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The rescue of microtubule-dependent traffic recovers mitochondrial function in Parkinson's disease ☆

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ABSTRACT

In Parkinson's disease mitochondrial dysfunction can lead to a deficient ATP supply to microtubule protein motors leading to mitochondrial axonal transport disruption. Compromised axonal transport will then lead to a disorganized distribution of mitochondria and other organelles in the cell, as well as, the accumulation of aggregated proteins like alpha-synuclein. Moreover, axonal transport disruption can trigger synaptic accumulation of autophagosomes packed with damaged mitochondria and protein aggregates promoting synaptic failure.

We previously observed that neuronal-like cells with an inherent mitochondrial impairment derived from PD patients contain a disorganized microtubule network, as well as, alpha-synuclein oligomer accumulation. In this work we provide new evidence that an agent that promotes microtubule network assembly, NAP (davunetide), improves microtubule-dependent traffic, restores the autophagic flux and potentiates autophagosome–lysosome fusion leading to autophagic vacuole clearance in Parkinson's disease cells. Moreover, NAP is capable of efficiently reducing alpha-synuclein oligomer content and its sequestration by the mitochondria. Most interestingly, NAP decreases mitochondrial ubiquitination levels, as well as, increases mitochondrial membrane potential indicating a rescue in mitochondrial function.

Overall, we demonstrate that by improving microtubule-mediated traffic, we can avoid mitochondrial-induced damage and thus recover cell homeostasis. These results prove that NAP may be a promising therapeutic lead candidate for neurodegenerative diseases that involve axonal transport failure and mitochondrial impairment as hallmarks, like Parkinson's disease and related disorders.

1. Introduction

There is accumulating evidence from *in vitro* and *in vivo* studies suggesting that mitochondrial abnormalities are a common event in sporadic Parkinson's disease (sPD) [1–4]. Moreover, defects in axonal and dendritic transport have also been linked to various neurodegenerative processes, including sPD pathogenesis. Indeed, sPD is characterized by a sequence of neuropathological events that arise due to a dying-back pattern of neuronal degeneration, namely early loss of synaptic terminals and axonopathy, before cell death [5–8]. Another central hallmark of PD is the presence of intracytoplasmatic aggregates, named Lewy Bodies that are mainly composed of alpha-synuclein (ASYN). Several studies suggest that larger ASYN aggregates may have reduced toxicity relative to their smaller-sized counterparts, the soluble oligomers [9–12]. ASYN inclusion dynamics results from an intricate process that may distress axonal transport, autophagic-lysosomal and ubiquitin-proteasome pathways.

Mitochondrial function and axonal transport are intimately connected. Mitochondria supply the energy for cytoskeleton motor proteins to transport them along cytoskeleton tracks to areas in the cell where energy demands are high and calcium buffering is required [13,14]. Importantly, alteration in microtubule dynamic may lead to a defective traffic of mitochondria inside the cell contributing ultimately to its dysfunction and to the disruption of protein transport, such as ASYN. ASYN, which is normally transported by fast axonal transport, can then accumulate potentiating the disruption of intracellular trafficking [15].

Since mitochondria are metabolically and functionally compromised in PD tissues it is important to evaluate mitochondrial contribution to microtubule transport failure. Axonal transport requires energy to allow molecular motors to move along microtubules and transport mitochondria, autophagic and synaptic vesicles [16]. Recently, Zala and coworkers [17] challenged the assumption that mitochondrial ATP was the principal source of energy for motor molecules. They showed that the transport of BDNF carrying vesicles was not affected by the inhibition of mitochondrial ATP production, but failed to demonstrate the

Abbreviations: PD, Parkinson's disease; ASYN, alpha-synuclein; CT cybrids, (control cybrids); sPD cybrids, (sporadic Parkinson's disease cybrids); mtDNA, mitochondrial DNA; MPP^+, 1-methyl-4-phenylpyridinium

☆ Conflict of interest: I. Gozes is a Director-Chief Scientific Officer at Allon Therapeutics Inc. the company that develops davunetide (NAP).

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same for mitochondrial transport, which is dependent on the hydrolysis of mitochondrial ATP by molecular motors.

In previous work we observed that sPD cybrids contain mitochondrial abnormalities, such as a complex I defect and ATP depletion [18–20]. This *ex-vivo* model results from the fusion of teratocarcinoma (NT2) cells that were depleted from their mitochondrial DNA (mtDNA) (NT2 rho0) with mitochondria isolated from platelets of age-matched healthy individuals or PD patients. Indeed, we and others claim that the differences observed in the mitochondrial pool between CT and PD cybrids are due to mtDNA variations. Although we agree that there is no demonstrated homoplasmic mtDNA mutation that associates with PD, we cannot rule out the presence of low abundance heteroplasmic mutations, epigenetic modifications to the mtDNA, or the possibility that polymorphic variation (rather than mutations *per se*) is responsible for the observed biochemical phenotypes. mtDNA polymorphic variation between individuals is high, which suggests that mtDNA between cybrid lines containing mtDNA from different individuals is certainly different. The great potential of this model is that these cells possess the same nuclear background but different mitochondrial background therefore allowing us to study mitochondrial role in several cellular pathways [21]. We also demonstrated that sPD cybrids present basal microtubule disruption with a concomitant accumulation of ASYN oligomers [22]. Hence, we showed that microtubule network alterations are due to mitochondrial deficits. We further proved that taxol, a known microtubule stabilizer, promoted microtubule network assembly and a decrease in ASYN oligomer content [22]. However, taxol is not neuron-specific, has limited brain bioavailability and in high concentration has the capacity to inhibit microtubule dynamics [23]. Thus, more specific, brain-penetrable microtubule-interacting drugs are required [24]. Herein, we decided to use a peptide known as NAP (davunetide) that is an eight amino acid peptide derived from an activity-dependent neuroprotective protein [25–27]. This peptide associates with tubulin and enhances proper microtubule assembly [28–30]. We demonstrate that the alterations induced by mitochondrial deficits on microtubule assembly and microtubule-mediated traffic in sPD cybrids are prevented by NAP. Correlated with this we observed that NAP promoted autophagic vacuole clearance by increasing autophagic flux and autophagosome–lysosome fusion in PD cells. Moreover, NAP restored mitochondrial distribution and mitochondrial membrane potential and was able to prevent the accumulation of Triton-soluble and insoluble ASYN oligomeric species in sPD cybrids.

Taken collectively, our results provide strong data that NAP is a promising therapeutic agent in the PD treatment.

2. Material and methods

2.1. Chemicals

Nocodazole, MPP^+ (1-methyl-4-phenylpyridinium), ammonium chloride (NH_4Cl), leupeptin, oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), rapamycin, ubiquinone and rotenone were obtained from Sigma (St. Louis, MO, USA). Rhodamine 123 was purchased from Molecular Probes (Eugene, OR, USA). NAP was generously donated by Dr. Illana Gozes from Tel Aviv University.

2.2. Human subjects

Subject participation was approved by the University of Kansas School of Medicine's Institutional Review Board. The mean age of the PD subjects ($n = 9$) who participated in this study was 64 ± 12.8 years, and for the control subjects ($n = 5$) it was 74.3 ± 5.5 years (Supplementary Table I). The PD subjects were followed regularly in a tertiary referral movement disorders clinic at the Kansas University Medical Center and met current criteria used to diagnose idiopathic PD in clinical and research settings [31]. The control subjects were participants of a longitudinal "normal aging" cohort that is characterized serially by the Brain Aging Program at the University of Kansas School of Medicine (see Supplementary Table I). After obtaining informed consent, sporadic PD and age-matched control subjects underwent a 10 ml or 60 ml phlebotomy using tubes containing acid-citrate-dextrose as an anticoagulant. The control subjects had no evidence of a neurodegenerative condition.

2.3. Preparation of platelet mitochondria

Following provision of informed consent, 60 ml of blood was collected through venipuncture in tubes containing acid–citrate–dextrose as an anticoagulant. Mitochondria were obtained from human platelets according to previously described methods [32]. Platelet mitochondrion protein concentrations were measured by the Bradford protein assay [33], in which bovine serum albumin was used as the standard.

2.4. Creation of cybrid cell lines

To create the cybrid cell lines for this study, we used NT2 (Ntera2/D1) cells, a teratocarcinoma cell line with neuronal characteristics (Stratagene, La Jolla, CA) [34,35]. These cells were depleted of endogenous mtDNA (NT2 rho0 cells) *via* long-term ethidium bromide exposure [36,37]. NT2 rho0 cells lack intact mtDNA, do not possess a functional electron transport chain, and are auxotrophic for pyruvate and uridine [38]. Consequently, platelet mitochondria from either PD or control subjects were isolated from the individual blood samples and were used to repopulate NT2 rho0 cells with mtDNA as previously described [36,37]. We generate control and disease cell lines at the same point in time and only compared a disease series with a control series that had been made at the same time and with the same immediate stock of NT2 rho0 cells. Briefly, NT2 rho0 cells were co-incubated in polyethylene glycol (Merck Chemicals) with platelets from the human subjects [38]. After that the resulting mixture was grown in Rho0 medium in T75 flasks. Seven days after plating, untransformed cells were removed by withdrawal of pyruvate and uridine from the culture medium and substitution of dialyzed, heat inactivated fetal calf serum for non-dialyzed, heat inactivated fetal calf serum [39,21]. Maintaining cells in selection medium removes rho0 cells that have not repopulated with platelet mtDNA. Moreover, "mock fusions" in which NT2 rho0 cells were not incubated with platelets were plated and maintained in selection medium in parallel with the true fusions. During the selection period all cells from the mock fusions died. After selection was complete, the resultant cybrid cells were switched to cybrid growth medium. Cells were grown in 75 cm^2 tissue culture flasks maintained in a humidified incubator at 37 °C and 5% CO_2.

2.5. Cell line culture and experimental treatments

Optimem and Dulbeco's modified Eagle's medium (DMEM) were obtained from Gibco-Invitrogen (Life Technologies Ltd, UK). Non-dialyzed and dialyzed Fetal Bovine Serum (FBS) was obtained from Gibco-Invitrogen (Life Technologies Ltd, UK). NT2 rho0 cell growth medium Optimem was supplemented with 10% non-dialyzed FBS, 200 μg/ml sodium pyruvate from Sigma (St. Louis, MO, USA), 100 μg/ml uridine from Sigma (St. Louis, MO, USA) and 1% penicillin–streptomycin solution. NT2 cybrid selection medium consisted of DMEM supplemented with 10% dialyzed FBS and 1% penicillin–streptomycin solution. Cybrid growth medium consisted of Optimem supplemented with 10% non-dialyzed FBS and 1% penicillin–streptomycin solution. Prior to experiments, cell lines were maintained in the cybrid growth medium. For Western blotting analysis and mitochondrial respiratory chain complex I activity, cybrid cell lines were seeded in petri-dishes at a density of 0.5×10^6 cells/well. For immunoblotting analysis, cybrid cell lines were grown on coverslips in 12-well plates at a density of 0.1×10^6