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# Mitochondrial calcium function and dysfunction in the central nervous system

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## Abstract

The ability of isolated brain mitochondria to accumulate, store and release calcium has been extensively characterized. Extrapolation to the intact neuron led to predictions that the *in situ* mitochondria would reversibly accumulate $Ca^{2+}$ when the concentration of the cation in the vicinity of the mitochondria rose above the 'set-point' at which uptake and efflux were in balance, storing $Ca^{2+}$ as a complex with phosphate, and slowly releasing the cation when plasma membrane ion pumps lowered the cytoplasmic free $Ca^{2+}$. Excessive accumulation of the cation was predicted to lead to activation of the permeability transition, with catastrophic consequences for the neuron. Each of these predictions has been confirmed with intact neurons, and there is convincing evidence for the permeability transition in cellular $Ca^{2+}$ overload associated with glutamate excitotoxicity and stroke, while the neurodegenerative disease in which possible defects in mitochondrial $Ca^{2+}$ handling have been most intensively investigated is Huntington's Disease. In this brief review evidence that mitochondrial $Ca^{2+}$ transport is relevant to neuronal survival in these conditions will be discussed.

### Keywords

mitochondria; brain; calcium; excitotoxicity; permeability transition; Huntington's Disease

Isolated, respiring mitochondria accumulate $Ca^{2+}$ when the concentration of the cation in their immediate environment rises above the 'set-point' at which the rates of uptake via the $Ca^{2+}$ uniporter and efflux via the mitochondrial $Na^+/Ca^{2+}$ exchanger are in balance [1] (Fig. 1a). With isolated brain mitochondria under plausibly physiological conditions in the presence of external adenine nucleotides and phosphate the set-point is close to 0.5μM [2], Fig. 1a, and this closely corresponds to values estimated for net $Ca^{2+}$ accumulation by mitochondria *in situ* within cultured neurons [3-6]. When more than 10nmol of $Ca^{2+}$ /mg protein has been taken up, the parallel accumulation of phosphate leads to the formation of a calcium phosphate complex in the matrix which effectively buffers the free matrix $Ca^{2+}$ ($[Ca^{2+}]_m$) at about 0.2 to 2μM[7,8], Fig. 1c, similar values being reported for *in situ* neuronal mitochondria [9]. The calcium phosphate complex is maintained by the high matrix pH [8] and its composition has been investigated by Kristian et al. [10] who performed X-ray spectral analysis of calcium phosphate precipitates in rapidly frozen $Ca^{2+}$-loaded brain mitochondria and reported a variable calcium to phosphate ratio depending on loading conditions, suggesting that the composition of the complex is somewhat flexible. This matrix $Ca^{2+}$ complexation in turn clamps the activity of the $Na^+/Ca^{2+}$ exchanger at a value that is independent of any further increase in total matrix $Ca^{2+}$ [11], Fig. 1b, and under these conditions the set-point is independent of the total matrix $Ca^{2+}$. The result is that the isolated mitochondria can act as perfect 'buffers' of free $Ca^{2+}$ in their vicinity, responding to multiple additions of $Ca^{2+}$ to the

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medium by lowering the extra-mitochondrial free $Ca^{2+}$ concentration ($[Ca^{2+}]_e$) back to the set-point after each addition (Fig. 1d). This can continue until in excess of 500nmol $Ca^{2+}$ /mg protein has been accumulated and the onset of bioenergetic collapse associated with the permeability transition [8].

The inference from these *in vitro* studies is that mitochondria within intact neurons will act as temporary reversible stores of $Ca^{2+}$, accumulating the cation when the cytoplasmic free $Ca^{2+}$ ($[Ca^{2+}]_c$) is above the set-point, for example following a train of action potentials, and releasing the cation back to the cytoplasm when the plasma membrane $Ca^{2+}$-ATPases succeed in pumping down $[Ca^{2+}]_c$ to below the set-point in the subsequent resting phase. As will be discussed below, there is convincing evidence that this occurs in intact neurons, lowering peak values of $[Ca^{2+}]_c$ but extending the duration of elevated cytoplasmic $Ca^{2+}$, and modulating cell signaling and transmitter release.

For this cytoplasmic buffering to occur with no deleterious consequences for the mitochondria and hence the cell, it is clearly essential that the period during which $[Ca^{2+}]_c$ is elevated above the set-point is brief to avoid mitochondrial $Ca^{2+}$ overload. If however a tonic activation of $Ca^{2+}$ entry pathways across the plasma membrane is sufficient to retain $[Ca^{2+}]_c$ above the set-point, then an inexorable accumulation of $Ca^{2+}$ within the matrix will occur until maximal capacity is exceeded and the permeability transition ensues, initiating rapid necrotic cell death. Conversely, in a quiescent neuron where $[Ca^{2+}]_c$ is close to 0.1μM the mitochondrial matrix will be largely depleted of $Ca^{2+}$, the calcium-phosphate complex will not form and $[Ca^{2+}]_m$ will vary as a function of $[Ca^{2+}]_c$. Isolated mitochondria smoothly transition into the regulatory mode when the total matrix $Ca^{2+}$ is decreased below 10nmol/mg [8]. This ‘regulatory’ rather than ‘storage’ range can control the activity of the TCA cycle in many cells (reviewed in [12]).

Under conditions of respectively, low, intermediate and excess matrix $Ca^{2+}$ accumulation, mitochondria in neurons may therefore be involved in intra-mitochondrial metabolic regulation, in controlling the extent and duration of cytoplasmic $Ca^{2+}$ elevations and in the extreme case in the very life or death of the neuron. Studies on the roles of mitochondrial $Ca^{2+}$ transport in the brain may thus be divided into the ‘physiological’ where the uptake of $Ca^{2+}$ into the matrix is reversible and serves a regulatory role and the ‘pathological’ that generally involves activation of the mitochondrial permeability transition. These will now be considered separately.

## 1. ‘Physiological’ transport of $Ca^{2+}$ by brain mitochondria

Mitochondrial $Ca^{2+}$ uptake in neurons may play multiple roles, including facilitating the uptake of $Ca^{2+}$ through voltage and receptor activated $Ca^{2+}$ channels and decreasing feedback inhibition by a sub-membrane ‘calcium cloud’ [13]. Mitochondria will also compete for cytoplasmic $Ca^{2+}$ with the plasma membrane and endoplasmic reticulum $Ca^{2+}$-ATPases for cytoplasmic $Ca^{2+}$. Mitochondria could also prolong periods of elevated $[Ca^{2+}]_c$ in stimulated neurons by reversibly sequestering and releasing the cation. David and Barrett [14,15] have investigated how inhibition of mitochondrial $Ca^{2+}$ transport affects quantal release of transmitter during repetitive stimulation of mouse motor nerve terminals. Comparing responses in the presence of oligomycin with those after oligomycin plus antimycin A (to control for bioenergetic effects) the authors concluded that mitochondrial $Ca^{2+}$ uptake prevented desynchronization of quantal release and minimized depletion of releasable vesicles during tetanic stimulation. In contrast, inhibition of e.r. $Ca^{2+}$ uptake with cyclopiazonic acid had minimal effects on $[Ca^{2+}]_c$ or quantal release.

In investigating the physiology and pathology of neuronal mitochondrial $Ca^{2+}$ transport, however, it is important to take a holistic view of the mitochondrion and its *in situ* bioenergetics,

rather than focusing purely on the transport of the cation. Thus an elevation in $[Ca^{2+}]_c$ sufficient to allow net mitochondrial $Ca^{2+}$ accumulation imposes a dual energetic demand on the mitochondrial proton circuit. Firstly the elevation in $[Ca^{2+}]_c$ will activate plasma membrane and endoplasmic reticulum $Ca^{2+}$-ATPases with a resultant increase in ATP demand. Secondly, the direct uptake of the cation into the matrix utilizes the proton circuit and directly competes with mitochondrial ATP synthesis. Indeed it was shown many years ago that a high rate of $Ca^{2+}$ uptake could even lower the protonmotive force sufficiently to cause a transient reversal of the ATP synthase [16].

### 1.1 Isolated mitochondria

Brain mitochondrial preparations are inherently heterogeneous [7], as well as differing in $Ca^{2+}$-sensitivity between strains [17] and brain regions [18,19]. 'Non-synaptic' mitochondria obtained by density gradient centrifugation include mitochondria from neuronal cell bodies and glia. Treatment of the crude mitochondrial pellet with low concentrations of digitonin liberates pre-synaptic mitochondria from the associated synaptosomes [20]. Alternatively, mitochondria can be prepared from cultured neurons and glia by nitrogen cavitation [21].

When determining $Ca^{2+}$ uptake by mitochondria, a clear distinction must be made between the free and total cation. Determination of total matrix $Ca^{2+}$ with isolated mitochondria is straightforward, defined amounts of $Ca^{2+}$ can be added to the incubation while monitoring $[Ca^{2+}]_e$ to ensure uptake into the matrix [22]. Alternatively $Ca^{2+}$ can be released into the incubation by addition of a protonophore, although experiments with isolated mitochondria suggest that not all the $Ca^{2+}$ accumulated in the presence of phosphate is readily released by protonophore [10].

### 1.2 Mitochondrial $Ca^{2+}$ transport in intact neurons

Early functional imaging studies of neurons with cytoplasmic free $Ca^{2+}$ ($[Ca^{2+}]_c$) indicators [23] demonstrated that the neuronal cytoplasm can experience large elevations in $[Ca^{2+}]_c$ under a variety of stimulation conditions including elevated KCl [24], high frequency field stimulation [4] and NMDA receptor activation [25]. It was therefore predicted that the *in situ* neuronal mitochondria would reversibly accumulate $Ca^{2+}$ in response to elevated $[Ca^{2+}]_c$ [26]. Werth and Thayer [4] obtained evidence that mitochondria in cultured rat dorsal root ganglion neurons reversibly accumulated calcium in response to high frequency field stimulation, and this was subsequently extended by different groups to KCl and glutamate induced calcium loading [13,27,28]. It is relevant to note that the 'set-points' determined for isolated mitochondria [2] correspond closely to those reported for intact neurons [4], and that the values for $[Ca^{2+}]_m$ reported for $Ca^{2+}$ -loaded *in situ* neuronal mitochondria [29] also closely correspond to values obtained with isolated mitochondria [8]. This suggests that neuronal mitochondria do not utilize adjacent endoplasmic reticulum to facilitate their $Ca^{2+}$ accumulation, and may thus differ from mitochondria in cell lines with extensive e.r. [30].

As in the case of isolated mitochondria, it is important to maintain the distinction between total and free matrix $Ca^{2+}$ in intact neurons. David et al. [9], see also [31,32], monitored $[Ca^{2+}]_m$ in lizard motor terminals during trains of action potentials, using the low-affinity matrix-located indicator rhod-5N ($K_d$ 320μM). $[Ca^{2+}]_m$ increased to a plateau close to 1μM. Importantly, increasing the frequency of stimulation increased the rate at which $[Ca^{2+}]_m$ rose to this plateau, but did not affect the plateau itself, suggesting that the matrix has a large capacity to buffer $Ca^{2+}$ at this value. This closely corresponds to the data obtained with isolated mitochondria [8], where the calcium phosphate complex maintained $[Ca^{2+}]_m$ at about 2μM over a 50-fold range of total $Ca^{2+}$ [8]. In contrast values for $[Ca^{2+}]_m$ reported using targeted aequorins tend to be considerably higher, e.g. [33]. The reasons for these discrepencies remain to be elucidated.

The specific, focused manipulation of mitochondrial $Ca^{2+}$ in intact neurons is not trivial. Early studies where protonophores were employed to depolarize the mitochondria [6,34] of course dramatically affected the cell's bioenergetics. In cells with a high glycolytic capacity, the role of mitochondrial $Ca^{2+}$ transport can be investigated by comparing cell function in the presence of oligomycin with that in the presence of oligomycin plus an electron transport chain inhibitor [13]. Since ATP generation is already glycolytic following oligomycin, the further addition of an electron transport inhibitor will allow $\Delta\psi_m$ (and hence mitochondrial $Ca^{2+}$ transport) to decay with no further effect on the cell's bioenergetics. Even the widely employed $Ca^{2+}$ ionophore ionomycin must be used with great care, since it induces a dissipative $Ca^{2+}$ cycling across the inner mitochondrial membrane [35]. Since $[Ca^{2+}]_c$ (and hence $Ca^{2+}$ uniport activity) is elevated by the ionophore's action at the plasma membrane, this induces a potent mitochondrial uncoupling manifested by increased respiration [36]. Indeed low micromolar concentrations of the ionophore can collapse the mitochondrial membrane potential ($\Delta\psi_m$) in cultured neurons leading to rapid necrotic cell death [35]. It would be instructive to determine the proportion of the 20,000 references in the literature utilizing such $Ca^{2+}$ ionophores where this underlying bioenergetic effect is an unrecognized complication. It is generally accepted that the hexavalent cation ruthenium red is impermeant across the plasma membrane of most cells and that it acts by inhibiting $Ca^{2+}$ uptake into the cell (reviewed in [37]). Ru360 has been proposed to be a more selective, cell permeant uniport inhibitor [38], but again there is considerable uncertainty as to its ability to enter cells and act specifically at the mitochondrial $Ca^{2+}$ uniporter [15,37].

## 2. Pathological' transport of $Ca^{2+}$ by brain mitochondria

### 2.1 Isolated mitochondria

As will become apparent in this review, much of the investigation into the role of mitochondrial $Ca^{2+}$ transport in CNS diseases has focused around the induction of the permeability transition. There has been some controversy over the nature or even existence of the permeability transition in isolated brain mitochondrial preparations. In part this is because the light-scattering changes of isolated brain mitochondria during $Ca^{2+}$ loading differ from e.g. liver mitochondria. The initial stages of $Ca^{2+}$ accumulation by non-synaptic brain mitochondria produces an increase in light-scattering, probably due to the increased refractive index of the $Ca^{2+}$loaded matrix [8]. An early study [39] showed that cyclosporine A (CsA) failed to protect cultured hippocampal neurons against mitochondrial swelling and depolarization associated with $Ca^{2+}$-ionophore-induced $Ca^{2+}$ loading, although as discussed above this could have been a consequence of the unrecognized bioenergetic consequences of the ionophore addition. Subsequently, the ability of CsA to decrease the $Ca^{2+}$-sensitivity of isolated brain mitochondria has been shown by several groups [18,40,41]. In the presence of adenine nucleotides, it was found that the capacity of non-synaptic brain mitochondria to accumulate $Ca^{2+}$ during the slow continuous infusion of the cation could be greatly increased (from 600 to 1500nmol/mg protein) in the presence of CsA [8].

The nature and regulation of the mitochondrial permeability transition have been extensively reviewed recently [42] and discussion here will be limited to the brain. The calcium loading capacity of isolated brain mitochondria has traditionally been determined by sequential bolus additions of the cation while monitoring the ability of the mitochondrial incubation to restore a low free $Ca^{2+}$ concentration in the incubation. However, since each bolus addition creates a transient energy demand on the mitochondria while affecting mitochondrial membrane potential and matrix pH these may distort an accurate estimation of loading capacity which can be avoided by slowly perfusing calcium into the medium while monitoring the external free calcium concentration [8]. Under these conditions loading capacity can be separated from the bioenergetic demands that accompany bolus additions.

There is disagreement in the literature as to the ability of cyclosporine to delay the permeability transition during massive calcium loading. In the author's laboratory 1 μM cyclosporin A increased the calcium loading capacity of rat non-synaptic mitochondria from 800 to 1600 nmol/mg protein [8]. Species differences are frequently ignored, but it is significant that non-synaptic brain mitochondria from C57Bl/6J mice are able to accumulate approximately twice as much $Ca^{2+}$ as the corresponding mitochondria from Sprague-Dawley rats before induction of the permeability transition [17]. Synaptic mitochondria prepared from synaptosomes are more sensitive to $Ca^{2+}$, have a higher level of cyclophilin D and are more vulnerable to the permeability transition unless cyclosporine A is present [43-45]. Knock-down of cyclophilin D in PC12 cells decreases their sensitivity to the permeability transition [43] and mitochondria from CypD-knockout mice are resistant to the permeability transition [46]. Some of the variation in reported CsA sensitivity was explained by the observation of Bambrick et al. [47] that only astrocytic (but not neuronal) mitochondria displayed a CsA-sensitive $Ca^{2+}$ accumulation capacity. In addition permeabilized astrocytes could accumulate more $Ca^{2+}$ than permeabilized granule neurons on a per-cell or mitochondrial protein basis. Thus a failure to observe cyclosporin protection in neurons cannot be taken as evidence against a permeability transition. A number of cyclosporine A analogues have been developed that are more potent against the permeability transition while being less active as immuno-suppressants and calcineurin inhibitors [48-51] and can therefore provide less ambiguous information on the permeability transition in intact cells than CsA.

Even though the permeability transition is likely to be a stochastic process at the level of the individual mitochondrion, the release of $Ca^{2+}$ from an individual mitochondrion will increase the $Ca^{2+}$ concentration in its neighborhood. In a very dilute incubation (e.g. 0.05mg protein/ml in a perfusion study [8]) the likelihood is that $Ca^{2+}$ released by the neuronal sub-fraction will be accumulated by the astrocytic mitochondria and that the overall incubation will show the CsA sensitivity characteristic of the latter. However, in a relatively concentrated isolated mitochondrial incubation, and even more so in the intact cell, the probability of a chain reaction of $Ca^{2+}$ overload following an initial permeability transition in a sub-population will increase, in which case the mixed population might show the CsA-independent characteristics of the neuronal sub-population.

### 2.2 Monitoring the permeability transition in intact neurons and in vivo

The permeability transition in isolated mitochondrial preparations can be monitored by decreased light scattering, release of cytochrome c, $Ca^{2+}$ and low molecular weight matrix components and collapse of $\Delta\psi$. In the intact cultured neuron mitochondrial swelling can readily be detected by confocal imaging as a thread-like to globular shape transition reflecting matrix $Ca^{2+}$ loading [52,53] but this cannot automatically be equated with the permeability transition since it can in many cases be reversed with no apparent loss of function [52,54]. Mitochondria that have undergone the permeability transition are depolarized and do not accumulate membrane potential indicators, and are thus not visible in fluorescent microscopy. Elmore et al. [55] have employed the loss of fluorescence resonance energy transfer (FRET) between the membrane potential-independent MitoTracker Green and $TMRM^{+}$ and the resulting increase in direct MitoTracker fluorescence emission to provide a positive signal reflecting membrane potential collapse. An alternative method has involved the cytoplasmic loading of calcein, which leaves dark ‘holes’ corresponding to the mitochondria. Activation of the permeability transition leads to entry of calcein into the matrix and the disappearance of the holes [56].

## 3. Mitochondrial Calcium transport in CNS Disease

Neuronal dysfunction can be modeled by reproducing the actual insult in the intact animal (e.g. vessel occlusion in stroke studies) followed by isolation of mitochondria or synaptosomes.

Alternatively, cell cultures can be exposed to stresses related to those experienced *in vivo* (e.g. glutamate exposure of neuronal cultures to simulate the glutamate excitotoxicity associated with ischemia). The limitation with this approach is that the cell cultures are neonatal and may thus lack the critical age-related changes that play such a major role in the incidence of most neurodegenerative diseases. In addition the normal physiological interconnections and glial interactions are usually missing, while in the specific case of calcium transport, the extracellular volume in cell culture is vast compared with *in vivo* and the essentially infinite extracellular $Ca^{2+}$ compartment may exaggerate the magnitude of $Ca^{2+}$ accumulation occurring *in vivo*.

The role of mitochondrial $Ca^{2+}$ in this context can be approached at several levels, starting with the isolated mitochondria, and ranging through cell lines (including cybrids), primary neuronal cultures, isolated nerve terminals (synaptosomes), acute and organotypic tissue slices to *in vivo* rodent models. Except in the few cases where human material is available the neuronal dysfunction has to be created, for example by mouse genetic manipulation. If there is sufficient evidence to implicate an alteration of mitochondrial function, for example impaired Complex I function in Parkinson's Disease (PD), then the consequence of this can be investigated by *in vitro* or *in vivo* addition of Complex I inhibitors, although of course the relevance of the model is entirely dependent upon the strength of the *in vivo* evidence, and this approach gives little indication of the upstream or parallel events that might have caused the dysfunction in the disease itself. However, a similar pathology between the human disease and a chemical intervention is encouraging - for example the demonstration that systemic rotenone in rodents can cause a similar striatal pathology to the disease [57].

While the focus of this review is on $Ca^{2+}$ and its involvement in CNS dysfunction, it is essential to bear in mind that mitochondrial $Ca^{2+}$ transport, oxidative stress and bioenergetics form a closely inter-related network. Deficient ATP generation in a cell can lead to a failure of plasma membrane $Ca^{2+}$ pump activity and $Ca^{2+}$ overload; oxidative stress can restrict ATP generating capacity. $Ca^{2+}$ uptake into the cell and its further transport into the mitochondrion can put a heavy load on the mitochondrial proton circuit, in some cases leading to neuronal cell death induced by an energy crisis [58]. While it is frequently stated that $Ca^{2+}$ accumulation by *in situ* mitochondria results directly in oxidative stress, there is no known mechanism to cause this, and indeed a decrease rather than an increase in $H_2O_2$ is seen in respiring isolated rat brain mitochondria as they accumulate $Ca^{2+}$, consistent with a lowered $\Delta\psi$ [59].

As previously mentioned, much of the literature on mitochondrial $Ca^{2+}$ and neurological disease has focused on the permeability transition,. These studies have recently been extensively reviewed in relation to excitotoxicity [60], ischemia [61], traumatic brain injury [62], and Parkinson's and Alzheimer's Diseases [63]. A central question must be posed about any *in vitro* experiment in this context, and that is whether the proposed involvement of the permeability transition holds for the *in vivo* condition, rather than being an *in vitro* phenomenon, and if it does, whether it is directly mediated by some dysfunction unique to the disorder, of whether it is a more general manifestation of excitotoxicity. Finally, even if excitotoxicity is implicated as the underlying mechanism in the neuronal cell death, is it in turn caused by altered $Ca^{2+}$ handling by the mitochondrion, by oxidative stress (e.g. [64-66]), by impaired mitochondrial metabolism or energetics (e.g. [58,67,68]) or mitochondrial trafficking in the neuron (e.g. [69]), by altered ion transport at the plasma membrane or by some to other subtle prime cause? These questions of course determine whether it is worthwhile to look for changes in $Ca^{2+}$ handling of isolated brain mitochondria from disease models or whether cell culture or *ex vivo* preparations such as synaptosomes are more appropriate.

In this author's opinion, there is convincing evidence for the involvement of the permeability transition in excitotoxicity from the level of the cultured neuron to *in vivo* in association with

stroke, ischemia, traumatic brain injury and other conditions associated with receptor-mediated cell $Ca^{2+}$ overload. With the possible exception of Huntington's Disease (HD) evidence for a defect at the level of isolated mitochondrial $Ca^{2+}$ transport is sparse. The remainder of this short review will therefore focus on *in vitro* and *in vivo* excitotoxicity and models of HD.

### 3.1 Glutamate Excitotoxicity and Stroke

The strongest evidence for a patho-physiological role the permeability transition occurs in the context of neuronal cell death in the aftermath of stroke as a result of glutamate excitotoxicity. Chronic NMDA receptor activation by glutamate released from a focal infarct underlies the progressive expansion of the volume of necrotic tissue in the aftermath of stroke [70]. The characteristics of the NMDA receptor that are important for the primary roles of the receptor in learning and memory, namely $Ca^{2+}$ as well as $Na^{+}$ transport and only partial desensitization in the continued presence of the ligand, are potentially lethal for the neurons in the penumbra surrounding the infarct. The mechanism of the resulting cell death as a consequence of 'glutamate excitotoxicity' has been extensively studied at the level of the cultured neuron and some of the earliest studies from the group of Choi [71-74] established the essential role of $Ca^{2+}$ in cell death resulting from chronic NMDA receptor activation. Cortical neurons exposed to glutamate in the absence of calcium underwent extensive osmotic swelling, and in the presence of the cation this was followed by a delayed neuronal disintegration [72]. Choi proposed that calcium entry through the NMDA receptor played a dominant role in the subsequent cell death. Randall and Thayer [25] monitored real-time change is in cytoplasmic free calcium in hippocampal neurons exposed to glutamate and found that cell necrosis was preceded by a delayed massive rise in cytoplasmic free calcium concentration. This was further investigated by Tymianski and colleagues [75] who extensively examined the conditions for this stochastic 'delayed calcium deregulation' and concluded that the power play of calcium entry was equally or more important than the total calcium uptake, although subsequent studies with low affinity calcium probes [76] have revised this view.

The studies with isolated mitochondria discussed above led to predictions that the intra-neuronal mitochondria would be major sinks of calcium entering the neurons under conditions of extensive calcium loading and elevated cytoplasmic free calcium. In particular, if the non-desensitizing conductance of the NMDAR in the presence of exogenous glutamate is sufficient to maintain $[Ca^{2+}]_c$ above the $Ca^{2+}$ set-point for the *in situ* mitochondria, then the organelles will inexorably load with $Ca^{2+}$ until their capacity is exceeded [13]. It is notable that an inverse relationship is seen at a single cell level in a population of cultured neurons exposed to glutamate between the extent of the immediate slight$\Delta\psi_m$ depolarization reflecting the rate of $Ca^{2+}$ accumulation into the mitochondria, and the survival time before the neurons undergoes delayed $Ca^{2+}$ deregulation [77]. The extent of delayed calcium deregulation was found to be proportional to the time for which mitochondria remained polarized, suggesting that a critical factor was the amount of calcium accumulated by the mitochondria [78]. Furthermore, cells with active glycolysis could be protected against delayed calcium deregulation by inhibiting mitochondrial calcium accumulation with the combination of rotenone and oligomycin [13]. Interestingly, bulk cytoplasmic free calcium elevations in response to glutamate were depressed by this combination of inhibitors in these cells [78]. Since no decrease in calcium entry was detected, it was concluded that inhibition of calcium uptake by mitochondria in close apposition to the plasma membrane calcium ATPase could remove a competition between the mitochondria and the ion pump for calcium and enhance net calcium extrusion from the cell.

Cell death in response to mitochondrial calcium loading immediately suggests the involvement of the permeability transition. However, no consistent protection was been reported for cultured cerebellar granule neurons exposed to glutamate in the presence of inhibitors of the permeability transition pore such as cyclosporine A [78], while mitochondria isolated from

these cells showed no protection by cyclosporine A [79]. This is in contrast to the extensive protection afforded by the inhibitor during calcium loading of isolated brain mitochondria [8]. However, the report of cyclosporine A-sensitive $Ca^{2+}$ accumulation by mitochondria prepared from the synaptosomal fraction [80] indicates that there remain questions concerning the cellular and sub-cellular localization of permeability transition-prone mitochondria.

Mitochondria in glutamate exposed cultured neurons undergo an extensive swelling that is associated with a cessation of trafficking within the neurite and dendritic beading [53,81-82]. Synchronous with the elevations in cytoplasmic free calcium mitochondria undergo a massive depolarization [77]. The matrix pH changes associated with glutamate exposure are particularly revealing; from a starting pH close to eight a transient alkalinization is seen, followed by an acidification of the matrix to pH of about 7.1. At the same time a cytoplasmic acidification occurs and the Delta pH across the membrane is largely retained [83]. Delayed calcium deregulation is associated with a further matrix acidification and the abolition of the proton gradient. It is notable that the matrix acidification will lead to a destabilization of the extensive matrix calcium phosphate precipitate, and would be predicted to greatly increase the matrix free $Ca^{2+}$ [8]. Since the membrane potential and pH gradient collapse during the cytoplasmic calcium deregulation, and respiration is inhibited [84] this is consistent with a permeability transition.

While much of the focus in excitotoxicity is on $Ca^{2+}$ transport, it must be born in mind that the NMDAR allows the entry of both $Ca^{2+}$ and $Na^{+}$. Indeed, even though a typical NMDAR may have a $Ca^{2+}/Na^{+}$ selectivity of about 10, the concentration of $Na^{+}$ in the external medium is 100-fold higher than $Ca^{2+}$ with the result that $Na^{+}$ entry dominates. Recent studies on the bioenergetic demand of the resultant activation of the $Na^{+}/K^{+}$-ATPase to extrude the cation [85] have shown that under some circumstances the entire ATP generating capacity of the *in situ* mitochondria can be diverted to provide ATP for the $Na^{+}/K^{+}$-ATPase and accordingly that a variety of stresses that are in themselves non-lethal, such as partial Complex I inhibition [85], 'mild uncoupling' [86] and oxidative stress following glutathione depletion [84] can result in a bioenergetic deficiency that leads to rapid necrotic cell death. It is therefore important to bear in mind that $Ca^{2+}$-sensitivity in ischemic models can be greatly amplified by factors that limit ATP generation, as first proposed by Henneberry [87], see also [67].

Cyclosporine A has been shown to be neuroprotective *in vivo* in both focal and global ischemia [88,89]. The ultimate test for the involvement of the permeability transition in glutamate excitotoxicity accompanying cerebral ischemia is to investigate the *in vivo* consequences of Cyp D knockout. Liver, heart and brain mitochondria from Cyp-D deficient mice are resistant to $Ca^{2+}$ -induced swelling and show a capacity to accumulate $Ca^{2+}$ equal to those from control mice in the presence of cyclosporine A [46,90,91]. Importantly, the infarct volume 24hr after middle cerebral artery occlusion was reduced to less than 50% of control values [90].

### 3.2 Mitochondrial $Ca^{2+}$ transport in models of Huntington's Disease

Huntington's disease is the most studied of the group of autosomal-dominant trinucleotide repeat expansion diseases that include at least 10 poly-Q disorders (reviewed in [68,92,93]). The affected protein is huntingtin (htt) and the threshold for pathology occurs at >36Q repeats, corresponding to the appearance of aggregated htt. The severity and age of onset of symptoms are related to the repeat length which can be up to 120Q. Htt is an essential protein that is widely expressed in cells; thus the homozygous knockout mouse dies in embryo and heterozygous deletion of the htt gene results in neurodegeneration [93]. Despite this, its normal function is still debated. There is evidence that it functions as a nucleo-cytoplasmic shuttling protein involved in the regulation of transcription [92], and that it affects trafficking in neurons [94], while mitochondrial interactions have been shown [95]. HD is an aging-related disease and it has been proposed that a change in function of the expanded htt accelerates normal aging-

related changes in the brain [92]. A variety of transgenic mouse models of HD have been developed. Those expressing truncated human htt (e.g. R6/2 and R6/1) have a severe and widespread pathology and are severely diabetic, while those expressing full-length expanded human htt on a yeast artificial chromosome, such as the YAC128 mouse, show more selective pathology. Knock-in models producing CAG-expanded endogenous mouse htt generally display a mild phenotype [96].

Post-mortem analysis of mitochondrial respiratory chain complex activities from HD patients revealed a greater than 50% reduction in Complex II and Complex III activity, relative to protein or citrate synthase activity in the caudate, with a lesser decrease in Complex IV and no change in Complex I[97]. The putamen showed a more than 60% reduction in complex activities (again with the exception of Complex I) [98]. Cybrid technology, where platelets from HD patients were fused with SH-SY5Y neuroblastoma cells, failed to reveal any bioenergetic differences from control samples suggesting that nuclear DNA can correct any deficiencies in the platelets' mitochondria[99]. As in the case of Parkinson's Disease there is therefore persuasive evidence for respiratory chain dysfunction in the mitochondria of the surviving cells. It is therefore important in the present context to establish whether any apparent change in mitochondrial $Ca^{2+}$ handling reflects a primary action on mitochondrial transport or storage, or is a downstream consequence of the inhibited bioenergetic function.

The close association between restricted bioenergetic function and enhanced susceptibility to excitotoxicity has been discussed above. Indeed there is extensive evidence (reviewed in [100,101]) for the involvement of NMDAR function and excitotoxicity in cell death associated with HD. Significantly, a YAC transgenic mouse expressing 72 CAG repeats in htt showed enhanced glutamate excitotoxicity *in vivo* and in cultures of striatal medium spiny neurons [102], while the NMDAR antagonist MK801 reduced malonate-induced striatal cell death *in vivo* although it was without effect on lesion size in transgenic R6/1 mice [103]. The excitotoxic cascade discussed above may thus contribute to the neuronal susceptibility in HD, although the high susceptibility of the background strain (FVB/N) to excitotoxicity [104] means that the findings cannot automatically be extrapolated to the human disease. Fernandez et al. [101] monitored $[Ca^{2+}]_c$ and $\Delta\psi_m$ in parallel in single medium spiny neurons prepared from wild-type and YAC128 mice and observed that maximal NMDAR activation induced DCD (collapse of $\Delta\psi_m$ and failure to restore basal $[Ca^{2+}]_c$ after removal of NMDA) in the YAC128 neurons but not wild-type. No significant difference was observed in NMDAR currents and it was concluded that the mitochondria in the YAC128 cells were more sensitive to the permeability transition. A similar conclusion was reached in a study with immortalized striatal cells derived from Q111 mice [105], however again caution must be exerted in implying an excitotoxic mode of neuronal death in the disease.

While experimental Complex II deficiency with malonate or 3-nitropropionic acid (3NPA) can reproduce some of the pathology of the disease [106-108], using these inhibitors to ‘model’ the disease *in vitro* will not take account of any parallel or upstream consequences of the *in vivo* pathology and should be interpreted with caution.

The synergism between impaired mitochondrial ATP generation and susceptibility to glutamate excitotoxicity discussed above in the context of stroke is also apparent in striatal cultures exposed to 3NPA [109]. However somewhat surprisingly in view of the impaired mitochondrial bioenergetics implicated in the *in vivo* disease, the R6/1 and R6/2 mice show an increased resistance to glutamate excitotoxicity induced by quinolinic acid *in vivo* [110,111] which could not be ascribed to decreased NMDA receptor expression but which correlated with the appearance of nuclear inclusions. It was proposed that mutant htt causes a sub-lethal metabolic stress that causes cell defense mechanisms to be up-regulated against subsequent excitotoxic insult [103]. Thus medium spiny neurons from R6/2 mice showed an increased

basal $[Ca^{2+}]_c$ while at the same time being more resistant to quinolinate-induced delayed $Ca^{2+}$ deregulation [111].

Many studies have been performed with isolated brain mitochondria, with the constraints due to heterogeneity discussed above. In particular, since there is evidence that a normal physiological role of htt is in the control of anterograde/retrograde transport in neurons [94] isolated mitochondrial preparations will not be able to detect bioenergetic defects that are a consequence of an altered distribution of mitochondria throughout the neuron. A study by Panov et al. [112] investigated $Ca^{2+}$ transport in mitochondria prepared from transformed lymphocytes of control and HD patients and observed that the latter were depolarized by 12mV relative to the controls and showed a significant reduction in their $Ca^{2+}$ accumulation capacity in the absence of exogenous adenine nucleotides. Similar reductions in $\Delta\psi$ and $Ca^{2+}$ accumulation capacity in the presence of ADP and cyclosporine A were seen in brain mitochondria from transgenic mice expressing a yeast artificial chromosome with 72, but not 18, CAG repeats [112]. No difference in basal $[Ca^{2+}]_e$ was shown, but the YAC72 mitochondria took up $Ca^{2+}$ more slowly than those from YAC18 mice. In view of the reported respiratory chain defects it is possible that the slowed $Ca^{2+}$ uptake, and the partial initial depolarization reflects an inhibited respiratory chain. Respiratory parameters were not quantified in this study [112] but a subsequent study revealed no differences in respiratory rates between lymphoblast mitochondria derived from control and HD subjects [113].

Interestingly some effect was shown from the direct addition of 5μM htt fusion proteins with long but not short Q-repeats [112]. It was however subsequently reported that the fusion proteins exerted a direct inhibitory effect on the respiration of liver and lymphoblast mitochondria [95].

A decreased $Ca^{2+}$-accumulation capacity was reported for mitochondria prepared from clonal striatal cell lines containing expanded htt [114], but a detailed study by Brustovetsky et al. [18] presented different conclusions - no increase in $Ca^{2+}$-sensitivity was observed between striatal mitochondria from control and HD mice (knock-in and R6/2). Indeed in young mice expanded htt produced resistance to $Ca^{2+}$ [18]. Similarly, Oliveira et al. [115], using the slow $Ca^{2+}$ infusion technique [8] with isolated mouse forebrain mitochondria in the presence of adenine nucleotides, albumin and oligomycin to optimize mitochondrial stability observed either no reduction in $Ca^{2+}$ accumulation capacity (Hdh150 knock-in mice) or a statistically significant increase (R6/2 and YAC128 mice).

The variable and somewhat confusing data on mitochondrial $Ca^{2+}$ handling obtained with the isolated organelles has favoured investigations with intact cultured neurons. The advantages are that pure neuronal mitochondria are present in their physiological milieu, that artifacts associated with the mitochondrial preparation are absent and that the mitochondria can be exposed to patho-physiologically relevant stresses. The limitation of course is that studies are restricted to neonatal preparations and that the age-related component of the disease cannot be modeled. This review has emphasized the importance of considering mitochondrial bioenergetics and $Ca^{2+}$ transport in parallel. A major experimental deficiency until recently has been the lack of sensitive techniques to monitor the respiration of cultured neurons. This was resolved with the development of the cell respirometer [116] which allows the respiration of coverslip-attached cells to be monitored in parallel with single cell fluorescent functional analysis. In our experience the small amount of material means that it is difficult to prepare ‘well coupled’ isolated mitochondria from specific brain regions of single transgenic mice. Indeed, in the HD literature is difficult to find details of the basic respiratory control parameters for the various preparations used for $Ca^{2+}$ transport studies, raising the suspicion that, as in our hands, the preparations may be functionally compromised. By avoiding isolation artifacts, the cell respirometer allows the respiratory parameters to be determined *in situ*.

A decrease in 'spare respiratory capacity', the ability of *in situ* mitochondria to increase their respiration in response to an increased ATP demand has been proposed to be an important factor defining the long-term survivability of neurons in the face of a variable energy demand [58]. In view of the evidence that Complex II may be deficient in HD it was somewhat surprising that analysis of the respiration of striatal neurons from wild-type ($Hdh^{+/+}$) and heterozygous knock-in ($Hdh^{150/+}$) litter mates revealed no significant difference in maximal respiratory capacity in the presence of the protonophore FCCP [115] and that in the basal state the mitochondria were only respiring at about 20% of their respiratory capacity, half of which drove the endogenous mitochondrial proton leak. In the basal state only 10% of the total ATP generating capacity was used by the wild-type mitochondria and 14% by the heterozygous knock-in. Thus, it is likely that *in situ* neuronal mitochondria present more robust respiratory properties than commonly isolated brain mitochondria. Despite this, parallel determination at single cell resolution of $[Ca^{2+}]_c$ using the low-affinity Fura-5F and mitochondrial membrane potential with $TMRM^+$ showed a statistically significant increase in the proportion of $Hdh^{150/+}$ neurons undergoing DCD during a 10min exposure to NMDA relative to control $Hdh^{+/+}$ neurons [115].

## 4. Conclusions

The four basic principles of isolated brain mitochondrial $Ca^{2+}$ transport, net accumulation when $[Ca^{2+}]_c$ rises above the 'set-point', storage in the alkaline matrix as a calcium-phosphate complex, controlled release when a low $[Ca^{2+}]_c$ is restored by the plasma membrane and finally catastrophic dysfunction associated with $Ca^{2+}$ overload and oxidative stress, can each be observed in intact neurons. However while all mitochondria can be induced to undergo a permeability transition, pathological conditions where such $Ca^{2+}$ overload might occur are more restricted, with the strongest evidence being the association with chronic NMDA receptor activation in glutamate excitotoxicity. Since mitochondrial $Ca^{2+}$ transport is driven by the proton circuit and thus competes with ATP synthesis it is essential to consider the interactions between these two processes, particularly since NMDA receptor activation induces a heavy cellular ATP demand. Finally, the severe limitation in interpreting studies with isolated brain mitochondria in models of neurodegenerative diseases such as Huntington's means that studies with intact neurons are more likely to provide some insight into the contribution of mitochondrial $Ca^{2+}$ transport to the pathology of the disease.

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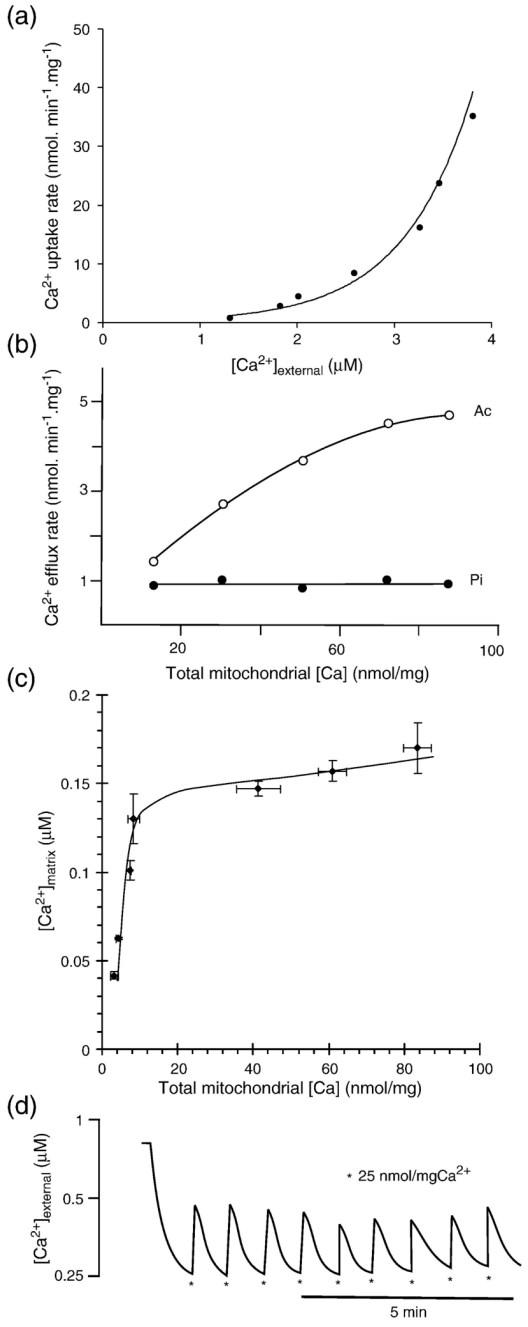


**Fig. 1.**

$Ca^{2+}$ transport by isolated mitochondrial. (a) Kinetics of $Ca^{2+}$ uptake via the $Ca^{2+}$ uniporter in rat liver mitochondria as a function of external free $Ca^{2+}$ concentration. (b) Kinetics of $Ca^{2+}$ release via the $Ca^{2+}/2H^{+}$ exchanger in rat liver mitochondria as a function of matrix $Ca^{2+}$ load; Ac, phosphate depleted mitochondria in the presence of 5mM acetate, $P_i$, mitochondria in the presence of 3.3mM phosphate. The efflux rate in $P_i$ is independent of total matrix $Ca^{2+}$ because free matrix $Ca^{2+}$ is buffered by the $Ca^{2+}$-phosphate complex. (c) Rat liver mitochondrial matrix free $Ca^{2+}$ concentration as a function of matrix $Ca^{2+}$ load. Note the transition from varying to buffered free $Ca^{2+}$ at 10nmol $Ca^{2+}$/mg when the $Ca^{2+}$ phosphate

complex starts to form. (d) Rat brain mitochondria buffer the external free $Ca^{2+}$ concentration close to 0.25μM in $Mg^{2+}$-free media. For experimental details see [2,8,11]