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Trends in Mitochondrial Therapeutics for Neurological Disease

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Abstract

Neuronal homeostasis is critically dependent on healthy mitochondria. Mutations in mitochondrial DNA (mtDNA), in nuclear-encoded mitochondrial components, and age-dependent mitochondrial damage, have all been connected with neurological disorders. These include not only typical mitochondrial syndromes with neurological features such as encephalomyopathy, myoclonic epilepsy, neuropathy and ataxia; but also secondary mitochondrial involvement in neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's disease. Unravelling the molecular aetiology of mitochondrial dysfunction opens new therapeutic prospects for diseases thus far lacking effective treatments. In this review we address recent advances on preventive strategies, such as pronuclear, spindle-chromosome complex, or polar body genome transfer to replace mtDNA and avoid disease transmission to newborns; we also address experimental mitochondrial therapeutics aiming to benefit symptomatic patients and prevent disease manifestation in those at risk. Specifically, we focus on: (1) gene therapy to reduce mutant mtDNA, such as anti-replicative therapies and mitochondria-targeted nucleases allowing favourable heteroplasmic shifts; (2) allotopic expression of recoded wild-type mitochondrial genes, including targeted tRNAs and xenotopic expression of cognate genes to compensate for pathogenic mutations; (3) mitochondria targeted-peptides and lipophilic cations for *in vivo* delivery of antioxidants or other putative therapeutics; and (4) modulation of mitochondrial dynamics at the level of biogenesis, fission, fusion, movement and mitophagy. Further advances in therapeutic development are hindered by scarce *in vivo* models for mitochondrial disease, with the bulk of available data coming from cellular models. Nevertheless, wherever available, we also address data from *in vivo* experiments and clinical trials, focusing on neurological disease models.

1. INTRODUCTION

Neuronal physiology is intricately linked with mitochondrial bioenergetics and dynamics. It comes therefore at no surprise that mitochondrial failure eventually leads to neuronal demise. Mitochondrial dysfunction may in fact affect multiple organs or tissues, but the energetic needs and the non-dividing nature of neurons place the nervous system at particularly high risk. Neuronal ion balance continuously demands ATP from an active mitochondrial respiratory chain [1], which integrates five multi-subunit complexes embedded in the inner mitochondrial membrane (IMM). With the exception of complex II, fully encoded by nuclear DNA (nDNA), all other respiratory complexes have a dual genetic origin, with some subunits encoded by nDNA and others by mitochondrial DNA (mtDNA). Respiratory complexes require nDNA-encoded factors for proper assembly and addition of prosthetic groups. In fact, the nucleus encodes all proteins involved in mtDNA replication, transcription, translation and repair, as well as mt-tRNA synthetases, mitochondrial ribosomal proteins, and proteins involved in maintaining the mitochondrial deoxynucleoside triphosphate pool [2]. Multiple Mendelian-inherited nDNA mutations may thus cause respiratory chain dysfunction.

Primary mitochondrial dysfunction also arises from mutations in mtDNA, which encodes 13 respiratory chain subunits plus 2 rRNAS and 22 tRNAs required for intra-mitochondrial protein synthesis. Specific features of mitochondrial genetics include the (practically) exclusive maternal inheritance of multiple mtDNA copies, which can be identical (homoplasmy) or different within the same cell (heteroplasmy). Also, random mitotic segregation of mtDNA, variable heteroplasmy, potential for clonal expansion, and diverse mutational thresholds, condition the differential expression of biochemical defects across cells and tissues [3]. Such idiosyncratic features contribute for a wide variability of mitochondrial disease manifestation and for the complex clinical maze of mitochondrial neurology [4].

Neurological symptoms accompany several diseases resulting from mtDNA mutations, namely, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS); myoclonic epilepsy and ragged red fibres (MERRF); neuropathy, ataxia, and retinitis pigmentosa (NARP); Leber's hereditary optic neuropathy (LHON); and maternally inherited Leigh syndrome, among others [5]. It is instructive that complex I deficiency in Leigh syndrome may stem not only from mutations in mtDNA but also in the nDNA-encoded subunits and assembly factors [2]. In fact, even when mtDNA is free from pathogenic mutations and all nuclear-encoded respiratory complex subunits are correctly expressed; multiple 'indirect hits' can still cause mitochondrial-associated neurological disease. Mutations in respiratory chain ancillary proteins are key examples of such indirect hits. Also, mutations in the mt-RNA translation machinery, in enzymes governing the IMM lipid milieu, and in coordinators of the mitochondrial fission-fusion equilibrium are increasingly identified as causes for neurological disorders [4]. The latter are typically illustrated by mutations in the pro-fusion proteins OPA1 and Mfn2, respectively associated with dominant optic atrophy and Charcot-Marie-Tooth type 2A neuropathy. Loss of function mutations in PINK1 and Parkin, causing hereditary Parkinson's disease (PD), provide another key link between abnormal mitochondrial dynamics, in this case mitophagy, and neurodegeneration. Even when mitochondrial damage is a secondary pathological event, it still plays a prominent role in key neurodegenerative disorders such as Alzheimer's disease (AD), Huntington's disease (HD) and spinocerebellar ataxias, amyotrophic lateral sclerosis, and several others [6, 7]. Interestingly, differences in mitochondrial physiology across brain regions may contribute, together with other cellular and environmental factors, for differential vulnerability patterns to neurological disease [8, 9].

Experimental models of mitochondrial disease are primarily cell-based. Cytoplasmic hybrid cells (cybrids; generated by depleting host cells from endogenous mtDNA and repopulating with mtDNA from patient cells), are widely used to address the relative contribution of nuclear *vs*. mitochondrial backgrounds for a given disease [10]. The yeast *Saccharomyces cerevisae* is also widely used due to the feasibility of mitochondrial genetic transformation in this organism [11]. Still, neither yeast nor cybrids model neuron-specific traits, much less model the complex features of a whole organism. To address these issues, mouse embryonic stem cells cybrids containing mtDNA mutation have been differentiated into neurons [12], and multiple techniques are available for the study of mitochondrial function in live neurons [13]. Mitochondrial disease caused by some nDNA mutations has already been modelled in mice by nuclear transgenesis, but mtDNA mutations are more challenging to model in animals [14]. Still, progresses are being made in the development of mouse models of mtDNA defects [15], and models of mitochondrial disease are also being developed in zebrafish, drosophila and *Caenorhabditis elegans* [16-19].

A systematic review of clinical data on mitochondrial therapeutics revealed that although statistically significant clinical improvements are reported in low-quality studies, they were not confirmed by subsequent high-quality studies. Currently prescribed drugs, consisting mostly of vitamin supplements, metabolic precursors and antioxidants lack convincing

evidence for efficacy other than that expected for placebo. Further, 'symptomatic improvements' due to normal growth and development (in the case of affected children), and regression to mean symptoms (in diseases where symptoms randomly fluctuate), are not always adequately controlled for in available trials [20]. There is therefore a great need for persistent improvement in clinical trial design, identification of reliable biomarkers, and further basic research on disease mechanisms and experimental therapeutics. In this review, we address recent advances on preventive strategies associated with assisted reproduction techniques, such as pronuclear, spindle-chromosome complex, or polar body genome transfer. We also address experimental mitochondrial therapeutics aiming to benefit symptomatic patients and prevent disease manifestation in those at risk. We focus on advances in different modalities of gene therapy, mitochondria-targeted drugs, and modulators of mitochondrial dynamics. The bulk of current data comes from cell-based experiments, nevertheless, wherever available, we address data from *in vivo* experiments and clinical trials focusing on neurological disease models.

2. MITOCHONDRIAL THERAPEUTIC STRATEGIES

2.1. Preventing transmission by mitochondrial gene replacement

Assisted conception strategies have the potential to prevent or at least reduce the risk of transmitting mitochondrial disease. Afflicted families can benefit from genetic counseling, but this is particularly challenging and associated with high level of uncertainty in the case of mtDNA mutations [21]. Pre-implantation genetic diagnosis (PGD) can assist selection of embryos without disease-linked nuclear DNA mutations. Mutations in mtDNA, however, are solely maternally inherited and may in principle be passed to all offspring, albeit in uncertain proportion. PGD to select embryos with very low mutant mtDNA levels reduces risk but does not eliminate the possibility that mitochondrial disease might manifest, or that asymptomatic girls will be carriers in their oocytes. Moreover, PGD is not helpful when homoplasmic mutations or high levels of heteroplasmic mtDNA mutations are already present in oocytes [22]. Development of other options for preventing transmission of mtDNA disease has thus been actively explored, primarily nuclear genome transplantation into the healthy mtDNA background of donor zygotes/oocytes via pronuclear or spindle transfer techniques [23, 24].

Pronuclear transfer into enucleated zygotes has been successfully applied in mice with pathogenic mtDNA mutations, yielding an F_0 progeny free of respiration defects throughout their lives [25]. Proof of principle experiments with the same technique were conducted in abnormally fertilized human zygotes, achieving less than 2% carryover of mtDNA and thus supporting its potential to prevent mtDNA disease transmission in humans [26]. Another technique for mtDNA replacement, spindle-chromosome complex transfer, has been successfully applied to non-human primates generating healthy offspring without detectable spindle-donor mtDNA [27]. A recently-published 3-year follow up on those primates has shown that they continue to develop normally, with comparable growth and metabolic status to age-matched controls, and without significant changes in mtDNA carryover and heteroplasmy in blood and skin samples [28]. In the same publication, authors demonstrated the feasibility of replacing mtDNA in human oocytes via spindle transfer. Normally fertilized human oocytes developed comparably to controls into blastocysts, and the derived embryonic stem cell lines exhibited normal euploid karyotypes, with undetectable carryover mtDNA [28]. Still, the current frequency of premature activation/abnormal fertilization of human oocytes with the spindle transfer technique is far from ideal [28], and methodological changes have been recently proposed [29]. In spite of major and encouraging advances, further

efficacy and safety studies are required before either pronuclear or spindle transfer techniques can be introduced into clinical practice ([30]; see also the 2014 update of the UK's Human Fertilisation and Embryology Authority report at: www.hfea.gov.uk/6372.html). As recently cautioned, however, pronuclear and spindle transfer may only benefit a small group of female mtDNA mutation carriers, whilst prenatal diagnostic testing can be used for all Mendelian mitochondrial disorders and for the majority of mtDNA mutations [31].

Polar body genome transfer has been recently proposed as an alternative technique for mtDNA replacement. This procedure, so far tested in mice, consists in transferring the genetic information of a polar body from a donor oocyte into a recipient oocyte with normal mitochondria [32]. Key advantages are that fewer mitochondria are carried over during transfer and, combined with spindle transfer, a single oocyte could offer two donor genome sources for a mtDNA replacement procedure, reducing the number of necessary donor oocytes [33]. Evidently, while all these techniques may prove useful for preventing mitochondrial disease transmission to newborn children, different approaches must be developed in order to treat symptomatic patients and to prevent disease in those already at risk [34].

2.2. Gene therapy to reduce or compensate for mutant mtDNA

Pathogenic mtDNA mutations are most often heteroplasmic, meaning that they coexist with wild-type mtDNA. Some gene therapy strategies thus aim to shift the balance towards wild-type mtDNA by reducing mutant mtDNA below the disease threshold. These include antigenomic approaches to halt replication of mutant mtDNA or mitochondrial-targeted nucleases to selectively degrade mutated mtDNA [35]. Other strategies compensate for mutant mtDNA via allotopic expression of the wild-type genes, or via xenotopic expression of cognate genes from other species [36]. Proof of principle in cellular models has been established for most of these strategies, but there is still very limited *in vivo* evidence for their safety and efficacy. Problems with delivery of the therapeutic agents to affected tissues remain main hindrances to drug development, together with the scarcity of good animal models for pre-clinical testing. Key strategies and their recent advances are summarized below.

Antigenomic (or anti-replicative) therapies have been tested with sequence-specific nucleic acid derivatives, designed to bind mutant mtDNA and inhibit its replication, whilst allowing wild-type mtDNA propagation. Peptide nucleic acids (PNAs) showed promising *in vitro* results [37], but PNAs have so far failed to modulate heteroplasmy in intact cells, as a result of their inability to cross the IMM and access mtDNA [35]. Recently, RNA vectors based on 5S rRNA or tRNA were reported capable of delivering anti-replicative oligoribonucleotides to mitochondria by exploring RNA import pathways. The same study reported a transient 15-35% decrease in mutant mtDNA levels in Kearns Sayre syndrome cell models using such anti-replicative RNAs [38]. Attempts to increase these agents lifetime by chemical modification of the oligonucleotides have so far resulted in loss of efficacy [39, 40].

Restriction endonucleases fused to a mitochondria-targeting (mito-) sequence were shown to selectively reduce mtDNA containing the recognized restriction site [41]. Experiments with a heteroplasmic cell line showed that mito-SmaI selectively depleted pathogenic m8993T>G mtDNA allowing for repopulation with wild-type mtDNA [42]. *In vivo* experiments with NZB/BALB heteroplasmic mice showed that adenoviral vectors injected in brain and muscle efficiently delivered mito-ApaLI, which selectively degraded BALB mtDNA [43]. Systemic delivery of mito-ApaLI via cardiotropic (AAV6) or hepatotropic (Ad5) adeno-associated virus increased NZB mtDNA in target tissues [44], whereas AAV9-mediated delivery of

mito-ApaLI to newborn mice reduced mtDNA heteroplasmy in all striated muscles, including the heart [45]. Endonuclease-mediated heteroplasmy shift is thus rapidly emerging as one of the most promising therapeutic approaches for mtDNA mutations. While the use of bacterial restriction nucleases requires a unique site in mutated mtDNA (but see the 'differential multiple cleavage site model'; [46]), other nucleases are more versatile. Zinc-finger nucleases (ZFNs) can be engineered to bind a specific DNA sequence, and mito-ZFNs were shown to selectively degrade mutated mtDNA in heteroplasmic cells [47, 48]. The subsequently developed transcription activator-like effector nucleases (TALENs) are an interesting alternative to ZFNs [49]. Transient mito-TALENs expression in patient-derived cells reduced deletion or point-mutant mtDNA, fueling the expectation that a few mito-TALEN administrations may suffice to correct heteroplasmy and rescue oxidative phosphorylation defects [50]. Recently, novel obligatory heterodimeric mito-ZFNs have shown improved heteroplasmy shifting and rescue of mitochondrial respiration, meeting the efficiency of mito-TALENs, with the advantage of being smaller constructs and thus more amenable to packaging in viral vectors [51]. Further development of these strategies, namely in vivo studies, are currently hindered by the size of the constructs, and challenged by the risk of severe mtDNA depletion, particularly in tissues containing high proportions of the targeted mutant mtDNA [34, 52].

Allotopic expression relies on nuclear-cytosolic synthesis of a recoded wild-type copy of the mutated mtDNA gene, plus its functional incorporation into mitochondria by means of a removable targeting signal. Feasibility was demonstrated in yeast, where cytoplasmically synthesized subunit 8 was successfully integrated into the mitochondrial ATPase complex [53]. Attempts in mammalian cells yielded contradictory results, and thus far lack unequivocal demonstration that the allotopically-expressed subunits assemble into the respective holoenzymes and are responsible for improved function [54]. Cytoplasmically synthesized ATPase 6 was reported to incorporate into mitochondria and rescue defective ATP synthesis in human cell lines with the m8993T>G mutation [55, 56]. Independent reformulation of these experiments, however, found neither evidence for ATPase 6 incorporation into the ATP synthase holoenzyme, nor for improvement in mitochondrial function, suggesting that earlier studies might have been biased by random clonal variation in ATP synthesis within the aneuploid cell lines [57]. Similarly, successful allotopic expression of the ND4 subunit to rescue the m11778G>A LHON mutation in human cells [58] was contradicted by subsequent studies [59]. Experiments with mtDNA-encoded genes in mammalian cells showed that ATPase 8 could be allotopicaly expressed and localized to mitochondria, whereas the more hydrophobic ND4 and apocytochrome b formed aggregates and induced loss of mitochondrial membrane potential [59].

To overcome hidrophobicity issues, 3'UTR sequences were engineered into the mRNAs to target them near mitochondria and optimize co-translational translocation of the allotopically-expressed gene. This optimized strategy was reported successful for the allotopic expression of ND1, ND4 and ATP6 to rescue mtDNA mutations affecting complex I and V in patients fibroblasts [60, 61]. Also, optimized allotopic expression of human ND4 was reported to prevent blindness in rats whose eyes were previously electroporated with human ND4 harboring the m11778G>A LHON mutation [62]. Clinical trial recruitment of LHON patients carrying the m11778G>A mutation has started [63]. The trial identified as NCT01267422 reports the random treatment of seven patients by single intravitreal injection of recombinant AAV2-ND4 (Clinicaltrials.gov, last processed on August 24, 2014). While some recent studies are supportive of the importance and strategies of these ND4 expression trials [64] [65], other studies caution that: (i) available data on allotopic expression is insufficient to initiate clinical trials in humans; (ii) reports of successful allotopic expression by phenotypic recovery in cells with mtDNA mutations may be explained by selection of revertants, without

mitochondrial internalization and/or holoenzyme incorporation of the expressed subunit; (iii) the conceptual validity of the mouse LHON model is questioned given the use of human mtDNA sequences, plus the possibility that ND4 hydrophobicity causes retinal toxicity by collapsing the mitochondrial import machinery [54].

Targeted tRNAs have been tested as a strategy to overcome mutations in mitochondrial tRNA (mt-tRNA) genes. Yeast $tRNA^{Lys}$ derivatives expressed in patient fibroblasts with the m8344A>G MERRF mutation were imported to mitochondria, correctly aminoacylated, and shown capable of rescuing mitochondrial translation and respiratory chain activity [66]. Interestingly, tRNA import appears more widespread than previously thought, with both rat and human mitochondria being capable of importing nucleus-encoded $tRNA^{Gln(CUG)}$ and $tRNA^{Gln(UUG)}$ *in vivo*, by a distinct mechanism from protein import [67]. A wide range of tRNAs and mRNAs may be targeted to mitochondria by appending the RNA component of human RNase P, which interacts with polynucleotine phosphorylase (PNPase) that facilitates delivery of the hybrid RNA into the mitochondrial matrix. Further appending a 3'-UTR localization sequence, however, was essential for efficient translocation of corrective mt-tRNAs, shown capable of rescuing mitochondrial translation and respiratory defects in two human cell lines with mutant mt-tRNA genes [68].

Overexpression of aminoacyl tRNA synthetases is an alternative strategy to rescue cognate pathogenic mt-tRNA mutations and, more recently, non-cognate mutations [69]). Accordingly, modification of the mt-tRNA binding domain of human mitochondrial phenylalanyl-tRNA synthetase significantly improved the aminoacylation efficiency of mt-$tRNA^{Phe}$ carrying the m611G>A MERRF mutation [70]. Overexpression of human mitochondrial valyl- or leucyl-tRNA synthetases increased, respectively, the steady-state levels of mutant mt-$tRNA^{Val}$ [71] or mt-$tRNA^{Leu(UUR)}$ [72], consistent with increased stability of the charged mt-tRNA. Also, overexpression of mitochondrial leucyl-tRNA synthetase rescued mitochondrial translation and respiration in cells carrying the A3243G MELAS mutation in mt-$tRNA^{Leu(UUR)}$ [73]. A related strategy consists in the allotopic expression of recombinant and importable tRNAs with altered aminoacylation identity. Yeast tRNAs with artificially changed identity, from cytoplasmic lysine to mitochondrial leucine, partially rescued mitochondrial translation, steady-state levels of mtDNA-encoded respiratory chain subunits, and cellular respiration in human cells with the A3243G MELAS mutation in mt-$tRNA^{Leu}$ [74]. More recently, human mitochondrial leucyl tRNA synthetase, particularly a carboxy terminal fragment, was reported to rescue respiratory deficiency induced by pathogenic mt-tRNA mutations [69, 75]. Mechanisms, particularly those involved in the suppression of non-cognate mutations, remain uncertain but unlikely to involve aminoacylation (domain absent from the carboxy terminal fragment), and are possibly related to stabilization of the mutant tRNAs. These findings raise the appealing possibility of developing a universal suppressor molecule for multiple mt-tRNA mutations [76].

Xenotopic expression of non-mammalian genes that can function as single respiratory chain subunits provides an encouraging alternative to allotopic expression. The rationale is that functional single subunits would obviate the need for integration into assembled multi-protein complexes, which has been the crux of allotopically expressed mammalian subunits. The *Ciona intestinalis* single subunit alternative oxidase (AOX) or the *Saccharomyces cerevisae* single subunit NADH oxidase (Ndi1) have the potential to alleviate, respectively, deficiencies in cytochrome *c* oxidase (COX) or in complex I [36]. *C. intestinalis* AOX expression in human cells conferred cyanide resistance to mitochondrial substrate oxidation [77], and corrected decreased cell respiration, glucose and pyruvate dependency in COX15-deficient fibroblasts derived from a patient with early fatal cardiomyopathy [78]. Xenotopically expressed AOX integrates the mammalian mitochondrial respiratory chain where it can transfer electrons from the reduced ubiquinone pool to oxygen, thus bypassing

downstream blockades. Unlike downstream complex III and IV, however, AOX is not a proton pump, and thus it is crucial to establish whether the advantages of a potential superoxide-limiting bypass outweigh the disadvantages of energy dissipation. AOX was safely expressed in mice (MitAOX) without obvious detrimental effects upon major physiological parameters, which suggests that AOX does not severely limit energy production when integrated in an otherwise normal respiratory chain. The increased survival of MitAOX mice exposed to lethal cyanide supports the possibility that AOX may counteract downstream respiratory chain impairment [79]. MitAOX mice are thus a promising tool for further evaluation of the therapeutic potential of AOX in mitochondrial disease.

Expression of yeast Ndi1 restored NADH dehydrogenase activity in human cells lacking complex I activity due to ND4 gene mutation [80]. Ndi1 insensitivity to complex I inhibitors such as rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) promoted its testing in PD or LHON models induced with such compounds. Viral delivery of Ndi1 (rAAV-NDI1) by unilateral injection to the substantia nigra protected mice from neuronal injury induced by either MPTP [81] or rotenone [82], providing *in vivo* evidence for functional complementation of complex I by Ndi1. Also, long term Ndi1 expression (>8months) was neuroprotective in a chronic (1-4 weeks) MPTP/probenecid mouse model [83]. Ndi1 expression was also protective in rotenone-induced mouse models of mitochondrial optic neuropathy, preserving retinal function and ameliorating vision [84, 85]. Concerning *in vivo* genetic models, a study with drosophila PINK1/Parkin mutants showed that Ndi1 rescued PINK1-associated defects, but failed to rescue any of the Parkin mutant phenotypes. Significantly, PINK1 mutants exhibited decreased complex I activity, unlike Parkin mutants [86]. Further *in vivo* studies including mammalian genetic models of mitochondrial disease would help define the therapeutic potential of xenotopic expression.

2.3. Lipophilic cations and mitochondria-targeted peptides

Lipophilic cations and mitochondria-targeted peptides are important agents for *in vivo* drug delivery, allowing enhanced mitochondrial accumulation of otherwise poorly taken up molecules. These delivery strategies increase potency of the targeted compounds, decreasing the required dose as well as extramitochondrial metabolism, inactivation and side effects. These strategies have two important limitations: (i) they lack organ-specificity, leading to preferential accumulation in mitochondria-rich tissues; and (ii) the typically used chemicals tend to accumulate in the matrix and the matrix-facing surface of the IMM, by comparison with other important mitochondrial compartments [87].

Mitochondrial membrane potential ($\Delta\psi_m$) drives accumulation of lipophylic cations such as triphenylphosphonium (TPP^+) derivatives, whose concentration within mitochondria increases approximately 10-fold for every 60 mV of $\Delta\psi_m$ as described by the Nernst equation. The TPP^+-modified ubiquinone, MitoQ, has been extensively investigated as a mitochondria targeted-antioxidant, and shown protective in multiple disease models [88]. Oral MitoQ improved survival and grip strength in the $SOD1^{G93A}$ mouse model of amyotrophic lateral sclerosis, in correlation with slower decline of mitochondrial respiration, reducing oxidative markers, astrogliolis and neuronal loss in the spinal cord [89]. MitoQ was also reported protective in rodent models of MPTP-induced PD [88]. A clinical trial in 128 patients treated for 12 month with MitoQ, however, failed to show any difference versus placebo in PD progression (NCT00329056 at Clinicaltrials.gov; [90]). Still, MitoQ was well tolerated and it remains to be ascertained whether early pre-symptomatic treatment, preceding overt dopaminergic degeneration, might prove beneficial in delaying the onset of PD.

Szeto-Schiller (SS) peptides are described to selectively target mitochondria and concentrate in the IMM, independently of $\Delta\psi_m$, by yet uncertain mechanisms (but see [91]) even though the peptides contain a net positive charge at physiological pH [92]. Potential-independent mitochondrial uptake would offer a significant advantage since dysfunctional mitochondria are likely to exhibit lower $\Delta\psi_m$, thus excluding or limiting the uptake of $\Delta\psi_m$–dependent drugs such as lipophilic cations. SS peptides display antioxidant activity, with SS-31 exerting protective effects in animal models of amyotrophic lateral sclerosis [93], PD [94], and in primary neurons derived from AD mice – an *in vitro* model where MitoQ was also found protective [95]. SS-31 was also reported to improve mitochondrial energetics and muscle performance in aged mice [96]. The ongoing and future clinical trials with a modified form of SS-31 [92] should help establish its therapeutic potential.

Hemigramicidin-TEMPO conjugates are antioxidants targeted to mitochondria via peptides derived from the membrane-active antibiotic, gramicidin S, which carry the stable free radical 4-amino-2,2,6,6-tetramethylpiperidine-*N*-oxyl (4-NH_2-TEMPO) as a ROS scavenger [97]. XJB-5-131 is one of the most promising compounds in this class. The mitochondrial targeting moiety is an alkene peptide isostere modification of the Leu-D-Phe-Pro-Val-Orn gramicidin S segment, whose β-turn motif inserts into the mitochondrial membrane. This mitochondrial targeting strategy is $\Delta\psi_m$-independent, avoiding exclusion of XJB-5-131 from depolarized mitochondria. XJB-5-131 was recently reported to enhance survival of primary neurons, reduce oxidative damage to mtDNA, improve mitochondrial function, and suppress motor decline in a HD mouse model [98].

Peptide-based-multi-walled carbon nanotubes are being developed as an alternative delivery system for therapeutic oligonucleotides against mtDNA mutations. Mitochondrial-targeting of these nanotubes is promoted by functionalization with a 25 amino acid peptide derived from the N-terminal region of human COX subunit VIII [99]. While these carbon nanotubes can clearly be internalized by cultured phagocytes and colocalize with their mitochondria, the *in vivo* pharmacokinetics and the bioenergetic consequences of inserting possibly $\Delta\psi_m$-collapsing tubes into mitochondrial membranes remain to be investigated.

2.4. Modulation of mitochondrial dynamics

2.4.1 Biogenesis and fission-fusion

The induction of mitochondrial biogenesis is being actively explored as a strategy to overcome mitochondrial dysfunction. The peroxisome proliferator-activated receptor (PPAR) and its co-activator α (PGC-1α) play key regulatory roles in biogenesis activation, highlighting the PPAR/PGC-1 pathway as a druggable target. Transgenic expression of PGC-1α or treatment with the PPAR agonist bezafibrate induced mitochondrial biogenesis *in vivo*, preventing mitochondrial myopathy in mice lacking an essential COX assembly factor in skeletal muscle [100]. Bezafibrate, however, failed to induce mitochondrial biogenesis in other COX deficient models [101], in contrast with PGC-1α overexpression or its indirect activation with the AMPK agonist AICAR [102]. Still, bezafibrate restored PGC-1α levels, induced mitochondrial biogenesis, and exerted neuroprotective effects in mouse models of either HD [103] or tau pathology [104]. Moreover, bezafibrate increased mitochondrial proteins and ATP generating capacity, being neuroprotective in a mouse model of mitochondrial encephalopathy [105]. Thus, whilst there is conflicting data on bezafibrate effects in skeletal muscle, data from neurological disease models suggest that it might be a promising compound for the treatment of mitochondrial-associated neurodegenerative disease.

PPAR-γ agonists, such as the thiazolidinediones rosiglitazone and pioglitazone, exhibited promising effects in pre-clinical models of neurodegeneration, but data from clinical trials are unsupportive of their use in AD patients [106], and their potential in the context of PD is still uncertain [107]. A tentative pre-clinical evaluation of the bioenergetic intermediate oxaloacetate, as potential treatment for AD and other neurodegenerative disorders, reported increased PGC-1α mRNA, activated mitochondrial biogenesis and hippocampal neurogenesis in C57Bl/6 mice [108].

Lysine deacetylases (KDACs), including the NAD^+-dependent sirtuins, are reported to regulate mitochondrial biogenesis via modulation of PGC-1α activity and changes in gene expression, suggesting that KDAC modulatory drugs may provide a strategy to boost mitochondrial biogenesis [109, 110]. Increasing NAD^+ availability through administration of the precursor nicotinamide riboside was reported to increase PGC-1α-dependent mitochondrial biogenesis, presenting beneficial effect in the Tg2576 AD mouse model [111]. Recently, nicotinamide riboside or MRLB-45696 (an inhibitor of the NAD^+ consuming enzyme poly(ADP-ribose) polymerase – PARP1) were reported to increase mitochondrial biogenesis with promising results in mitochondrial myopathy models [112-114]. Still, further studies on dosage regimens and pharmacokinetic data are still required for a more detailed evaluation of the therapeutic potential of these NAD^+ modulating strategies [115].

Exercise is a well-known activator of mitochondrial biogenesis by upregulating PGC-1α [116]. In addition to its effects on skeletal muscle, exercise training was shown to increase mitochondrial biogenesis in the brain [117], highlighting exercise as a complementary strategy for neuroprotection and management of mitochondrial-associated neurological disease [118].

Restoration of the mitochondrial fission-fusion equilibrium appeals as a therapeutic strategy for mitochondrial dysfunction, given the association between excessive mitochondrial fission and/or defective fusion in neurodegenerative disorders [119, 120]. The hydrazone M1 was recently identified as a small molecule mitochondrial fusion promoter, capable of inhibiting mitochondrial fragmentation and enhancing survival of MPP^+-treated SH-SY5Y neuroblastoma cells. M1 dose-dependently increased mitochondrial fusion in Mfn1 or Mfn2 single knockouts (KO), but not in Mfn1/2 double KO or OPA1 KO cells, suggesting it requires basal fusion activity, albeit the proposed mechanism of action is increased ATP5A/B expression levels [121]. Small molecule inhibitors of mitochondrial fission are also under investigation [122]. The selective Drp1 inhibitor mdivi-1 rescued abnormal mitochondrial dynamics induced by human mutant PINK1 in dopaminergic N27 neuronal cells [123], and was reported neuroprotective in rodent models with induced seizures [124] or ischemic injuries [125, 126]. A novel peptide inhibitor of Drp1, P110, selectively inhibited Drp1 activation and mitochondrial Drp1-Fis1 interaction only under stressed conditions in cultured neuronal cells. P110 reduced mitochondrial fragmentation in SH-SY5Y cells treated with MPP^+ or mitochondrial uncoupler (CCCP), and reduced neurite loss in primary dopaminergic neurons challenged with MPP^+ to model PD neurodegeneration [127]. P110 was also reported to reduce mitochondrial fragmentation in HD cells, decreasing mitochondrial dysfunction, neuropathology and mortality in R6/2 HD mice [128].

2.4.2. Movement and mitophagy

Stimulating mitochondrial movement is a possible strategy to overcome deficits in mitochondrial distribution, particularly in neurons where mitochondria must travel extensively to meet the variable energy and Ca^{2+}-buffering needs of distant synapses. AD and HD brain samples present decreased tubulin acetylation and cellular models of these diseases exhibit impaired microtubule-dependent mitochondrial transport [129-131]. Inhibition of the

microtubule deacetylase HDAC6 with tubacin increased α-tubulin acetylation, promoting motor recruitment and enhancing bidirectional transport in HD cellular models [130]. Another HDAC6 inhibitor, tubastatin A, rescued mitochondrial transport in amyloid-β (Aβ) treated hippocampal neurons [131], as did HDAC6 deletion, which also improved memory function in AD mice without affecting Aβ plaque load [132]. HDAC6 inhibitors also corrected mitochondrial transport and reversed axonal loss in a mouse model of peripheral neuropathy [133]. The normally nuclear-located HDAC1 was suggested to play a critical role in axonal damage and abnormal mitochondrial transport, following neuroinflammatory stimuli (glutamate + TNFα) capable of evoking the Ca^{2+}-dependent nuclear export of HDAC1 [134]. Further concerning Ca^{2+}-dependent effects, pan-KDAC inhibitors were shown to improve neuronal Ca^{2+} recovery following glutamate receptor activation [135]. Moreover, HDAC1 inhibition with MS275, but not HDAC6 inhibition with tubacin, prevented cytosolic HDAC1 from disturbing mitochondrial transport following neuroinflammatory stimuli [134]. Available evidence therefore suggests that different KDACs may impair neuronal mitochondrial dynamics depending on the pathogenic insult. KDAC inhibition as a therapeutic strategy for rescuing mitochondrial transport must, nevertheless, be balanced against the possible interferences with other roles, namely that of HDAC6 in autophagy/mitophagy [109].

Mitophagy dysregulation, either defective or excessive, is associated with neurodegeneration [136]. Experiments in yeast suggest that mitophagy can be mechanistically separated from other forms of autophagy [137], prompting the search for selective mitophagy triggers in mammals. The PINK1/Parkin pathway appears to be a prominent player in mammalian mitophagy. Briefly, damaged mitochondria with collapsed $\Delta\psi_m$ fail to import and process PINK1, which accumulates outside mitochondria recruiting the ubiquitin-ligase Parkin. Ubiquitin-binding adaptors (e.g. p62, HDAC6) recognize ubiquitinated mitochondria and facilitate the interaction with the autophagosomal protein LC3, igniting a cascade that culminates in autophagosome-lysosome fusion and mitochondrial digestion [138]. The post-mitotic nature of neurons and their extreme morphology pose a unique challenge for mitochondrial turnover, being unlikely that neurons follow the same mitophagy rules as most mammalian cells. Interestingly, it was recently reported that at least some neurons shed axonal mitochondria for transcellular degradation by adjacent astrocytes [139]. Also, major bioenergetic differences between neurons and cell lines raise doubts on the relevance of the PINK1/Parkin pathway in neuronal mitophagy [140] [141], but other studies are supportive of that pathway in neurons [142, 143], where mitochondrial deubiquitinase USP30 was recently reported to oppose parkin-mediated mitophagy [144]. Currently, the (patho)-physiological triggers for neuronal mitophagy and the mechanisms for handling damaged mitochondria in distant synapses remain insufficiently understood [138], thus posing an outstanding challenge for developing mitophagy-related therapeutic strategies. Still, a study with heteroplasmic cybrid cells reported a selection against a deleterious mtDNA mutation by means of Parkin overexpression, thus suggesting that this strategy of enhancing mitophagy may ameliorate certain mitochondrial diseases [145].

3. CONCLUDING REMARKS

Currently available therapies for mitochondrial-associated neurological diseases are mostly palliative. Advances in understanding the molecular aetiology of mitochondrial dysfunction, together with breakthroughs in assisted conception, gene therapy and mitochondrial drug targeting strategies, fuel the expectation that effective preventive and disease-modifying therapeutics might be available in the near future. To achieve this goal, it is critical to develop

good *in vivo* models for pre-clinical studies, organize patient cohorts and registries, establish reliable biomarkers, and improve clinical trial design. As always, and particularly in the case of complex mitochondrial disorders, clinical research will benefit tremendously from the unravelling of molecular pathogenesis afforded by basic mitochondrial research.

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5. AUTHOR CONTRIBUTIONS

ALR performed the majority of literature search and wrote the first draft. BRP contributed to section 2.3. PGD contributed to section 2.4. JMAO coordinated the work and wrote the paper. All authors read and approved the final version.

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