progresses (Guegan et al., 2001). However, there are differences in the neuropathology of transgenic mouse strains having different copy numbers of the mSOD gene. For instance, mice with fewer copy numbers of the G93A SOD1 gene do not show the vacuolar changes or swelling of mitochondria seen with high copy numbers (Stieber et al., 2000). This observation suggests that the mitochondrial pathology is related to the high mSOD copy number and that such changes may not be evident in patients with FALS with lower levels of the mSOD protein. In the G93A transgenic mouse with high copy number, the pro-apototic protein bax has been reported to translocate from the cytosol to the mitochondria. Cleavage of caspases 7 and 9 as well as the X-linked inhibitor of apoptosis protein (XIAP) are seen as the disease progresses from presymptomatic to end stages, indicating at least some activation of apoptotic cascades in the mSOD transgenic animal (Guegan et al., 2001). Further evidence for involvement of these apoptotic cascades in mSOD mice is provided by the data showing that overexpression of the anti-apoptotic protein, Bcl-2, or overexpression of a dominant negative mutant of caspase 1, delay disease progression (Friedlander et al., 1997; Kostic et al., 1997). However, typically motoneuron nuclei do not exhibit apoptotic features (Bendotti et al., 2001). The degree to which apoptotic mechanisms contribute to the neurodegenerative disorders in human disease remains controversial.

10. Coda

The involvement of mitochondria as targets of pathophysiological events seems to be clearly established. In glutamate toxicity and the pathological processes that accompany

the evolution of a stroke, mitochondrial [Ca^{2+}] overload triggers mitochondrial pathology, which in turn initiates the progression to cell death. Changes in $[Ca^{2+}]_c$ and in mitochondrial function are inextricably linked in the evolving pathology. Mitochondrial pathology has been increasingly implicated as a contributing factor in most of the major neurodegenerative diseases. In this area, the primacy of mitochondrial dysfunction, the precise part played by mitochondria in the evolution of disease and its relationship to cellular $[Ca^{2+}]_c$ signalling or other aspects of cellular pathophysiology remain poorly understood. This remains a field of study very much in its infancy in which the next few years are likely to bring exciting developments.

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