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Serial Review: The powerhouse takes control of the cell:
The role of mitochondria in signal transduction
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The powerhouse takes control of the cell: Is the mitochondrial permeability transition a viable therapeutic target against neuronal dysfunction and death?☆

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Abstract

Stroke and neurodegenerative disease exert an increasing large toll on human health at the levels both of the individual and of society. As an example of each, in the United States, stroke is the major single cause of overall morbidity and mortality, and the financial costs of Alzheimer's disease alone dwarfs the entire federal medical research budget. It has been long recognized that mitochondrial energy production is essential for the second to second functions of the central nervous system (CNS), and that severe mitochondrial impairment is incompatible with normal cerebral function. The last decade, however, has brought a growing understanding that mitochondria play an even greater role than previously suspected. Increased understanding of the role of mitochondria in antioxidant defense and calcium homeostasis further solidified the importance of mitochondria in CNS function — just as increased understanding of mitochondrial roles in calcium-mediated toxicity and production of reactive species further exemplified the Janus role of mitochondria — as mediators of CNS dysfunction. Perhaps most unexpected, however, was the evidence that mitochondria serve as the dominant integrators, checkpoints, and amplifiers of the cell death signals in the CNS. The mechanism of propagation of cell death cascades by mitochondria remains controversial. In this review, we focus on the evidence that *supports* the involvement of an event termed the mitochondrial permeability transition that (i) occurs (patho)physiologically; (ii) occurs in the CNS, and; (iii) is a potential target for pharmaceutical intervention against CNS dysfunction, injury, and cell loss resulting from stroke, trauma, and neurodegenerative disease.
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Keywords: Mitochondria; Apoptosis; Permeability transition; Stroke; Alzheimer's disease; Parkinson's disease; Huntington's disease

Contents

Abbreviations: CNS, central nervous system; mPT, mitochondrial permeability transition; AIF, apoptosis-inducing factor; DOPEGAL, 3,4-dihydroxyphenylglycolaldehyde; DOPAC, 3,4-dihydroxyphenylacetic acid; DOPET, 3,4-dihydroxyphenylethanol; MAO, monoamine oxidase; HD, Huntington's disease; CsA, cyclosporine A.

☆ This article is part of a series on "The powerhouse takes control of the cell." The full list of papers may be found on the home page of the journal.

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Why care about mitochondria in the CNS? The personal and societal cost of CNS impairment

Impaired CNS[1] function is a devastating problem at all levels. CNS impairment occurs in multiple situations, including, but not limited to, the psychiatric disorders, multiple sclerosis, delirium, inborn errors, traumatic injury, stroke, and the four "major" neurodegenerative diseases (ALS, Huntington's, Parkinson's, and Alzheimer's). This review will focus primarily on the mitochondrial permeability transition (mPT) as a possible causal contributor to cell death in the CNS, especially as related to traumatic injury, stroke, and neurodegenerative disease. These diseases and conditions directly affect roughly 30–40% of individuals at one point in their lifetime. Given that incidence of the three most prevalent — stroke, Alzheimer's, and Parkinson's — display generally if not entirely age-related incidence, it is reasonable to expect that, without intervention at the public health level, these diseases will continue to increase in overall incidence as the population ages. These diseases exert untoward effects on caretakers and relatives, with effects that often persist past the death of the directly affected individual. Similarly, these diseases exert substantial costs on society. The estimated overall economic cost of stroke-related disability and death in the United States is $50 billion/year; the cost of Alzheimer's disease $100 billion/year; this in comparison to an NIH budget of ~$27 billion, and combined budget for NIA and NINDS of ~$2 billion.

The overall impact of these disorders underlies initiatives, again at all levels, toward understanding and treating these maladies. One of the oldest concepts, put forth initially by Quastel in 1933, is that failure to produce energy would impair CNS function [86]. More recent work has included understanding the roles of signal propagation in the CNS, ranging from signaling molecules such as calcium ions to the changes in membrane potential and ionic gradients that underlie neuronal signaling. Signal transduction is also critically dependent on neurotransmitter synthesis and turnover. Research has also addressed the involvement of free radicals and reactive oxygen species in both normal physiology and in the pathology of these diseases, and the maintenance of antioxidant species and their critical roles in the maintenance of cellular function and health. Perhaps the most rapidly growing field is that of cell death, understanding, and trying to prevent, the cascades that lead to caspase-dependent and independent cell death cascades.

Each of these issues, from the oldest to the most recent, from energetics to calcium transport to cell death cascades, runs through the mitochondria. Mitochondria respond to calcium influx by up-regulating three of the tricarboxylic acid dehydrogenases and thus increase the capacity for energy production. The production of energy — here defined as ATP production — is essential for the maintenance and usage of membrane electrical and ionic gradients, and the production of energy — here defined as the production of a mitochondrial membrane potential ($\Delta\Psi$) — is essential for the synthesis and turnover of neurotransmitters, particularly acetylcholine [38]. Mitochondrial respiration is the primary source of reactive oxygen species, and mitochondrial respiration is also central for the regeneration of antioxidants such as ubiquinol — both directly through complex I and the NADH-NADPH transhydrogenase) and ketoglutarate dehydrogenase and indirectly (through maintenance of cellular redox status) involved in the regeneration of glutathione [15,16,108] This review will focus on mitochondrial roles in cell death in the CNS, particularly a role for an event termed the mitochondrial permeability transition, or mPT.

Mitochondria

While the importance of the role mitochondria in the propagation of neuronal cell death is well-established [7,32,39,69,71,74,92,98,112], the mechanisms remain unclear. Mitochondria serve to integrate and amplify upstream cell death signals. The process of cell death can be initiated in the CNS by multiple signals. These signals include the loss of critical growth factors (e.g., NGF), activation of cell death receptors, or the biochemical processes subsequent to events such as ischemia-reperfusion, which includes elevated concentrations of free radicals and other reactive oxygen and nitrogen species and an increase in intracellular Ca^{2+} and other cations, such as Zn^{2+}. While the initiators may vary, it is clear that one of the central decision points in neuronal cells is the mitochondrion. Here signals are integrated and amplified, clarifying the cellular reaction to a go/no go response.

Thus, as a starting point, it is worth considering that, when cells are stressed, at least three types of signals propagate, an indication of this stress to that cell's mitochondria (Fig. 1). These signals include proteins, reactive species, and divalent

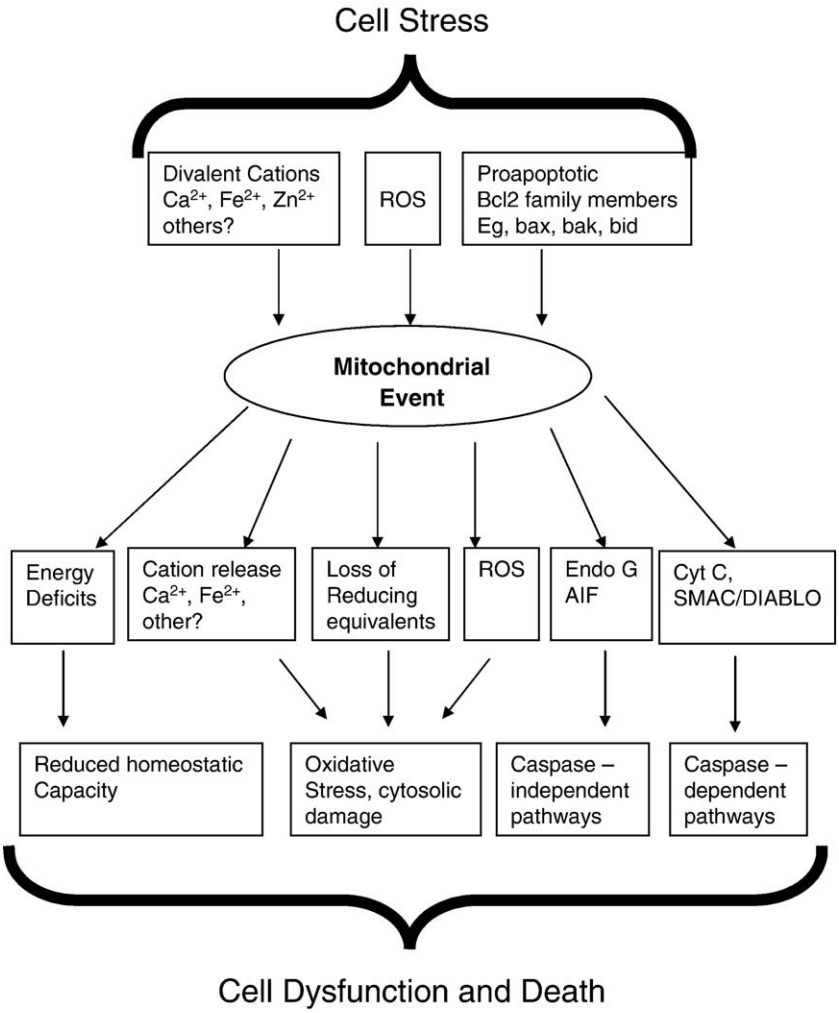

Fig. 1. Schematic showing mitochondrial roles as a checkpoint and signal transformer in cell death.

cations. The protein signals include the proapoptotic members of the bcl-2 family, such as *bid, bax,* and *bak*. Reactive compounds include free radicals, reactive species such as peroxynitrite, and damage by-products that are themselves damaging, such as the hydroxyalkenals [34].

If the stress is sufficient, mitochondria in turn respond by releasing a series of protein factors (Fig. 1) [28,68,99–101]. These factors include cytochrome *c* and SMAC/DIABLO, which act to initiate and facilitate (respectively) the downstream stages of the caspase-dependent cell death cascades. Other factors released include apoptosis-inducing factor (AIF) and endonuclease G, which then initiate the downstream stages of caspase-independent cell death cascades. Mitochondrial events, in part discussed below, may also contribute to cell death by producing further reactive species, releasing sequestered cations (Ca^{2+}, Fe^{+2}), and by compromising energy production pathways.

The biochemical events that link the upstream receipt of prodeath signals coming into mitochondria with the mitochondrial response have been a major area of interest across broad fields of interest ranging from cancer to neurodegeneration to mitochondrial physiology and evolutionary biology. Broadly, we will divide the potential mechanisms into two groups. The first group includes those mechanisms that appear to occur when the stress signal propagated to mitochondria involve only a protein signal, such as *bax*. Under these conditions, such as appear to occur during growth factor deprivation, protein factors are released, but mitochondria appear to retain their integrity and retain an active membrane potential. The second group includes those mechanisms that result, most commonly, from the signal to mitochondria involving a supraphysiological calcium signal. Here the mechanism appears to involve an event termed the mitochondrial permeability transition (mPT).

The mPT has been classically defined in isolated liver mitochondria as a cyclosporin A-sensitive, Ca^{2+}- and oxidant-mediated induction (opening) of a pore in the inner mitochondrial membrane allowing free diffusion of solutes under 1500 Da [40,116]. That said, there are clear cases where an event that is reminiscent of the mPT is cyclosporin A insensitive [42], and where induction does not require Ca^{2+} [66]. An argument has also been made that events typically assigned to the mPT may not always involve a

specific proteinaceous pore [42]. The consequences of induction include (i) the loss of oxidative phosphorylation capacity due to the failed integrity of the inner membrane; (ii) the conversion of complex V (ATP-synthase) from an energy-producing to an energy-consuming complex (glycolytic ATP can be consumed), resulting in further compromise of cellular energetics; (iii) inhibition of complex I due to loss of NADH; (iv) efflux of matrix Ca^{2+} stores, potentially leading to Ca^{2+}-dependent cytosolic abnormalities (e.g., aberrant protease activation).

mPT induction may also propagate both caspase-dependent and caspase-independent cell death cascades by release of apoptogenic factors such as cytochrome *c*, AIF, and SMAC/DIABLO [27,45,85,99,115]. These findings link mPT to the release of the direct mediators of the downstream caspase-dependent and -independent cell death pathway, and they provide further insight into the biochemical events underlying reports proposing that induction of an mPT has been linked to neurotoxicity following pathological insults such as stroke and excitotoxicity [37,71,77,92,93], trauma [91], and hypoglycemia [36].

A comparison of observations in isolated liver and brain mitochondria suggests that "mPT-like" events do occur in brain mitochondria, even if the characteristics of the brain mPT differ in detail from that which occurs, for example, in liver mitochondria [12,13,31,36,50,55,71,77,115]. Data from studies at the level of isolated mitochondria, cells, and intact animal models support the existence of "PT-like" events in the nervous system [35,43,55,77,92,106,115], and equally compelling data, including data from our group and others, suggest that PT-like events in the nervous system must be significantly different than those in the brain, and that cell death in the CNS may often be "PT independent" [1,4,33,43,55,70,115]. Addressing this discrepancy is complicated by the lack of an accepted, well-defined model for the mPT in isolated brain mitochondria.

Thus, the question of whether mPT is a (patho)physiologically important mechanism of cell death remains the holy grail of this area of research. Our laboratory has been considering this question in the context of a series of subquestions: (i) Does the mPT occur in mitochondria isolated from tissues in the CNS? (ii) Are compounds that facilitate mPT induction associated with neurodegenerative disorders or stroke? (iii) Is mitochondrial resistance to mPT altered by physiological conditions? (iv) Are compounds that are known to be neuroprotective active against mPT? (v) Are mPT inhibitors neuroprotective?

(i) Does the mPT occur in mitochondria isolated from tissues in the CNS?

The mPT is well-characterized in isolated liver mitochondria [116], but mPT and "mPT-like events" are not as well understood in isolated brain mitochondria. This question shapes the debate on the role of mPT in vivo because of the difficulty in otherwise determining what is a definitive experiment. For this reason, we initially focus here on studies in isolated mitochondria, because of the necessity of initially defining the phenomena at its most biochemically basic form.

It is worthwhile to begin by considering at least some of the many differences between liver and brain mitochondria. (i) Population differences: Liver mitochondria are derived from both hepatocytes and other cell populations present in the liver, but the mitochondrial populations isolated appear to functionally behave as a single distribution. Brain mitochondria are also isolated from a mixed cell population that includes, among others, glia, neurons, and endothelial cells. Mitochondria isolated from different regions of the brain have considerable biochemical distinctions [10,35]. Synaptosomal and nonsynaptosomal mitochondrial populations likewise have different properties [61,62]. Thus, there is considerable heterogeneity within the populations [59]. (ii) Isolation considerations. While multiple different liver mitochondrial preparation techniques exist, most give or all give populations that appear qualitatively similar. In contrast, different techniques for the isolation of brain mitochondria (e.g., Percoll gradients [50,94], Ficoll gradients [55,61,62], or digitonin [2,14] are needed to eliminate contaminating myelin and synaptosomes) have been argued to give different relative yields from these cell types, and the preparations have different properties [14]. (iii) In contrast to liver, brain mitochondria also require 100 mM KCl for optimal respiration [75]. (iv) In contrast with liver, the primary mechanism in the release of Ca^{2+} from neuronal mitochondria is considered to be Na^+/Ca^{2+} exchange rather than H^+/Ca^{2+} exchange [40,41,105,107].

The initial demonstration of mPT induction in mitochondria and cells isolated from the CNS was reported by one of us (B.S.K.) in collaboration with Dubinsky [50]. Elevated Ca^{2+} and inorganic phosphate produced mPT-like swelling in Percoll-purified isolated brain mitochondria and in digitonin-permeabilized cultured astrocytes. The observed mitochondrial swelling was reduced or prevented by classic mPT inhibitors like cyclosporine A (CsA) (although much less effective than in liver mitochondria), Mg^{2+}, thiol reagents DTT, *N*-ethylmaleimide. The ability of ruthenium red and CGP 3715 (Na^+/Ca^{2+} -exchange inhibitor) to prevent Ca^{2+}-induced mitochondrial swelling suggested a role for Ca^{2+}-cycling in mPT induction in these mitochondria. In contrast, the ionophore-mediated introduction of Ca^{2+} into the mitochondrial matrix in the context of blockade of both the uniporter and the Na^+/Ca^{2+} exchange to prevent calcium cycling showed that cycling was not mandatory for all effects. These data indicated that in the presence of elevated mitochondrial calcium loads, both Ca^{2+}-cycling-dependent and -independent events could induce mPT-like events. While time has led to a better understanding of the limitations of this study, such as the swelling assay used, the basic observation of the existence of an mPT-like event in isolated brain mitochondria has remained, and has been extended. These initial studies

showed the relative resistance of mPT in brain to oxidants, the relatively poor protection conferred by CsA, and the reduced swelling response of brain vs liver, all properties that have been consistently observed and appear robust. One initial study of basic properties showed that isolation in Ficoll gradients gave qualitatively similar results [55]. These data also suggested that CsA protection is notably more complete in the presence of complex I substrate [55]. Some other studies demonstrating existence of mPT-like events in isolated brain mitochondria are as follows.

Ca^{2+} accumulation by brain mitochondria induced the release of up to 40% of total cytochrome *c* and matrix glutathione. These events were cyclosporin A insensitive and were not accompanied by mitochondrial swelling and membrane potential breakdown. mPT was also observed under different conditions. Thus, the nature of the release mechanism can be highly condition dependent [1,2].

Friberg et al. showed that sensitivity to Ca^{2+}-induced PT varied across the brain, with cerebellum showing the lowest sensitivity and hippocampus the highest [72]. Differences correlated with selective vulnerability and were ascribed to the differences in the mitochondrial concentration of adenine nucleotides, which in turn was related to the density and structure of the adenine nucleotide translocator, which regulates the uptake and turnover of adenine nucleotides. Mitochondria from hippocampus had the lowest content of adenine nucleotides and were significantly more sensitive to mPT than mitochondria from the cortex and cerebellum.

Brustovetsky and Dubinsky [11] proposed the existence of two different mechanisms of PT operating in brain mitochondria, a high- and low-conductance mode.

Berman et al. [4] extended the work showing that brain mitochondria are relatively resistant to the classical PT-induced agents, particularly oxidants, and that they were less prone to undergo the mPT-mediated swelling than liver mitochondria. Brain mitochondria also better retained glutathione.

Kushnareva et al. [60] demonstrated that brain mitochondria are quite sensitive to a variant "permeability transition" induced by mitochondrial signal peptides (human cytochrome oxidase subunit iv signal peptide). Permeability transition induced by these peptides differs in several characteristics from the classical mPT and represents a cyclosporine A-insensitive, swelling-independent mechanism, associated with disruption of the outer membrane and release of intermembrane proteins, including cytochrome *c*.

Kristian et al. [57] demonstrated that acidosis can promote mPT induction in isolated brain mitochondria — a contrast from the liver system [6,76]. In another report, Kristian et al. [58] further revealed the heterogeneity of the isolated brain mitochondrial population. Specifically, repeated Ca^{2+} pulses (40 nmol/mg of protein) saturated the uptake system, and brain mitochondria then failed to release the accumulated Ca^{2+}. The first Ca^{2+} pulse, however, was accompanied by a moderate release of Ca^{2+}, relatively marked depolarization, and swelling. CsA eliminates the Ca^{2+} release associated with the first Ca^{2+} pulse. These data suggest that, under the conditions tested, the mitochondrial population gives a heterogeneous response to Ca^{2+} exposure with only some part of the population undergoing an mPT.

Chinopoulos et al. [24] found that in the presence of adenine nucleotides and Mg^{2+}, Ca^{2+}, an mPT-like event in brain mitochondria is insensitive to several classical mPT inhibitors, including CsA, antioxidants, inhibitors of phospholipase A_2, or nitric oxide synthase, but showed sensitivity to bongkrekic acid and 2-aminoethoxydiphenyl borate.

These data, and others like them, suggest that mitochondria isolated from the brain can undergo an event that echoes the mPT, although there are considerable differences between this event and liver mitochondria.

(ii) Are compounds that facilitate mPT induction associated with neurodegenerative disorders or stroke?

The mPT has been most commonly described as the calcium and oxidant-mediated induction of a specific proteinaceous pore in the inner mitochondrial membrane [5,40,116]. That said, cofactors may help facilitate mPT induction, thus lowering the required Ca^{2+} load. Cofactors might also provide disease specificity or underly selective vulnerability. We have therefore been interested in identifying those compounds that exist physiologically and which might be involved in facilitating mPT induction. For example, there is little doubt that Ca^{2+} overload is a reasonable candidate contributing to cell death cascades. The role and targets of other divalent cations, however, are less clear. One intriguing example is Zn^{2+}, which has been implicated as a neurotoxin in vivo [47] and an mPT inducer in vitro [44]. Another intriguing example is ganglioside GD3, which is necessary and sufficient to induce apoptosis in some T-cell lines [26] and, from theoretical grounds, may be indirectly implicated in stroke through ceramide toxicity [95]. We showed that this compound can facilitate mPT induction, whereas related gangliosides that are nonneurotoxic do not [48]. mPT is also commonly recognized as involving an oxidant-mediated component, but the specific nature of the oxidant remains unclear. We have specifically focused on reactive aldehydes as potential mPT inducers.

Reactive aldehydes and mPT induction

At least two major sources or reactive aldehydes exist in the CNS, lipid peroxide-derived hydroxyalkenals, and monoamine-derived aldehydes. In each case, we have provided evidence that these reactive aldehydes are potential mPT inducers at concentrations that may occur locally at the mitochondria, especially under pathophysiological conditions.

Lipid peroxidation processes, which increase during, for example, ischemia-reperfusion injury associated with

stroke, are associated with production of aldehydes. Of these, malondialdehyde is probably the most commonly studied, but it has been recognized that the hydroxyalkenals are far more toxic [34]. Most of the work done to date has focused on 4-hydroxynonenal, but some work has examined 4-hydroxyhexenal as well. Given the prevalent associations between neurodegenerative conditions/stroke-related pathology and oxidative damage, we examined whether these lipid peroxidation by-products facilitated mPT induction [54]. Both 4-hydroxyhexenal and 4-hydroxynonenal facilitated mPT induction. Surprisingly, 4-hydroxyhexenal was active at femtomolar concentrations, whereas 4-hydroxynonenal required micromolar levels. Given that these molecules have essentially equal reactivity and are identical in the active side of the molecule, this result suggests that aldehyde effects on mPT induction appear to be highly structure specific, likely due to a requirement to reach a specific target site. Consistent with numerous observations of a relative insensitivity of nonsynaptosomal mitochondria to oxidant-induced acceleration of mPT induction, these aldehydes were inactive in in vitro brain mPT assays [50]. It remains unclear if this is a real aspect of the biology of these mitochondria, or if this is somehow an artifact of the systems used to study it.

We also examined the ability of monoamine-derived aldehydes to accelerate mPT induction — such aldehydes have been proposed as potential neurotoxins in both Alzheimer's and Parkinson's diseases [8]. For example, DOPEGAL [3,4], the monoamine metabolite of epinephrine and norepinephrine, had been implicated as a potential neurotoxin that might contribute to Alzheimer's disease [18]. We found that it was a moderate mPT accelerant [17]. We then looked at DOPAL.

DOPAL is the initial product of MAO (monoamine oxidase) activity on dopamine, and it is the precursor of 3,4-dihydroxyphenylacetic acid (DOPAC) and 3,4-dihydroxyphenylethanol (DOPET). Several pieces of indirect evidence suggested that it is a potent neurotoxin that may mediate events through the mPT [e.g., 20,63,64,79,80] In studies using isolated liver mitochondria and "NGF-differentiated" PC12 cells, we have identified DOPAL as a mPT inducer and cytotoxin [49]. Dopamine was >1000-fold less potent as an mPT inducer. Sensitivity to DOPAL was reduced ≥30-fold in fully energized mitochondria, suggesting that mitochondrial respiration may increase resistance to mPT induction by the endogenous DOPAL in the substantia nigra. These data help provide a potential mechanism addressing the apparent association between mitochondrial dysfunction and mPT induction of cell death, monoamine oxidase activity, and dopaminergic neurotoxicity.

β-Amyloid as an mPT inducer

Proteins may also facilitate mPT. In addition to effects of proteins such as bax, other proteins may also serve as director transducers of stress to mitochondria. One example is the growing evidence that β-amyloid may directly contribute to the well-established deficit in energy metabolism in Alzheimer's disease. β-Amyloid exposure of isolated mitochondria is associated with a decrease of mitochondrial enzyme activity [19,21,22], leakage of ROS [67,102], $\Delta\Psi$ collapse, induction of mPT,and cytochrome *c* release, and subsequent activation of caspase cascade [73,83,84,87]. As has been recently found in mitochondria of a transgenic mice model and AD patients all these events are promoted by direct molecular interaction of Aβ with mitochondrial Aβ-binding alcohol dehydrogenase [67].

(iii) Is mitochondrial resistance to mPT altered by physiological conditions?

Susceptibility to mPT induction is a product of the relative resistance of the mitochondria and the presence of specific activators and cofactors. The previous section addressed the question of whether the chance of mPT induction could be increased by the presence of cofactors at appropriate times. As seen, potential cofactors such as Zn^{2+} or the hydroxyalkenals are good candidates for cofactors whose increased prevalence may contribute to disease progression. The other side of the equation is whether the probability (or susceptibility) of events that link the exposure of mitochondria to a potential inducer to mPT inducer is themselves subject to physiological shifts.

The question is analogous to asking whether there are physiological changes that make lipid membranes more susceptible to peroxidation. At some level, the answer here is clearly yes; one can change the content of bis-allylic bonds or change the level of vitamin E in the membrane. Also, at some level, the answer is clearly no; a hypothetical study of oxidative damage to a single given isolated lipid of identical structure (i.e., two linoleic acid molecules) from two membranes of physiological interest should be equivalent. The key point here is the word isolated, i.e., the concept of context. The problem of context in questions about the mPT is not trivial because, at least in some ways, we still lack a sufficient understanding of what defines the most relevant context with respect to the mPT.

Several pieces of evidence suggest that mitochondrial physiology, and by extension, the status of an organism, does significantly impact susceptibility to mPT induction. The differences in mPT induction between liver and brain mitochondria provide an obvious example, in that they show that some aspect of the intact, isolated mitochondria system does influence the events subsequent to exposure to mPT inducers. Likewise, differences in mitochondria from different regions of the brain are consistent with some aspect of context being retained in the isolated brain mitochondria.

Age, diet, and disease (diabetes) have been shown to modulate susceptibility in isolated liver mitochondria. We found that mitochondria isolated from 6-month-old male Fischer 344 rats were significantly more resistant to mPT induction than those of middle or old age, and animals maintained on life-prolonging calorie restricted diets were

also far more resistant [56]. The differential sensitivity also showed inducer specificity, suggesting that multiple systems are being regulated. These effects seem strain specific, as we were unable to replicate it in Fischer x brown norway F1 rats (B.S. Kristal and B.F. Krasnikov, unpublished data). These data are consistent with a genetic basis for relative susceptibility. We further showed that liver mitochondria isolated from diabetic animals were more resistant to mPT induction [53]. This initially surprising result appears due to alterations in several mitochondrial parameters, such as reduced membrane potential and increased ROS scavenging capacity, that likely follow from the electron leak at Center P of the electron transport chain that occurs in mitochondria isolated from diabetic animals [51,52].

Huntington's disease

Studies in mitochondria isolated from humans with Huntington's disease (HD) and from transgenic mouse models of HD have extended this to neurodegenerative models. Mitochondria within the lymphocytes of HD patients were less resistant to depolarization and more susceptible to induction of apoptosis [90]. The depolarization was reduced by CsA, suggesting a potential involvement of the mPT. Purified mitochondria from these lymphocytes had lower resting membrane potentials and a reduced ability to sequester Ca^{2+} [82]. Similar defects were observed in brain mitochondria from mice harboring a full length mutant *Htt* (Huntington) transgene (YAC72) but not mice harboring a control transgene (YAC 18). Htt was seen to associate with mitochondrial membranes in brain-derived thin sections [82], and a loose association with the outer side of the outer membrane has been observed using subfractionation approaches as well [25]. Addition of mutant Htt was found to inhibit Ca^{2+}-uptake capacity and facilitate mPT induction [25,81,82]. Striatal cells carrying an HD transgene were more sensitive than controls to cell death following treatment with the complex II toxin 3-nitropropionic acid, and this cell death was reduced by either CsA or ruthenium red, consistent with a role for mPT [89]. Similarly primary striatal neurons carrying mutant Htt were also more sensitive to NMDA receptor activation-mediated cytotoxicity than control neurons. This cell death was reduced by either CsA or bongkrekic acid, consistent with a role for mPT [113].

Together these data suggest that the cellular environment of HD shifts the susceptibility of mitochondria to undergo mPT.

(iv) Are compounds that are known to be neuroprotective active against mPT?

If the mPT is involved in the causative pathway of cell death, then it is reasonable to predict that it is a potential therapeutic target. We, and others, therefore asked whether known neuroprotectants could have the mPT as a potential site of action.

Tauroursodeoxycholic acid is an endogenous bile acid that protects against stroke, but is known to modulate activities in three major pathways involved in ischemic damage, including mPT, activity of bcl-2 family members, and signal transduction pathways [88]. This complicates the use of this drug as a test for mPT involvement.

Creatine helps ensure adequate energy exchange and, as such, may help buffer the mitochondrial adenylate charge, the high energy adenine nucleotides are protective, and creatine kinase has been implicated as a potential part of the permeability transition pore complex in brain mitochondria [78]. In accordance with these views, liver mitochondria from mice transgenic for creatine kinase in the liver are more resistant to mPT in the presence, but not absence, of creatine [29],and feeding creatine reduced subsequent mPT induction in traumatic brain injury [97]. In contrast, neither Brustovetsky and Dubinsky [9] or our group [114] were able to show a protective effect on mitochondrial-Ca^{2+} interactions in isolated brain mitochondria, and we also did not observe differences in mitochondrial-Ca^{2+} interactions in brain mitochondria isolated from creatine kinase knock-out animals [46]. Thus, it appears that creatine does not mediate its protective effects by influencing susceptibility to mPT induction.

Minocycline, another broadly active neuroprotectant, also seemed a logical candidate because studies showed that it blocked caspase activation [115]. Minocycline is a second generation tetracycline antibiotic known to be protective in models of stroke [110,111], ALS [115], HD [23], spinal cord injury [65,104], and neonatal hypoxia-reperfusion injury [3]. In conjunction with Robert Friedlander's group, we showed that minocycline blocked mitochondrial cytochrome *c* release and that this release correlated with the ability to prevent mPT-induced swelling in both isolated brain and liver mitochondria [115]. The protection was found to be stoichiometric, and appropriate concentrations of mitochondria could be protected by 2 μM minocycline, comparable to levels reached in vivo. Minocycline also appears to block release of other factors such as SMAC/DIABLO in striatal cell line models of HD [103], linking minocycline's effects at the mitochondrial level to both caspase-dependent and independent-cell death cascades. Minocycline does exert multiple effects at the mitochondrial level, clearly complicating mechanistic studies. While this work links minocycline to prevention of mPT-mediated release of mitochondrially sequestered protein factors that facilitate both caspase-dependent and -independent cell death pathways [103,115], other actions of minocycline have been identified [110,111], and the use of minocycline to build a case for mPT involvement awaits a more mechanistic study of the actions of minocycline.

(v) Are mPT inhibitors neuroprotective?

The converse of the previous section is to ask whether mPT inhibitors are neuroprotective. Neuroprotection medi-

ated by CsA was initially cited as evidence for causal involvement of mPT in ischemic injury [77,93], but this is now appreciated to be problematic as CsA also affects calcineurin, the blockade of which itself has been shown to be neuroprotective [30]. Arguably, one of the best direct tests of the hypothesis that mPT lies on the causative pathway of clinically relevant cell death thus comes from the studies of N-Met-Val-CysA — a non-immunosuppressive analog of CsA reputed not to interact with calcineurin. This compound reduces infarct size in a rat model of transient focal ischemia [71]. These data are strengthened somewhat by evidence that the effects of CsA on protection against infarction may extend temporally in time from the effects of the calcineurin inhibitor FK506 [109]. The universal acceptance of mPT involvement in stroke remains limited, however, in part because of the reliance on data from a single drug [43], and the limited availability and characterization of its analog. Furthermore, CsA is not viewed as a long-term medical option, as the blood brain must be opened (e.g., by needle puncture) for any therapeutic efficacy [109].

To develop reagents that might help test the involvement of the mPT in neuropathology and to identify potential therapeutics, we screened a library enriched in FDA-approved drugs and identified the tricyclics as a potential class of useful reagents [96]. Based on initial results, we further evaluated 32 heterocyclic, tricyclic, and phenothiazine-derived compounds as potential mPT inhibitors. All but one of these compounds are clinically approved and many have been widely used as antidepressants, antipsychotics, or antihistaminics. In our assay, we employed three well-defined models of mPT induction and found that 28 compounds revealed protective effects under all conditions in the concentration range 10–30 μM. Further studies showed no alterations in basic mitochondrial functions such as respiration in state 4, respiration coupled to oxidative phosphorylation, and uncoupled respiration. Protection was not related to the clinical subclass of the agents or to their effects on calmodulin or phospholipase A_2. Four compounds — promethazine, methiothepin, flufenazine, and clomipramine, representatives of some of the structural and functional variety of tricyclics — were chosen for evaluation of their effect on mitochondrial membrane potential and calcium uptake/release capacity. Clinically relevant doses of one drug in this general structural class that inhibits mPT, promethazine, were protective in both in vitro and mouse models of stroke. Specifically, promethazine protected primary neuronal cultures subjected to oxygen-glucose deprivation and reduced infarct size and neurological impairment in mice subjected to middle cerebral artery occlusion/reperfusion. This work then enabled us to retrospectively reconsider the literature, where we were able to determine that 10 of the drugs now identified as mPT inhibitors are neuroprotective in in vivo models of stroke.

These data are consistent with the view that the mPT may be considered a validated target for neuroprotection, at least in the acute stroke pretreatment model. This model is relevant both for scientific consideration of the role(s) of mPT in neuropathology and the clinical use of these agents in patients at high risk for stroke, e.g., people undergoing carotid endartectomy.

Summary

In summary, determining whether or not the mPT plays a causative role in cell death in the CNS has a potential impact scientifically, clinically, and economically. Evidence at each of the biological levels addressed in this review suggests that (i) mPT, or something like it, can occur in the CNS; (ii) factors that can accelerate mPT induction are likely to be present in the CNS under pathophysiological conditions; (iii) the systems that protect mitochondria are both subject to and amenable to modification; (iv) at least some known neuroprotectants may reduce susceptibility to mPT induction; and (v) compounds identified on the basis of their ability to inhibit mPT can be neuroprotective. These compounds include several FDA-approved compounds. Together, these data and others like them support a tentative assignment of the mPT as a validated target for neuroprotection in the CNS.

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