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Mitochondria, Cybrids, Aging, and Alzheimer's Disease

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Abstract

Mitochondrial and bioenergetic function change with advancing age and may drive aging phenotypes. Mitochondrial and bioenergetic changes are also documented in various age-related neurodegenerative diseases, including Alzheimer's disease (AD). In some instances AD mitochondrial and bioenergetic changes are reminiscent of those observed with advancing age, but are greater in magnitude. Mitochondrial and bioenergetic dysfunction could, therefore, link neurodegeneration to brain aging. Interestingly, mitochondrial defects in AD patients are not brain-limited, and mitochondrial function can be linked to classic AD histologic changes including amyloid precursor protein processing to beta amyloid. Also, transferring mitochondria from AD subjects to cell lines depleted of endogenous mitochondrial DNA (mtDNA) creates cytoplasmic hybrid (cybrid) cell lines that recapitulate specific biochemical, molecular, and histologic AD features. Such findings have led to the formulation of a "mitochondrial cascade hypothesis" that places mitochondrial dysfunction at the apex of the AD pathology pyramid. Data pertinent to this premise are reviewed.

Keywords

Aging; Alzheimer's disease; bioenergetics cybrids; mitochondria

Introduction

Mitochondria were identified as cell organelles over 100 years ago [1]. Considerable time elapsed before their functions were fully appreciated. During the 1960's it was determined that mitochondria contained their own genome, the mitochondrial DNA (mtDNA)[2, 3], and

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that they generated ATP according to a process defined as the chemiosmotic hypothesis [4]. The membranes that delineated these organelles were also identified, and the fact that these membrane boundaries created compartments that allowed for particular chemical reactions and indeed even pathways to reside was appreciated.

In the second half of the 20th century, a potential role for mitochondria in aging was widely postulated [5]. While mitochondria are neither central nor essential components of all aging hypotheses [6], their contribution to the aging process as either a primary or downstream contributor to this phenomenon is suspected under a variety of current paradigms [7]. The idea that mitochondria might also contribute to neurodegenerative diseases followed the emerging appreciation of their putative role in aging. This general concept was fueled by the observation that the more common neurodegenerative diseases are “age-related,” such that prevalence and incidence for diseases such as Alzheimer’s disease (AD) increase with advancing age [8]. Over the past three decades the contribution of mitochondria to neurodegenerative diseases in general, and to AD specifically, has been hotly debated with views ranging from a potential primary role to a mechanistically irrelevant artifact of cell death that arises due to other factors [9]. During this time, though, the debate has taken a notable turn and at this point the main question seems not so much whether mitochondrial dysfunction is important and relevant in selected neurodegenerative diseases, but rather how critical mitochondrial dysfunction will turn out to be in these selected neurodegenerative diseases [10].

This chapter will address ways in which perceptions of how mitochondria influence brain aging and neurodegeneration have evolved over time. In particular, it will focus on an evolving appreciation of how mitochondria may contribute to AD, and why modifying mitochondrial function is currently considered a viable AD therapeutic target.

Mitochondria and Aging

Overview

In the first half of the 20th century it was observed that caloric restriction enhanced rodent lifespan [11–15]. This contributed to the emerging view that metabolism in general, and perhaps energy metabolism specifically, could help to regulate lifespan. An appreciation of the idea that metabolism could influence aging came to form the basis of the “Rate of Living Hypothesis” that evolved through the early twentieth century [16, 17], which essentially stated species with higher metabolic rates had shorter life spans than species with lower metabolic rates. For example, rodents with high metabolic rates could survive for only a few years, while some reptiles such as turtles with presumably slow metabolic rates could live for many decades. This hypothesis, of course, could not account for long lived species with apparent high metabolic rates, such as birds.

The Rate of Living Hypothesis was eventually succeeded by the more mechanistically specific Free Radical Theory of Aging that assumed the production of oxygen radicals as a byproduct of biochemical reactions should be elevated in species with higher metabolic rates [18]. These free radicals, in turn, would react with molecules within cells and in doing so

alter their structure and function. It was further believed that this accumulation of metabolism-generated oxidative stress would drive an aging phenotype.

By the 1970's it was appreciated that within cells, mitochondria were a leading site of free radical generation. With this realization some circles rebranded the Free Radical Theory of Aging as the "Mitochondrial Theory of Aging" [5]. As a corollary of this emerging mitochondria-centric approach to aging theory, some began to speculate mitochondria might possess some sort of aging clock [5, 19]. To this point mtDNA comprised a particularly attractive candidate, and by the late 1980's it was proposed that an accumulation of somatic mtDNA mutations over time functions as the suspected aging clock [20]. Essentially, mtDNA mutations would accumulate as a consequence of oxidative damage to mtDNA and a modification of its bases, which would in turn compromise the production of respiratory chain subunits through either a lack of production or else through a production of miscoded peptides. In support of this view, an age-associated accumulation of mtDNA mutations, in either the form of deletions or point mutations, was recognized [21].

Mitochondrial aging was (and is) not central to all theories of aging [6], and a positive correlation between mtDNA somatic mutations and advancing age could also potentially reflect a consequence of aging as opposed to a driver of aging. Investigators began to critically assess this possibility in the early 21st century in genetically modified mice that were designed to accumulate mtDNA mutations at an accelerated pace. This was accomplished by creating mice with a mutated mtDNA polymerase gamma (mtPOLG), in which the innate proofreading ability of the enzyme was perturbed [22, 23]. This allowed for a rapid accumulation of somatic mtDNA mutations across a range of tissues. Phenotype characterizations of these mice showed changes consistent with accelerated aging, and were accepted as support for the view that increasing levels of somatic, heteroplasmic mtDNA mutations could drive an aging phenotype.

Mitochondrial Function and Homeostasis in Advancing Age

The predominant current view is that the functional capacity of an organism's mitochondria declines with advancing age. Much of this thinking was informed by rodent studies, in which mitochondrial functional endpoints were assessed in mice or rats of different ages. For example, it has been reported that the maximum kinetic function of two mitochondrial respiratory chain enzymes, NADH: ubiquinone oxidoreductase (complex I) and cytochrome oxidase (COX; complex IV) declines with advancing age [24, 25]. Age-related declines in mitochondrial enzyme activities may represent a specific rather than generalized phenomenon, as the activities of other enzymes such as succinate dehydrogenase (complex II) appears to be preserved. In this respect it is perhaps of interest that complex I and IV are partially encoded by mtDNA, while complex II is entirely encoded by nuclear DNA.

An interesting parameter in the relationship between brain aging and mitochondria has to do with the number of mitochondria that are present, also referred to here as mitochondrial mass. Some studies have reported that in brains derived from subjects who were free of a neurodegenerative disease prior to death, mtDNA copy number increased with advancing age [26]. This increase in mtDNA was observed despite the fact that mRNA levels were reduced. Increased mtDNA content, therefore, was interpreted by the authors as potentially

reflecting a compensatory response to a reduction in mtDNA transcription efficiency. As part of a related finding, another study reported protein levels of an mtDNA-encoded COX protein subunit, COX2, were increased in the brains of aged individuals when compared to the brains of young individuals [27]. These findings in humans were essentially reflected in a more recent study from 5, 12, and 24 month old C57Bl/6 mice, in which synaptic mitochondria were found to demonstrate apparent adaptive changes at the protein level, which were arguably compensating for overall detrimental changes including an increase in mtDNA damage [28].

In general, relative to young organisms, mitochondria from aged organisms have been reported to show decreased ATP production, increased free radical production, depolarization of the mitochondrial membrane potential, and a reduced ability to buffer calcium [29]. Not all studies, though, have uniformly detected such changes, and to some extent attribute a possible preservation of mitochondrial functional indices to compensatory responses [30].

Mitochondria and Free Radical Production

Oxidative modifications of cell proteins are seen at increased levels in the aging brain, which could potentially reflect increased mitochondrial free radical production [31]. In corollary to this, levels of at least some mitochondrial antioxidant enzymes (for example manganese superoxide dismutase, mitochondrial catalase, and periredoxin) increase with advancing age, presumably in response to increased mitochondrial free radical production [28].

When considering the significance of oxidative stress in aging, it must be kept in mind that oxidative stress is not uniformly a toxic event. Up to certain levels, it appears, oxidative stress functions as a signal transducer [32, 33]. For example, it has been reported that oxidative stress can facilitate retrograde signaling from the mitochondria to the nucleus [34, 35]. Free radicals, in fact, may promote mitochondrial biogenesis in situations where compensatory increases in mitochondrial mass could prove beneficial [36–38].

Mitochondrial DNA and Somatic Mutation

MtDNA differs from nuclear DNA in several notable ways. One unique characteristic is heteroplasmy, which in some ways the mitochondrial correlate of nuclear heterozygosity. Nuclear heterozygosity infers one copy of a gene contains a particular variant while the other does not. MtDNA heteroplasmy is more complex because cells can each have hundreds to thousands of mtDNA. A state of homoplasmy exists when all mtDNA copies within a cell or organism are identical. Heteroplasmy is present when this is not the case and mtDNA copies diverge at a particular nucleotide or nucleotides. Heteroplasmy can be difficult to absolutely rule out, as doing so depends on the sensitivity of its ascertainment. For example, it appears that individual low-abundance mtDNA sequence deviations, or microheteroplasmies, are relatively common at the 1–3% level [39, 40]. They can be detected at levels even lower than 1%, although at extremely low percentages the reliability of the observed sequence deviation can become questionable. In other words, does an extremely low-abundance deviation represent a bona fide sequence deviation, or could it perhaps represent a sequencing artifact?

Regardless, mtDNA reportedly accumulates mutations at approximately ten times the rate of nuclear DNA[41]. This has been attributed to a number of factors, including the lack of protective histone proteins, but also to the fact that mtDNA resides in close proximity to electron transport chain-derived free radical production [42]. In one scenario, cytosine can undergo an oxidative deamination to uracil, which pairs with an adenine rather than guanosine upon replication and results in a G-C base pair undergoing conversion to an A-T base pair (Figure 1A) [43, 44]. In another scenario, 2-deoxy guanosine is oxidized to 8-hydroxy-2-deoxy guanosine and then to 8-oxo-2-deoxyguanosine. 8-oxo-2-deoxyguanosine mismatches to an adenine nucleotide, eventually leading to the conversion of a G-C base pair to a T-A base pair (Figure 1B). 8-hydroxy-2-deoxy guanosine modifications increase with advancing age [45]. While substitutions in the mtDNA that are consistent with these patterns do appear to accumulate with advancing age, it is interesting to note that in one study of mtDNA POLG mutator mice, which over time accumulate such substitutions at an accelerated pace and show an accelerated aging phenotype, there was no evidence of an age-associated concomitant increase in oxidative stress [23].

As somatic mutations begin to accumulate they create heteroplasmies whose levels will likely initially reside below the level of detection. If they become fixed in the genome and are replicated over time, their percent of the total mtDNA copies increases and the percent heteroplasmy increases. Classically, it has been easier to detect somatic deletion mutations than it has been to detect somatic point mutations. Levels of some deletions, such as the ~5 KD common deletion, appear to increase in the brains of aging humans [46].

Could mtDNA Inheritance Affect Longevity?

It has been speculated that inherited mtDNA variations may influence aging and longevity. One prominent aging study, the Framingham Longevity Study, found that how many years either of an individual's parents lived correlated with how long that individual would live, but the mother's age at death correlated better with the age at death of the child [47]. One possible interpretation of this study is that a maternally inherited genetic factor, perhaps mtDNA, influences aging.

More specific mtDNA studies report particular mtDNA sequences also associate with life expectancy. An ND2 C5178A substitution is reportedly over represented in Japanese centenarians [48], while an ATPase6 G9055A substitution is reportedly over represented in French and Irish centenarians [49, 50]. Positive haplogroup association studies have also been published. MtDNA haplogroups are defined as patterns of nucleotide substitutions that tend to occur together within individuals, and which have arisen over the course of humanity and become fixed in different populations at different frequencies over the course of human migration and history [51, 52]. For example, the frequency of haplogroup J in Italian centenarians is reportedly higher than it is in the overall Italian population [53], and the frequency of haplogroups U and J is reportedly higher in Finnish centenarians than it is in the overall Finnish population [54].

Critical Questions about the Role Mitochondria Play in Aging

While there seems to be consensus that an organism's mitochondria and bioenergetic performance change over time, concerns about the place and role of mitochondria in aging are frequently raised. For the case of somatic mutations, it can be pointed out that correlation and association do not prove causation; it is possible that the observed accumulation of mtDNA mutations over lifespans, including point mutations and deletions, represents a consequence of aging and are not actually driving aging. MtDNA POLG mutator mice experiments were done with the purpose of addressing this question [22, 23], but some have raised questions about how well this system mechanistically models actual human aging, as well as to how rigorously the phenotype changes observed in these mice truly reflect normal physiologic aging [55, 56]. For those accepting the mtDNA POLG mutator mice as a good model of aging more nuanced questions have spurred debate, such as whether point mutations or deletions are primarily responsible for driving age-related changes in the mice [57, 58].

In particular, questions have been raised about whether the magnitude of functional mitochondrial changes seen in aging organisms in general, and in humans specifically, is predictably profound enough to induce physiologic changes. As a corollary to this, it has certainly been reported that compensatory mechanisms are initiated in the face of declining mitochondrial function and it must be considered how well these compensations mitigate the potential consequences of age-related changes in mitochondrial function and cell bioenergetics [30].

Finally, animal experiments have been reported in which the pharmacological or genetic induction of mitochondrial dysfunction actually associated with increased lifespan [59]. At the very least, this suggests that even if mitochondria are a major driver of human aging, the overall picture of how and why they drive aging may turn out to be quite complex.

Mitochondria and Alzheimer's Diseases

Overview

During the 1980's fluorodeoxyglucose positron emission tomography (FDG PET) brain scans revealed cortical glucose utilization is reduced in AD subjects [60, 61]. Decreased glucose utilization was featured in neuroanatomically discrete regions, including the posterior temporal and parietal cortices, as well as the posterior cingulate-precuneus region (Figure 2). The underlying basis for this observation has to date remained uncertain. Proposed possibilities have postulated these declines may reflect an artifact of neuron loss, or a loss of synaptic connectivity. However, when glucose utilization is studied in homogenized brain tissue, where synaptic connectivity is no longer maintained and the amount of material in the assay is standardized, reduced glucose utilization still remains [62]. This raises the possibility that reduced glucose utilization on FDG PET could also possibly reflect perturbed glycolysis flux.

Mitochondria are also different in AD patients than they are in age-matched control subjects. Overall mitochondrial size is reduced, although this is punctuated by the increased presence of overly swollen mitochondria with misshapen cristae [27, 63]. Activities of certain

mitochondria-localized enzyme are also reduced. This includes a reduction in the activity of the Krebs's cycle enzyme α -ketoglutarate dehydrogenase complex (KGDHC) and of pyruvate dehydrogenase complex [64–66], which gates the entry of glycolysis-derived, pyruvate-based carbon into the Krebs's cycle. Interestingly, the activity of some Krebs cycle enzymes, specifically the activity of enzymes in the second half of the cycle, have been reported to be increased in brains from AD subjects [67].

COX activity also tends to be lower in AD subjects than it is in age-matched control subjects [68]. Interestingly, this COX activity reduction is not limited to the brain. In fact, it was first reported to be present in platelet mitochondria derived from AD subjects [69–74], and only after that was it assayed in the brains of AD subjects, where its activity was similarly and consistently found to be reduced [70, 75–84]. In addition to platelet and brain mitochondria, AD COX activity has also been reported in fibroblast cultures derived from sporadic AD subjects [85].

Determinations of mitochondrial number in AD are to some extent complicated. For the most part, in AD brain the number of normal mitochondria per neuron appears to be reduced, although the amount of mitochondrial material that seems to be undergoing digestion within autophagosomes is increased [27]. Mitochondrial homeostasis is further perturbed in that there is an apparent shift towards increased mitochondrial fission, mitochondria are less likely to radiate from the perikaryon to neurite projections, and messenger RNA and protein levels of the transcriptional coactivator peroxisome-proliferator activated receptor γ coactivator 1 α (PGC1 α), which facilitates mitochondrial biogenesis, are reduced [86–90].

By some parameters mtDNA in AD subjects also seems to differ from that of age-matched control subjects. The amount of intact mtDNA is reportedly reduced, even though the amount of autophagosome-localized mtDNA may be increased [27, 91–93]. There appears to be increased numbers of some types of presumably somatic mutations, such as deletions and potentially also specific, presumably acquired point mutations [27, 92, 94–98]. Levels of mtDNA nucleotide oxidative damage are also higher in AD subjects than they are in control subjects [99].

Some studies claim inherited mtDNA variations are found in higher or lower frequencies in AD subjects as compared to non-AD subjects. Association studies have variably reported particular mtDNA haplogroups are statistically over or under-represented in AD cohorts, and that particular mtDNA single nucleotide polymorphisms (SNPs) are statistically over or under-represented in AD cohorts [100–113]. Results across different mtDNA association studies in AD have not been consistent across studies, though, which has led to some concern about the reliability of individual reports [114–118].

Could A β or APP Account for Differences in AD Mitochondria?

The plaques observed in AD subject brains contain a large percentage of the beta amyloid (A β) protein. A number of investigators have reported that A β localizes to mitochondria (in brains from human AD subjects and in APP transgenic mice), where it can bind to and interfere with the function of different intra-mitochondrial proteins including cyclophilin D and the A β -binding alcohol dehydrogenase (ABAD) protein [119–127]. A β has also been

shown in experimental systems to interfere with mitochondrial respiratory chain function, and to specifically inhibit COX activity [122, 128–131].

When added to neuronal NT2 teratocarcinoma cells, A β induces increased oxidative stress and cell death. However, when A β is added to NT2 teratocarcinoma cells that have been depleted of their endogenous mtDNA (p0 cells), that do not produce mtDNA-encoded respiratory chain subunits and are unable to successfully perform oxidative phosphorylation, these toxic A β effects are not observed [132]. This suggests A β toxicity in *in vitro* systems is at least to some extent mediated through its effects on cell respiration.

A β also impacts other aspects of mitochondrial homeostasis. It appears able to reduce mitochondrial movement [133], and to shift the mitochondrial fission-fusion balance towards the fission end of the spectrum [86, 87, 90].

The A β protein derives from a larger parent protein called the amyloid precursor protein (APP). An elegant series of experiments has demonstrated APP itself localizes to mitochondria [134–137]. APP in fact contains an N-terminal mitochondrial targeting sequence of strategically placed, positively charged amino acids that lead it to enter mitochondria through the translocase of the outer mitochondrial membrane (TOMM) and translocase of the inner mitochondrial membrane (TIMM) protein import apparatus [134, 136]. However, APP also contains a peptide sequence that causes the process of mitochondrial import to prematurely arrest, leaving an intra-mitochondrial N-terminal end and a long extra-mitochondrial C-terminal end [134]. The presence of APP at the mitochondria appears to in general interfere with normal mitochondrial function, and to specifically reduce COX activity [134].

Much of the data showing A β -mitochondria and APP-mitochondria physical associations derives from model systems and model organisms, such as transgenic mice that over express a mutant human APP transgene [119–122, 124, 125, 127]. However, physical associations have also been demonstrated in brains from deceased AD subjects [119, 126, 134]. Still, it is not immediately clear how mitochondria-localized APP or A β might interfere with the function of mitochondria outside the brains of affected individuals.

Evidence of a Maternal Inheritance Contribution to AD

Epidemiologic studies suggest that although an individual's AD risk is determined by both parents, maternal influence seems to be more profound than paternal influence [138–141]. This has been shown, for example, by the study of Edland et al. who found that among AD probands who also had a demented parent, the demented parent was more than twice as likely to be the mother [140]. Importantly, this relationship was observed even when the age of parental dementia onset was relatively young. This finding, therefore, is unlikely to simply reflect an artifact caused by greater longevity in women versus men, a factor that needs to be considered since survival of mothers to older ages than fathers might also increase their chance of developing a dementing disorder such as AD.

Endophenotype studies also support the presence of an AD maternal inheritance bias. Endophenotypes are incomplete manifestations of a disease; they can be defined by disease-

consistent characteristics that are insufficient by themselves to qualify one for a diagnosis, or by biomarkers. AD endophenotype studies have consistently shown that the non-demented adult children of AD mothers are more likely to have AD-like biomarker changes than the non-demented children of AD fathers. The first of these studies was that of Mosconi et al., which reported that FDG PET scans from mostly middle aged children of AD-affected mothers were more likely to manifest AD-like glucose utilization patterns than the mostly middle aged children of AD-affected fathers [142]. Similarly, an arterial spin labeling (ASL) study found the middle aged children of AD-affected mothers were more likely to show decreased perfusion patterns than the middle aged children of AD-affected fathers [143]. Studies have also shown the children of AD-affected mothers tend to have more cerebral amyloid than the children of AD affected fathers, and are also more likely to have increased levels of cerebrospinal fluid oxidative stress markers [144–147]. Several studies have found children of AD mothers also tend to have greater amounts of neuroanatomically specific cerebral atrophy than do the children of AD fathers [148–152]. One study found platelet mitochondria COX activity was lower in the children of AD mothers than it was in the children of AD fathers [153]. Finally, it was reported in the Framingham Longevity study that middle aged, non-demented APOE4 carriers who also had an AD-affected mother had lower scores on a memory test than did middle aged, non-demented APOE4 carriers who also had an AD-affected father [154].

Could APOE Influence AD Risk by Affecting Mitochondrial Function?

The APOE gene, located at locus 19q13.2, represents the most extensively studied sporadic AD genetic risk factor [155–157]. The APOE gene encodes a protein, apolipoprotein E, that plays a role in lipid and cholesterol transport [158]. There are three relatively common polymorphism-defined APOE alleles, the APOE2, APOE3, and APOE4 variants [159]. The APOE4 version is associated with an increased lifetime risk of developing AD [155]. Several hypotheses have been proposed in an attempt to mechanistically explain the association between APOE variants and AD risk. One hypothesis has arisen from the observation that the apolipoprotein E4 isoform folds differently than the other versions, and that this folding difference causes it to be proteolyzed into a smaller peptide that displays a functional mitochondrial targeting sequence [160, 161]. This apolipoprotein E 4-derived peptide appears to have toxic effects on mitochondria, and in cell culture it even seems to reduce COX activity [162]. Human brains from young deceased APOE4 carriers were also found to have reduced cortical COX activity; this was observed despite an absence of concurrent A β accumulation [163].

As was pointed out in the previous section, a cognitive-based endophenotype study of the Framingham Longevity cohort found that among non-demented, middle-aged APOE4 carriers individuals with an AD-affected mother had less robust performance on a test of memory than did subjects with and AD-affected father [154]. If an apolipoprotein E4 degradation product truly functions as a mitochondrial toxin, and inherited mtDNA features do turn out to influence AD risk, it could be the case that APOE4 and particular mtDNA sequences could interact to give rise to a “double” mitochondrial hit that creates a particularly elevated AD risk. Potentially consistent with this possibility are reports that

certain mtDNA haplogroups appear to modify the APOE4-associated increase in AD risk [106, 164].

While a reasonably strong case has been made that APOE genotype influences AD risk, it is nevertheless important to point out that the translocase of the outer mitochondrial membrane 40 kilodalton (TOMM40) subunit gene sits immediately adjacent to the APOE gene [165]. TOMM40 polymorphic variants have also been reported to associate with AD risk [166–174], although some of these TOMM40 variants are in linkage disequilibrium with the critical APOE isoform-defining variants [175]. This genetic confounding makes it difficult to prove or disprove whether the TOMM40 gene, through the TOMM40 protein, independently contributes to AD risk or contributes at least to some extent to the AD risk currently attributed in most circles to the APOE gene and apolipoprotein E protein [176].

Evidence for a Somatic mtDNA Mutation Contribution to AD

Some have hypothesized an accumulation of somatic mtDNA mutations could play a major role in the development of AD, and perhaps even represent a primary driving cause [21]. Data addressing this possibility have been mixed, and studies are limited to some extent because prior to the introduction of next generation sequencing approaches, it was technically difficult to resolve somatic single nucleotide changes at the microheteroplasmy level.

Perhaps for this reason initial studies emphasized quantification of large deletions. One early study, that of Corral-Debrinski et al., reported that brains from AD subjects who were less than 75 years of age had higher levels of the ~5 kb “common deletion” than brains from age-matched control subjects [94]. Hamblet et al. also found increased levels of the 5 kb common deletion in AD brain [96]. This finding was also essentially corroborated by Cottrell et al. and Krishnan et al., who initially found using a histochemical approach that AD brains were more likely to show COX-perturbed neurons in specifically examined areas, and later reported COX-perturbed neurons in AD brains had higher amounts of various large scale mtDNA deletions [177, 178].

Chang et al. used a sequential PCR amplification and restriction enzyme digestion approach to screen for the presence of mtDNA point mutations in AD brains [95]. This study did report evidence of increased AD brain mtDNA point mutations, which were felt to likely represent a consequence of mtDNA nucleotide oxidative damage. Interestingly, though, increased levels of the 5 kb common deletion were not detected in the AD brains from this study.

In a study by Coskun et al, the frequencies of several specific D-loop mutations were found to be profoundly increased in the brains of AD subjects [92]. This study further reported expression levels of ND6 were decreased and that the mtDNA to nuclear DNA ratio was reduced in the presence of these mtDNA control region mutations, which was interpreted as support for the view that these mutations interfered with mtDNA transcription and replication. It was also later reported by these investigators that the D-loop mutations that were found to be increased in AD subject brains were also increased in AD subject blood and lymphoblastoid cell line mtDNA [179].

On the other hand, in their study of AD and control brains Lin et al. did not detect a quantitative difference in the burden of microheteroplasmic point mutations [180]. To perform this study, the authors used a clonal sequencing analysis approach of PCR-amplified COX1 amplicons; in addition to analyzing brains from AD and age-matched control subjects, brains from a younger control group were also evaluated. While the microheteroplasmic mutation burdens were comparable between the AD and control groups, this study nevertheless presented several interesting findings: 1) microheteroplasmic mutations were relatively frequent; 2) there was a positive correlation between advancing age and the number of mutations per subject, so that it did appear that the mutation burden did increase with age; 3) there was a negative correlation between advancing age and COX enzyme activity, so that it did appear that COX activity did decline with age; and 4) there was a negative correlation between COX enzyme activity and COX1 mutation burden, so that it did appear that as COX1 mutations accumulated, COX activity fell.

Evidence of a Mitochondrial Link to Classic AD Histopathology Changes

More than one cell culture-based study has reported toxin-induced mitochondrial dysfunction, including toxin-mediated COX inhibition, reduces the processing of APP by the non-amyloidogenic α -secretase degradation pathway. This conclusion is based on the finding that cells treated with toxins such as sodium azide (a COX inhibitor) generate reduced levels of soluble APP α (sAPP α) [181, 182]. It has been further inferred by such studies that decreased α secretase-mediated processing of APP could reflect a shift in APP processing towards its amyloidogenic, β -secretase mediated processing pathway [183]. The cell culture study of Gabuzda et al. to some extent supports this inference, as these authors found that sodium azide-treated cells produced higher levels of an 11 kD APP cleavage product that was suspected to contain an intact A β peptide sequence [183].

A number of studies utilizing APP transgenic mice more directly suggest pertinent connections between brain energy metabolism and AD histopathology do exist. In the study of Scheffler et al., the investigators used a strategic strain inter-breeding approach to create groups of APP transgenic mice that ultimately differed primarily in their mtDNA sequences [184]. It was found that groups of mice with different mtDNA sequences developed profoundly different amounts of amyloid plaques.

Two mouse studies found reducing the amount of COX holoenzyme actually reduced amyloid plaque deposition. For the first of these studies, mice with APP and presenilin 1 (PS1) mutations were crossed with mice engineered for a Cre-loxP-mediated knock-out of the cytochrome oxidase 10 (COX10) gene [185]. The COX10 gene encodes a farnesyltransferase that is required for the synthesis of COX heme; eliminating this farnesyltransferase results in a dramatic reduction in COX holoenzyme production. COX activity accordingly declines, as does measureable markers of oxidative stress. In the second of these studies, APP/PS1 mutant mice were crossed with mice designed to express a mitochondria-targeted restriction enzyme that cleaves mtDNA [186]. This led to mtDNA depletion without generating evidence of oxidative stress, and a reduction in levels of the COX1 protein subunit. Increased levels of the APP-derived β -C terminal fragment (β -CTF) were detected, which did suggest a potential change to APP processing, although no increase

in BACE activity was observed and similar to the findings of Fukui et al., plaque accumulation was reduced.

Kukreja et al. evaluated a different mouse model with a predictably different type of respiratory chain defect [187]. The mice in this study were generated by breeding mice expressing a mutant human APP transgene with mice designed to express a dysfunctional mtDNA polymerase γ (PolgA D257A mice) and which accumulate mtDNA mutations at an accelerated rate. In this model of accelerated aging, plaque accumulation was also accelerated. Plaque accumulation differences between the Korea et al. study and the studies of Fukui et al. and Pinto et al. are not entirely clear, although it seems reasonable to consider differences in the nature of the induced mitochondrial defects may prove pertinent. One potential distinction between these studies is that the Fukui et al. and Pinto et al. mice seem to have decreased amounts of respiratory chain enzymes (or at least decreased amounts of COX or COX protein subunits), while the Korea et al. mice may have made functionally abnormal respiratory chain enzymes rather than less respiratory chain enzymes.

Another relevant mouse study is that of Dumont et al. [188]. This study crossed transgenic mice that expressed a mutant APP with transgenic mice that over expressed PGC1 α . Contrary to what was perhaps initially expected, the irenic mice showed increased amyloid plaque deposition. The irenic mice also demonstrated evidence of perturbed mitochondrial function, as the activities of several mitochondrial-localized enzymes (complex I, succinate dehydrogenase, and citrate synthases) were diminished. Proteasome activity was also diminished in the irenic mice, and this was felt to play a role in the amyloid deposition increase.

Other data from transgenic mouse studies could be considered potentially consistent with a possible bioenergetics-amyloidosis relationship. It has been shown that in APP transgenic mice, A β secretion into brain interstitial fluid is higher when the mice are awake and lower when they are asleep [189]. When APP transgenic mice are manipulated into a state of sleep deprivation, interstitial fluid levels are further elevated [190]. Increasing the whisker stimulation of APP transgenic mice increases, while decreasing the whisker stimulation of these mice decreases, interstitial fluid A β levels [191]. When Yamamoto et al. used optogenetic stimulation to induce chronic neuronal excitability within the hippocampal performant pathways of APP transgenic mice, interstitial A β and A β plaque levels increased [192]. Taken together, studies such as these suggest synaptic activity increases A β production, and because synaptic activity creates a state of bioenergetic stress these data at least indirectly argue a link should exist between cell bioenergetics and APP processing/A β production.

In humans potential links between bioenergetics and APP processing/A β production are harder to establish, although a study of trauma victims did find that emergence from a coma state corresponded temporally with an increase in the brain's interstitial A β level [193]. This would seem to be consistent with mouse data that report synaptic activity correlates with A β production.

Other relevant human data may be inferred from the study of Vlassenko et al., who reported that areas in which plaques initially present are parts of the brain's default mode network (DMN)[194]. These regions show a unique bioenergetic pattern that features an increased reliance on aerobic glycolysis, defined by the authors as all glucose utilization that occurs in an adequately oxygenated tissue or adequately oxygenated cell that is not utilized in oxidative phosphorylation. Progressively lower amounts of glucose carbon released as CO₂ in this study were interpreted as being indicative of a progressively increased metabolism of glucose through aerobic glycolysis; non-oxidative phosphorylation uses of glucose include metabolism of glucose to lactate, incorporation into glycogen, a contribution of carbon to fatty acid or cholesterol synthesis, or the entry of glucose into the pentose phosphate shunt. This study stresses that in considering the potential relationship between bioenergetics and APP/A β , in addition to considering how much energy metabolism is present, what energy fluxes are present as well as how and why particular fluxes are occurring warrants consideration.

Relationships between neuro fibrillary tangles and the tau protein they contain are also reported. Toxic perturbation of cell bioenergetics is certainly recognized to influence the activities of kinases that phosphorylate tau, and to increase tau phosphorylation. This has been demonstrated in both cell culture and animal-based experiments [195–197]. For example, administering the COX inhibitor sodium azide to rats increases tau phosphorylation [196], as does exposing wild type mice and mice that express a mutant tau transgene to annonacin, a complex I inhibitor [198, 199]. Links between tau phosphorylation and metabolism are also suggested by a study that reports prolonged fasting in mice induces brain tau phosphorylation [200].

A recent study by Zhao et al. demonstrated a potential link between mitochondria and the aggregation of tau into tangles [201]. In this study the authors evaluated the effects of a gene polymorphism in the myelin-associated oligodendrocyte basic protein (MOBP) gene that was previously associated with the risk of developing progressive supranuclear palsy (PSP), a neurodegenerative disease that features tangle accumulation [202]. MOBP is located relatively close to the gene that encodes a protein called apoptosin, anuclear-encoded protein that localizes to the mitochondrial inner membrane and participates in heme synthesis. The authors found that the MOBP polymorphism influenced apoptosin expression, which increased in the presence of the PSP-associated MOBP polymorphism [201]. Higher amounts of apoptosin lead to increased heme production, which in turn lead to increased production of cytochrome c. This resulted in an increase in the amount of cytochrome c protein that leaked into the cytoplasm, which in turn activated caspase 3. Caspase 3 then cleaved tau protein at a caspase cleavage site, which generated a tau fragment that aggregated to form tangles and also induced synaptic dysfunction.

Mitochondrial uncoupling has also been demonstrated to induce tau paired helical filament formation [203]. Interestingly, fibroblasts cultures prepared from sporadic AD subjects also show altered mitochondrial function [85], and are more likely to bind an antibody that recognizes paired helical filament tau than are fibroblast cultures prepared from non-AD subjects [204].

AD Cytoplasmic Hybrid (Cybrid) Studies

Overview

The cytoplasmic hybrid (cybrid) technique makes it possible to transfer mtDNA from one cell to another, and to then perpetuate that transfer (Figure 3)[205]. In some ways it is similar to forming cell hybrids [206], with an important distinction being that the resulting cell product contains nuclear DNA from only one source. Further, when mtDNA is transferred it is not transferred in its pureform. Rather, it is contained within the mitochondria from the donor source. Whole mitochondria, therefore, actually serve as a transfer vessel. Finally, mitochondrial transfer can be accomplished using different approaches that to some extent determine whether additional cell constituents are also transferred. For example, isolated mitochondria can be injected into the recipient cell, or donor and recipient cells can be mixed in the presence of a detergent that disrupts membrane integrity and allows for a more extensive mixing of cytosolic contents [207]. Ideally, though, how the mtDNA transfer is accomplished should ultimately lead to the same product because as the resultant cell undergoes subsequent growth and division non-perpetuating materials should degrade over time, and should dilute over the course of repeated cell divisions. The only obvious transferred component that can replicate and therefore perpetuate is the mtDNA. Conceptually, it could be possible that a templating protein could also be transferred and perpetuate, such as a prion protein, but to date this has not been described in the cybrid literature.

Although simply introducing isolated mitochondria to cultured cells is accompanied by some degree of intracellular mitochondrial internalization, an event referred to as “transformation” [208], the first intentional transfer of mitochondria to recipient cells (in the mid-1970’s) featured fusion of enucleated cytoplasts with nucleated cells. The scientific goal of these early studies (which introduced the cybrid term) was to test whether chloramphenicol resistance, a characteristic of some cell lines, was an mtDNA-determined trait [209, 210]. The investigators found that when mitochondria-containing cytoplasts from a chloramphenicol-resistant cell line were mixed and fused in culture with a chloramphenicol-sensitive cell line, some of the chloramphenicol-sensitive cells acquired chloramphenicol resistance. This lead the investigators to conclude that chloramphenicol resistance was indeed an mtDNA-determined trait.

It is important to note that as a result of this approach the resulting cybrid cells, at least initially, were presumably heteroplasmic as they should have contained the mtDNA that was endogenous to the accepting cell line, as well as the mtDNA from the donor cytoplasm mitochondria. To refine the technique, King and Attardi subsequently developed the idea of using a $\rho 0$ cell line as the accepting cell line [207]. $\rho 0$ cells are cells that have undergone depletion of all detectable mtDNA. The development of $\rho 0$ cell lines, in turn, followed the efforts of several groups to mimic in cultured cell lines the previously observed ability of yeast cells to deplete their mtDNA content under conditions that favored glycolysis [211–213]. These mtDNA-depleting yeast cells were called ρ petites, since prior to its identification as mtDNA cytosolic DNA was initially referred to as ρ DNA [214]. By using

$\rho 0$ cells as the accepting cell line, investigators gained the ability to create cell lines that contained only mtDNA from the mitochondrial donor.

Moving forward using $\rho 0$ cell lines as the recipient cells, investigators began to study issues of heteroplasmy, threshold, and in general the biochemical consequences of known mtDNA mutations [215–220]. Mitochondria from human subjects with known homoplasmic or heteroplasmic mtDNA mutations were transferred to $\rho 0$ cells. The resulting cybrid cells were expanded in culture. In instances where heteroplasmic mutations were transferred, the expanding cybrid cells were isolated in order to facilitate the creation of cybrid clones, which ultimately could be shown to contain different ratios of mutant to wild type mtDNA. These clones with different heteroplasmic ratios were then analyzed biochemically to determine how much of a mutational burden was required for a particular mutation to cause a change in biochemical function, and thereby estimate the percent of mutation that had to be present to reach a phenotypic threshold.

Interest in the cybrid approach to address mtDNA-related questions further developed as more $\rho 0$ cell lines were created, and after it was shown that platelets could serve as mitochondrial donor cells [219, 221]. Platelets, which derive from megakaryocytes, lack nuclei and are easily accessed through routine phlebotomy. Through a simple procedure platelet-rich plasma can first be generated from a blood sample, and an enriched platelet fraction can then be prepared through centrifugation of the plasma. The enriched platelet fraction can then be mixed with the $\rho 0$ line of choice to generate cybrid cells.

In the mid-1990s the cybrid approach was first used for a somewhat novel application that involved the utilization of mtDNAs whose sequences were unknown [222]. It was reasoned that biochemical differences between cybrid cell lines prepared from different mitochondrial donors could be used to infer differences existed in their mtDNA sequences. From the perspective of whether mtDNA sequences do indeed vary between individuals applying such an approach did not carry much risk; it was already recognized that mtDNA sequences from individuals that did not derive from a common maternal lineage typically deviate at multiple nucleotide positions. Whether bona fide biochemical differences would be too subtle to detect, and produce false negative results, though, was a concern. A second concern was that variation in biochemical measures could lead to false positive results or incorrect conclusions about the association of particular mtDNA genomes with a particular characteristic. Regarding these two points it was anticipated that by creating large enough groups of cybrid cell lines from large enough groups of mitochondrial donors with a particular biochemical characteristic, one could accurately ascertain whether the specific biochemical characteristic within that group was influenced by the mitochondrial genome [205].

At the time this strategy was defined it was felt to also offer particular experimental strengths. In the mid-1990s DNA sequencing was a far more expensive endeavor than it currently is, and the sequencing approaches of that time were not able to reliably detect low abundance heteroplasmic deviations. Plus, given the high degree of mtDNA polymorphic variation that exists between individuals, unless a particular “smoking gun” sequence mutation that reliably segregated with a cohort was identified, without functional data it

would prove difficult to associate individual sequence deviations with a specific group. Doing so would presumably require analyzing very large numbers of individuals.

The first time this approach was utilized was in studies of cybrid cell lines prepared from a group of platelet mitochondria/mtDNA donors with Parkinson's disease (PD)[222]. As a group, PD patients were already recognized to have platelet mitochondria complex I activities that were lower than those measured in age-matched control subjects [223–225]. Twenty-four cybrid lines were generated from PD subjects, and 28 cybrid lines were generated from 28 age-matched, control subjects. Platelets served as the mitochondria/mtDNA donor source. A human neuroblastoma SH-SY5Y ρ 0 cell line that had previously been derived from the standard SH-SY5Y line served as the mitochondria/mtDNA acceptor [221]. The mean complex I activity was found to be ~20% lower in the PD subject-derived cybrid group (simply referred to as the “PD cybrid” group) than it was in the control subject-derived cybrid group (simply referred to as the “control cybrid” group). It was concluded that mtDNA, at least to some extent, contributes to reduced complex I activity in persons with PD.

AD Cybrid Experiments

The cybrid approach was deemed reasonable to address the specific question of why individuals with AD on average have a lower platelet mitochondria COX activity than age-matched, non-AD subjects [69–74]. Non-genetic explanations included the presence in the circulation of a factor that inhibits COX activity. Because COX contains 13 subunits, 10 of which are encoded by nuclear genes and 3 of which are encoded by mitochondrial genes, genetic explanations could alternatively implicate a nuclear DNA or mtDNA-dependent component. It was a priori hypothesized that mtDNA genes were more likely to contribute to lower COX activity in AD subjects than nuclear DNA genes, since late-onset AD (LOAD) rarely demonstrates recognizable Mendelian inheritance [226]. LOAD is generally considered to show sporadic epidemiology although nevertheless with a genetic influence, and in many ways the unique genetic rules of mtDNA, including heteroplasmy, threshold, mitotic segregation, and maternal inheritance uniquely position it to play a role in otherwise apparent sporadic diseases that also demonstrate altered mitochondrial function [226].

In terms of applying the cybrid technique to address this question, it was reasoned that if lower mean COX activities were caused by a circulating inhibitory factor, that factor would wash out over the course of expanding the cell lines generated using platelets obtained from AD subjects (herein referred to as “AD cybrids”). Presumably, low AD subject platelet mitochondria COX activity under this scenario would not perpetuate in culture as AD cybrid line COX activities would increase in culture to match that of the cybrid lines generated from platelets obtained from age-matched, control subjects (herein referred to as “control cybrids”). It was further reasoned that a nuclear DNA-dependent feature would be unlikely to account for a relative reduction in the AD cybrid COX activity because nuclei are not routinely transferred during the procedure, or if such a transfer did occur the transferred nuclei would be unlikely to perpetuate. Similar to toxin-induced activity reductions, nuclear DNA-dependent reductions in COX activity would predictably wash out after the transfer and selection process was completed.

The first published AD cybrid study was the one by Davis et al [227]. This study compared COX data from a group of 20 AD cybrid cell lines to a group of 45 control cybrid cell lines. Transferred mtDNA derived from subject platelet mitochondria, and the acceptor cell line was the SH-SY5Y p0 line. COX activity was ~20% lower in the AD cybrid group than it was in the control cybrid group. It is relevant to note that the Davis et al. report was subsequently retracted, although the reasons for the retraction were unrelated to the cybrid data that were presented [228].

Later in 1997 two other AD cybrid studies were reported. In the study of Sheehan et al., platelets served as the mitochondria/mtDNA donor source, and the acceptor cell line was the SH-SY5Y p0 line [229]. An ~50% lower COX activity in the AD cybrids was seen. In the other study, that of Swerdlow et al., platelets served as the mitochondria/mtDNA donor source and the acceptor cell line was an NT2 teratocarcinoma-derived p0 line [230]. Fifteen AD cybrid lines were compared to 9 control cybrid lines, and a relative 16% reduction in the AD cybrid group COX activity was observed.

Other studies of unique AD cybrid series have focused in particular on COX activity. In the study of Cardoso et al., the authors used platelet mitochondria to generate AD and control cybrid lines on an NT2 p0 nuclear background, and found that COX activity in the AD cybrid cell line (n=6) group was 22% lower than it was in the control cybrid cell line (n=5) group [231]. In the study of Silva et al, the authors used platelet mitochondria to generate AD and control cybrid lines on an SH-SY5Y p0 nuclear background, and found that COX activity in the AD cybrid cell line (n=8) group was ~30% lower than it was in the control cybrid cell line (n=7) group [232]. On the other hand, the study of Ito et al. also used COX activity as a primary endpoint, and found COX activity was comparable between the AD and control cybrid groups [233]. However, there are a number of notable methodologic differences between the Ito et al. study and the positive studies indicated above. The Ito et al. group used a HeLa cell p0 cell line to generate their cybrids, and the mitochondria/mtDNA donor source was mixed; four AD cybrid lines were prepared from platelet mitochondria, three control cybrid lines were prepared from platelet mitochondria, and two control cybrid lines were prepared from fibroblast mitochondria. Also included in the analysis were what were designated as an additional three AD cybrid lines, which were generated by mixing HeLa p0 cells with synaptosomes prepared from a brain that was acquired from a deceased AD subject after a 20-hour postmortem interval. The authors reported they were able to identify three cell colonies from this fusion that contained mtDNA, and COX activity data ascertained from each of these three colonies were individually included in the analysis. Due to these substantial methodologic differences, it is arguably difficult to conclude that the Ito et al. negative study contradicts the positive studies.

A number of AD cybrid studies have evaluated various other aspects of mitochondrial function as well as parameters influenced by mitochondrial function [227, 229–232, 234–252]. In many cases changes are reported that recapitulate changes that are seen in the brains of AD subjects themselves [68]. Relative to control cybrid cell lines, in AD cybrid cell lines oxidative stress markers are increased [229–231, 234, 236, 240, 241, 245, 248, 249], inflammatory and stress signaling pathways are activated [234, 236, 239–241, 245, 250], A β levels are increased [238, 241], glucose utilization is decreased [232], oxygen consumption

is decreased [232], there is a shift towards mitochondrial fission and a smaller average mitochondrial size [243, 247], numbers of ultrastructurally perturbed mitochondrial are increased [243, 252], PGC1 α mRNA levels are reduced [232], HIF1 α protein is reduced [232], mTOR protein is reduced [232], SIRT1 protein is reduced [232], and apoptotic markers are increased [231, 238–241, 245].

AD cybrids have also been used to model aspects of AD-specific, mitochondria-related function that are difficult or impossible to study in autopsy brain tissue [68]. For example, mitochondrial membrane potential analyses of AD cybrid lines shows a relative degree of depolarization [235, 238, 242, 252], and AD cybrid mitochondria appear to internalize less calcium and are less able to buffer calcium-mediated intracellular signaling activity than control cybrid lines [229]. AD cybrid ATP levels are reduced [231, 232]. It has been shown using differentiated AD cybrid cell lines that mitochondrial movement is relatively reduced [244]. AD cybrid cells are more sensitive to A β toxicity than are control cybrid cells [231, 250]. AD cybrids have also been used to screen the molecular effects of potential therapeutic interventions; pharmacologic inhibition of mitochondrial fission activity and antioxidants has been shown to benefit certain mitochondria-related functional parameters [241, 245, 247, 248].

Three studies, one performed using an NT2 ρ 0 cell background and two performed using an SH-SY5Y ρ 0 cell background, have reported mitochondria-relevant functional changes (including a reduction in COX activity) between cybrid lines generated from human subjects diagnosed with mild cognitive impairment (MCI; a frequent AD precursor state) and cybrids generated from age-matched control subjects [232, 246, 248]. To date, over 20 cybrid studies have been published that report at least one biochemical or molecular parameter that differs between groups of AD/MCI and control cybrid cell lines [227, 229–232, 234–252]. Most of these studies have in fact reported multiple divergent parameters. The only categorically negative AD cybrid study was that of Ito et al. [233], which evaluated just one biochemical parameter (COX activity), and which is notable for a variety of distinct methodologic differences that may have caused that study to differ from the other positive studies.

Implications and Limitations of AD Cybrid Studies

Data from AD cybrid studies support three important assumptions. First, they argue mtDNA accounts for, or at least to some extent contributes to, differences in mitochondrial function that reportedly exist between AD and non-AD subjects. Second, they argue that mitochondrial function can contribute to hallmark extra-mitochondrial histopathology changes, such as increases in oxidative stress markers [253] and A β production. Third, since cybrid cell lines in these studies have been almost exclusively generated through transfer of platelet mitochondria/mtDNA, the AD cybrid literature argues that at least at a molecular or biochemical level, AD changes are not strictly limited to the brain.

Cybrid studies also have limitations [205, 254]. Because mitochondria/mtDNA are transferred from platelets, it is possible that what drives mitochondrial dysfunction in AD cybrids is different from what drives mitochondrial dysfunction in AD brains. Given that the nature of mitochondrial dysfunction and its consequences seem to recapitulate so many biochemical, molecular, and physiologic features observed in AD brains, though [68], it

would seem a stretch to propose that mitochondrial dysfunction in AD cybrids is entirely unrelated to mitochondrial dysfunction in AD brain.

The acceptor p0 cell lines are derived from tumor cell lines, which limits their ability to rigorously model the characteristics of a human brain. Therefore, when using cybrid cell lines to model AD mitochondrial functional defects, it is probably best not to extrapolate interpretations too far beyond the level of fundamental observation.

One general criticism of the cybrid approach is that although the approach was initially adapted to address questions about the contribution of mtDNA to mitochondrial function in AD, and subsequently used to model aspects of AD subject-specific mitochondrial function, cytosolic components other than mtDNA are transferred during the procedure. This is relevant because any transferred perpetuating component could theoretically lead to sustained changes in mitochondrial function. To date, though, no such component other than mtDNA has been identified. Also, since whole mitochondria are transferred from platelets to acceptor cells, and platelet mitochondria in AD show unique biochemical characteristics, the possibility that a pre-existing mitochondrial defect simply did not have adequate time to wash out requires consideration. This possibility was empirically addressed in one of the early AD cybrid studies, which found that the six-week selection and expansion period later used in most of the AD cybrid studies appears to provide an adequate wash-out period [230]. Moreover, one study reported that with extended time in active culture, a number of altered mitochondrial functional parameters appeared to become more profound [252]. This occurred despite an apparent activation of compensatory adaptations.

While cybrid studies implicate an mtDNA contribution to AD-associated mitochondrial dysfunction, they do not indicate what specific mtDNA features, characteristics, or sequences contribute to that dysfunction. They could reflect an accumulation of somatic mutations; to date data pertinent to the question of whether putative somatic mutations accumulate in circulating blood cells is mixed. One study did not find an obvious age-related accumulation of large deletions in a general population sample [255], which suggests large mtDNA deletions do not commonly accumulate in circulating cells as they do in the brain. Data addressing the question of whether somatic point mutations accumulate in AD subject blood cells do, however, suggest this may indeed occur [97, 98, 179].

Alternatively, it is possible that inherited mtDNA sequence differences may partly or exclusively contribute to AD cybrid line differences in mitochondrial function. Currently, the nature of such potential inherited mtDNA changes, if they in fact exist, is not clear. MtDNA sequence studies performed on AD thus far have not identified a single mutation or variant that distinguishes AD-affected from AD-unaffected individuals, although various studies have claimed common inherited mtDNA signatures, such as those defined by the different mtDNA haplogroups, influence AD risk [113]. No mtDNA study to date has definitively addressed the question of whether rare mtDNA mutations or sequence variants associate with AD; such studies would require extensive mtDNA sequence data from large numbers of AD and control subjects.

Finally, a considerable degree of polymorphic variation has been demonstrated to exist within human nuclear respiratory chain subunits [256]. Because the cybrid approach neutralizes the potential functional contributions of a subject's nuclear DNA make-up, valuable information regarding the interplay between that individual's nuclear and mitochondrial genomes is likely to be lost. Substituting the nuclear background of a $\rho 0$ cell for the nuclear background of the mtDNA donor could theoretically magnify or mitigate a particular mtDNA sequence-associated functional consequence.

The Mitochondrial Cascade Hypothesis

Despite the aforementioned limitations of AD cybrid studies, it is tempting to try and integrate AD cybrid data with data generated from AD tissue, epidemiology, endophenotype, and genetic studies in order to define an overarching hypothesis of AD etiology. Because advancing age is the single greatest sporadic AD risk factor [8], it is recommended that any resulting hypothesis take into account applicable data and conceptual constructs generated through primary studies of human and animal aging.

A number of investigators have proposed mitochondrial dysfunction could play an important role in AD [21, 27, 62, 64, 69, 257–268]. Some have proposed mtDNA inheritance or an age-related somatic accumulation of mtDNA mutation could contribute [21, 226]. When these conceptual constructs are considered in conjunction with the observation that specific differences in non-brain mitochondrial function seem to exist between AD and non-AD subjects, and that differences in mitochondrial function and cell bioenergetics can influence AD histopathology changes, the case can be made that perhaps mitochondria and bioenergetic function play an upstream if not primary role in AD.

The “mitochondrial cascade hypothesis” represents one attempt to comprehensively synthesize data discussed throughout this chapter into a comprehensive hypothesis that tries to account for the development and progression of AD, as well as the relationship of AD to brain aging (Figure 4) [269–272]. The premise is that individuals inherit a baseline level of mitochondrial function and durability; this baseline is determined by genetic contributions from both parents, although the mother makes a greater contribution and has a greater influence because she contributes the mitochondrial genome [273]. Then, as the individual ages certain tissues, and especially the brain, either accumulates somatic mutations or else drifts towards increased levels of inherited microheteroplasmic mutations. The rate at which mutations accumulate would presumably be influenced by the individual's genetic make-up, and also by lifestyle factors. These age-related changes would result in declining mitochondrial function, which up to a point could probably be accommodated and addressed through adaptive molecular changes, and thereby define a period of compensated aging. Eventually, though, a threshold of mitochondrial/bioenergetic dysfunction could be reached in which accommodation and compensation are no longer adequate, thus introducing a period of uncompensated brain aging. At this point, an AD phenotype would begin to emerge.

The mitochondrial cascade hypothesis further presumes the classic histologic features of AD, including A β plaque and tau neurofibrillary tangle deposition, are a downstream consequence of changing mitochondrial and bioenergetic function. In its original form the

hypothesis speculated A β plaques accumulated only after the state of uncompensated brain aging was reached [271]. Recent neuroimaging data from a human cohort longitudinal study now shows, though, that most A β plaque deposition occurs during the run-up to the AD clinical syndrome and dramatically slows during the symptomatic stages [274, 275]. In conjunction with experimental studies that show A β is generated as a byproduct of synaptic activity [191, 192], this suggests the possibility that A β plaque deposition may primarily represent a byproduct of the compensatory changes that accompany age-related mitochondrial functional declines. If correct, this modification to the mitochondrial cascade hypothesis could potentially account for why a substantial percentage of older adults develop A β plaques but do not develop the clinical disease [8]; such individuals may avoid making the transition from bioenergetically compensated to uncompensated brain aging.

Conclusions

Decades ago the aging field began to specifically postulate mitochondria and bioenergetic function contributed to aging [5, 19–21]. This was originally predicated on correlative and descriptive data. More recent experimental data have emerged, though, that are consistent with this possibility [22, 23].

Over an almost five-decade period it has become increasingly clear that bioenergetic and mitochondrial physical and functional changes also occur in AD [21, 27, 62, 64, 69, 257–268]. In many cases changes observed in AD are reminiscent of those seen in aging, and in some ways differ primarily in their magnitude [68]. While mitochondrial and bioenergetic changes in AD were initially felt to represent a consequence of the disease, their potential relevance to disease progression has increasingly been considered, and the view that such changes represent valid therapeutic targets has emerged [276–279].

When considering the hierarchy of biochemical, molecular, and physiologic events that result in AD, some have pointed out that in AD subjects bioenergetic and mitochondrial differences are found outside the brain, and that changes in bioenergetic and mitochondrial function can alter how cells and tissues handle other AD phenomena, including how APP is processed to A β and A β plaque deposition [9, 280]. Additional data pertinent to these points have been reported from studies of cybrid cell lines generated through the transfer of AD subject platelet mitochondria/mtDNA to p0 cell lines; results from these studies are consistent with the view that mtDNA contributes at least in part to AD mitochondrial and bioenergetic changes, and that these changes can drive or at least contribute to a variety of biochemical, molecular, and histologic phenomena observed in AD subject brains [68].

Synthesizing a spectrum of data from the aging, AD, and cybrid literature supports a conceptual construct that places mitochondrial function and bioenergetics at the apex of AD-associated molecular changes [269–272]. MtDNA would to some extent influence relevant mitochondrial and bioenergetic functional parameters. These molecular changes would similarly play out during the basic process of aging, and in some cases differences observed in both aging and AD would differ mostly by degree, with deficits being more prominent when clinical AD is present. Under this scenario some of the key histologic changes we now associate with AD, such as processing of APP to A β and A β plaque deposition, would

represent downstream consequences of altered mitochondrial and bioenergetic function. Some of these histologic changes may arise during stages where declining brain mitochondrial and bioenergetic function could still be accommodated and compensated for, or could arise after declining brain mitochondrial and bioenergetic function have surpassed a critical level at which point successful compensation is no longer possible. In the brain, classic AD histology changes initiated by mitochondrial and bioenergetic dysfunction could in turn exacerbate failing mitochondrial and bioenergetic function. This mitochondrial cascade hypothesis makes testable predictions and suggests particular therapeutic strategies may be worth pursuing. It will be interesting to see how well the mitochondrial cascade hypothesis absorbs new current and future data generated by the AD research field.

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Figure 1. Oxidation-mediated mtDNA mutation

(A) A G-C pair is converted to a T-A pair following the oxidative deamination of a cytosine nucleotide to a uracil nucleotide. (B) A G-C-pair is converted to a T-A pair following the oxidative conversion of a guanosine to 8-hydroxy-2-deoxy guanosine and then to 8-oxo-2-deoxyguanosine.

Figure 2. Fluorodeoxyglucose positron emission tomography (FDG PET) scan from an AD patient

In the normal case the cortical region should show a consistent level of relatively high glucose uptake and, therefore, utilization. In in this FDG PET scan from an individual with AD there is attenuation of the high cortical glucose uptake/utilization signal (indicated by a red-orange color) in the region of the posterior temporal/inferior parietal cortical regions (indicated by a yellow-green color).

Figure 3. The cybrid technique

A cell line's endogenous mtDNA is removed to create a p0 cell line, which lacks respiratory-competence and must be maintained in medium supplemented with pyruvate and uridine. After mixing p0 cells with mitochondria-containing cytoplasts or platelets, and facilitating cytosolic mixing by addition of detergent, some p0 cells incorporate exogenous mitochondria and by extension their mtDNA. The transferred mtDNA allows for the restoration of respiratory competence, and the newly created cybrid cells can be selected for by removing pyruvate and uridine from the medium (leading to the removal of residual untransformed p0 cells). The cybrid cells that result from a single fusion can be grown as separate clonal colonies; in cases where the donor mtDNA carries a heteroplasmic mutation, the individual cybrid clonal lines can be analyzed to address issues of threshold. Alternatively, the cybrid cells that result from a single fusion can be expanded together, creating a single cybrid line that can be compared to other unique cybrid cell lines.

Figure 4. The AD mitochondrial cascade hypothesis

Inheritance from both parents determine an individual's bioenergetic set-point and durability, with the mother having the greater input due to her contribution of the mtDNA. Over time mitochondrial efficiency declines, likely due to accumulating damage to the mtDNA. At relatively low levels it is possible to compensate for this change (compensated brain aging), although the compensatory process may itself have consequences. More profound declines in mitochondria function, which may occur as further damage accumulates, can lead to a stage of uncompensated brain aging, which associates with other consequences as well as symptomatic AD.