The NAD+ Precursor Nicotinamide Riboside Rescues Mitochondrial Defects and Neuronal Loss in iPSC and Fly Models of Parkinson’s Disease

Highlights
- NAD+ metabolism and mitochondrial function are altered in GBA-PD neurons
- Human iPSC-derived neurons are responsive to NAD+ precursors
- Nicotinamide riboside improves mitochondrial function in GBA-PD iPSC neurons
- Nicotinamide riboside rescues neuronal loss and motor deficits in GBA-PD flies

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In Brief
Mitochondrial damage is a key feature in Parkinson’s disease. Schöndorf et al. demonstrate that nicotinamide riboside, an NAD+ precursor, boosts mitochondrial function in neurons derived from Parkinson’s disease patient stem cells and is neuroprotective in Parkinson’s disease fly models. These findings support use of NAD+ precursors in Parkinson’s and other neurodegenerative diseases.
The NAD+ Precursor Nicotinamide Riboside Rescues Mitochondrial Defects and Neuronal Loss in iPSC and Fly Models of Parkinson’s Disease

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SUMMARY

While mitochondrial dysfunction is emerging as key in Parkinson’s disease (PD), a central question remains whether mitochondria are actual disease drivers and whether boosting mitochondrial biogenesis and function ameliorates pathology. We address these questions using patient-derived induced pluripotent stem cells and Drosophila models of GBA-related PD (GBA-PD), the most common PD genetic risk. Patient neurons display stress responses, mitochondrial demise, and changes in NAD+ metabolism. NAD+ precursors have been proposed to ameliorate age-related metabolic decline and disease. We report that increasing NAD+ via the NAD+ precursor nicotinamide riboside (NR) significantly ameliorates mitochondrial function in patient neurons. Human neurons require nicotinamide phosphoribosyltransferase (NAMPT) to maintain the NAD+ pool and utilize NRK1 to synthesize NAD+ from NAD+ precursors. Remarkably, NR prevents the age-related dopaminergic neuronal loss and motor decline in fly models of GBA-PD. Our findings suggest NR as a viable clinical avenue for neuroprotection in PD and other neurodegenerative diseases.

INTRODUCTION

Mitochondrial dysfunction has been proposed as a key mechanism in many neurodegenerative diseases. Among others, Parkinson’s disease (PD) stands out due to the role of PD-linked genes in mitochondrial function and dynamics (Exner et al., 2012). Evidence for mitochondrial dysfunction in PD was first described in the 1980s by Schapira et al., who showed complex I (CI) defects in cells and tissues from PD patients (Schapira et al., 1989). Substantial progress has been made since, and genetic and biochemical studies now indicate that mitochondrial dysfunction and cellular energy failure are key to PD (Jansen et al., 2017). In this respect, recent studies have shown that the activation of pathways related to mitochondrial biogenesis and energy metabolism, such as the NAD+/sirtuin 1 (SIRT1) pathway, provides protection against aging-related disease (Rajman et al., 2018). Similar approaches could be easily translated into treatment for PD. However, it is still unclear whether mitochondrial defects are actual disease drivers and increasing mitochondrial biogenesis provides neuroprotection in PD. In addition, little is known about NAD+ metabolism and availability of NAD+ precursors in human neurons. Here, we have addressed these fundamental questions in an induced pluripotent stem cell (iPSC) neuronal model of PD bearing mutations in the lysosomal enzyme β-Glucocerebrosidase (GCase) gene (GBA-PD), the most common genetic risk for PD (Sidransky et al., 2009). β-Glucocerebrosidase (GCase) activity is reduced not only in mutation carriers but also in idiopathic PD and healthy individuals at older age (Gegg et al., 2012; Rocha et al., 2015), pointing toward a general role for GCase in brain aging and neurodegenerative processes. Importantly, patients with GBA mutations represent an etiologically homogeneous subgroup of PD, therefore providing the ideal cohort for precision medicine approaches.

The pathogenetic mechanisms involved in GBA-PD are only partially understood and include autophagic defects, increased α-synuclein aggregation, calcium dyshomeostasis,
and endoplasmic reticulum (ER) stress (Migdalska-Richards and Schapira, 2016). GCase is a lysosomal enzyme that catalyzes the hydrolysis of glucosylceramide (GlcCer), a membrane glycosphingolipid, to ceramide and glucose, and both loss and gain of its enzymatic function may contribute to disease. According to the loss-of-function hypothesis, GCase deficiency causes substrate accumulation that alters lysosomal function and promotes β-synuclein aggregation (Jo et al., 2000; Velayati et al., 2010; Mazzulli et al., 2011). GCase is glycosylated and folded in the ER and subsequently trafficked through the Golgi to the lysosome. According to the gain-of-function hypothesis, GCase mutations interfere with the folding process in the ER, leading to ER-associated degradation, ER stress, and activation of the unfolded protein response (UPR) (Maor et al., 2013; Fernandes et al., 2016). Interestingly, mitochondrial dysfunction has been described in experimental models of GCase deficiency (Osel-lame et al., 2013; Keatinge et al., 2015; Cleeter et al., 2013). However, whether mitochondrial function is altered in PD patients with GBA mutations (GBA-PD) and the mechanisms underlying such demise are still unknown. Furthermore, whether improving mitochondrial biogenesis and function represents an effective therapeutic strategy for PD needs to be investigated.

RESULTS

iPSC-Derived Neurons from GBA-PD Patients Show Defects in Mitochondrial Function

To investigate whether GBA is linked to mitochondrial function in human neurons, iPSC lines from PD patients with heterozygous GBA mutations (N370S, L444P, and RecNcil), as well as corresponding isogenic gene-corrected (GC) and unaffected controls (Schöndorf et al., 2014) (Table S1), were differentiated into dopaminergic (DA) neurons, and mitochondrial morphology was examined by transmission electron microscopy (TEM). Morphometric analysis revealed altered cristae morphology in GBA-PD neurons compared to isogenic GC and healthy controls (Figures 1A, 1B, and S1A). In addition, GBA-PD neurons showed a significant increase in mitochondrial diameter (Figure 1C). Next, we measured oxygen consumption rates (OCRs) and found that GBA-PD neurons displayed significantly reduced basal respiration and decreased maximal OCR as well as ATP-linked OCR and spare respiratory capacity (SRC) compared to isogenic controls (Figures 1D and 1E). Similarly, basal respiration, maximal OCR, and ATP-linked OCR and SRC were significantly reduced in GBA-PD neurons compared to unrelated unaffected controls (Figure S1B). Western blot analysis revealed an increase in the level of respiratory chain complex subunits in GBA-PD neurons compared to isogenic controls, but this increase did not reach statistical significance (Figures S1C and S1D). We next measured CI activity in enriched mitochondrial fractions from GBA-PD iPSC neurons and GC controls. CI activity was significantly reduced in GBA-PD neurons compared to isogenic controls (Figure 1F). Consistent with these findings, GBA-PD neurons produced significantly higher amounts of mitochondrial reactive oxygen species (mtROS) than isogenic controls (Figure 1G). However, mitochondrial membrane potential and mitochondrial mass were not significantly changed in GBA-PD neurons (Figures 1H and 1I). Of note, no significant difference in the degree of mitochondrial function was observed among different GBA genotypes, suggesting that the genotypes examined in this study (RecNcil, L444P, and N370S) equally affect mitochondria (Figures S1E–S1I). To get further insight into the mechanisms underlying mitochondrial dysfunction in GBA-PD, we examined the GCase substrate sphingolipid composition of mitochondria from GBA-PD neurons and isogenic controls by high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). To this end, mitochondria were isolated from iPSC neurons with a high degree of purity. Western blot analysis showed high level of enrichment of isolated mitochondria with a small amount of non-mitochondrial organellar contamination (Figure S1J). Quantification of individual species of GlcCer revealed the absence of GlcCer accumulation in patient mitochondria with only a significant increase of C16-GlcCer (Figure 1J).

iPSC-Derived Neurons from GBA-PD Patients Show Defects in Mitochondrial Dynamics

Next, we examined the level of mitochondrial fission (DRP1 and Fis1) and fusion (OPA1 and Mfn1) proteins in GBA-PD iPSC neurons and isogenic controls. Immunoblot analysis revealed a
Figure 2. Mitochondrial Dynamics in GBA-PD iPSC Neurons

(A and B) Western blot of DRP1, Mfn1, and Fis1 in isogenic GBA-PD (N370S, L444P) and gene-corrected (GC) iPSC neurons. Vinculin was used as a loading control. Quantification of western blots is shown in (B). Data are normalized to GC and presented as mean + SEM (n = 5).

(C and D) Western blot analysis of OPA-1 processing. The L-OPA1/S-OPA1 ratio is shown in (D). Data are presented as mean + SEM (n = 5; two-tailed t test).

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reduction in DRP1, OPA1, and Mfn1 levels in GBA-PD neurons (Figures 2A–2D). On the other hand, there was an increase of Fis1 in GBA-PD neurons (Figures 2A and 2B). However, these differences did not reach statistical significance. Interestingly, the ratio between the long and short form of OPA1 was significantly increased in GBA-PD neurons compared to isogenic controls, suggesting an impairment of mitophagy (MacVicar and Lane, 2014) (Figures 2C and 2D). GBA-PD neurons showed a significant reduction of mitochondrial-lysosomal co-localization compared to GC controls, as assessed by confocal microscopy and Amnis ImageStream flow cytometry (Figures 2E, 2F, S2A, and S2B). In line with these findings, the expression of the mitophagy adaptor protein BNIP3L/NIX was significantly reduced in GBA-PD compared to isogenic controls (Figure 2G).

Mitochondrial Function Is Altered in GBA Knockout iPSC-Derived Neurons

To assess the impact of loss of GCase enzymatic function on mitochondria in the absence of gain-of-function mechanisms, we generated GBA knockout (KO) iPSCs using clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 (Figures S3A–S3F). GBA was knocked out in two healthy control iPSC lines and one GBA-PD N370S iPSC line (Table S2). All GBA KO clones showed complete loss of GCase protein and its enzymatic activity (Figures S3E and S3F). No significant difference in DA neuronal differentiation potential was observed among GBA KO iPSCs and corresponding parental lines as assessed by quantification of βIII-tubulin- and tyrosine hydroxylase (TH)-positive neurons (>80% neurons, of which on average 40% of cells in both controls and GBA KO lines expressed TH). GBA KO iPSC-derived neurons exhibited accumulation of the GCase substrates GlcCer and glucosylsphingosine as revealed by HPLC-MS/MS (Figures S3G). A significant increase of C16-galactosylceramide was also observed (Figure S3I). No significant change in levels of other sphingolipids was detected (Figures S3H and S3I).

GBA KO clones were selected from each parental iPSC line and used for further experiments (Table S2). As observed in GBA-PD neurons, morphometric analysis revealed ultrastructural abnormalities in GBA KO iPSC-neurons compared to isogenic controls (Figures 3A and 3B). Similar to what we observed in GBA-PD neurons, GBA KO neurons showed significantly reduced basal and maximal respiration as well as ATP-linked OCR and SRC (Figure 3C). Despite the consistent trend for decreased CI activity in GBA KO neurons, the values did not reach statistical significance (Figure 3D). GBA KO neurons showed elevated levels of mtROS (Figure 3E). Furthermore, we did not observe significant changes of mitochondrial membrane potential (Figure 3F). HPLC-MS/MS analysis of mitochondria purified from GBA KO iPSC-derived neurons showed a significant accumulation of all subtypes of the GCase substrates GlcCer and glucosylsphingosine (Figure 3G).

GBA-PD, but Not GBA Knockout, iPSC-Derived Neurons Show Increased ER Stress and UPR

Interestingly, when comparing OCRs and CI activity in the isogenic lines (GBA-PD N370S, GC control and GBA KO), no gene dosage effect was found (Figures S3J and S3K). These data suggest that different mechanisms contribute to mitochondrial defects in heterozygous GBA-PD patient and GBA KO neurons. One of such mechanisms could be the UPR and ER stress that have been previously observed in GBA-PD (Fernandes et al., 2016). To dissect the contribution of gain- and loss-of-function of mutant GCase, we measured the levels of the ER chaperone immunoglobulin-binding protein (BiP) by western blot in GBA-PD N370S iPSC-derived neurons as well as isogenic controls and isogenic GBA KO neurons. Levels of BiP were increased in GBA-PD neurons compared to isogenic controls and isogenic GBA KO neurons (Figure 4A). Consistent with these findings, RNA levels of spliced X-box-binding protein-1 (XBP1s) were significantly increased in GBA-PD, but not in isogenic GBA KO neurons, suggesting activation of the IRE1 related branch of ER stress (Figure 4B). Healthy control-derived GBA KO neurons showed levels of BiP similar to their isogenic controls (Figure S3L). In addition, levels of phospho-eIF2α were significantly increased in GBA-PD, but not in isogenic GBA KO neurons, as compared to GC controls (Figure 4C). Consistent with these findings, levels of phospho-PERK were only increased in GBA-PD neurons as compared to GC controls (Figure 4D).

NAD+ Metabolism Is Altered in GBA-PD iPSC-Derived Neurons

Mitochondrial dysfunction and increased oxidative stress are hallmarks of aging and have been linked to the decline of intracellular levels of NAD+ (Mouchiroud et al., 2013). To examine whether GBA mutations lead to changes in NAD+ metabolism, we measured the expression of the NAD+ biosynthetic enzymes nicotinamide mononucleotide adenyltransferases (NMNATs), mRNA levels of NMNAT2 were significantly decreased in GBA-PD neurons compared to isogenic controls, whereas levels of NMNAT1 and NMNAT3 were unchanged (Figure 5A). NMNAT2 levels were unchanged in GBA KO neurons (Figure S3M). Levels of nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in the NAD+ salvage synthesis pathway, were similar in both groups (GBA-PD and GC controls), as assessed by qRT-PCR (Figure 5A). Next, we measured the adenosine and pyridine nucleotide pool in GBA-PD and isogenic control as well as GBA KO iPSC-neurons by targeted metabolomics using liquid chromatography-mass spectrometry (LC-MS). The intracellular NAD+ content was maintained in GBA-PD and GBA KO neurons (Figure S4). To exclude that the observed absence of significant changes of NAD+ levels could be linked to the absence of overt neurodegeneration in our stem cell-based model system, we also examined the NAD+ metabolism in adult gba+/−, gba−/−, and gba−/− whole zebrafish brains. Adult gba−/−...
zebrafish recapitulate the key pathological aspects of PD, including DA neuronal loss, early microglial activation, and mitochondrial dysfunction (Keatinge et al., 2015). Metabolomic analyses revealed that the NAD+ pool was maintained in brains of adult $gba^{+/+}$ and $gba^{-/-}$ zebrafish (Figure S5). However, a significant increase in nicotinamide mononucleotide (NMN) was observed in $gba^{-/-}$ compared to $gba^{+/+}$ zebrafish brains as well as in $gba^{+/+}$ compared to $gba^{-/-}$ brains (Figure S5), suggesting an alteration of NAD+ metabolism. To rule out the possibility that the heterogeneity of the cell culture and whole brain tissues influences the results, we then employed the biosensor Peredox to monitor the cytosolic NADH/NAD+ ratio in single iPSC-derived neurons with live-cell imaging (Hung et al., 2011). The NAD+/NADH redox state was significantly reduced in $gba$-PD iPSC neurons, suggesting reduced level of available NAD+ (Figure 5B). No significant change was observed in $gba$ KO neurons (Figure S3N).

The NAD+ Precursor Nicotinamide Riboside Rescues Mitochondrial Defects in $gba$-PD iPSC-Derived Neurons

Next, we tested the ability of different NAD+ precursors to increase NAD+ levels in human iPSC-derived neurons. Control iPSC-derived neurons were treated with nicotinamide (NAM), NMN, or nicotinamide riboside (NR), and NAD+ levels were measured using a NAD+ cycling assay. NR and NMN showed the strongest effect in boosting NAD+ levels (Figure S6A). As NR represents a promising approach, as shown by pharmacokinetic studies in healthy subjects (Trammell et al., 2016), we investigated the ability of NR to rescue $gba$-linked mitochondrial defects. NR significantly increased NAD+ and NADH levels in $gba$-PD neurons (Figures S6B and S6C). NR treatment resulted in an increase in expression of markers of mitochondrial biogenesis (TFAM) and mitochondrial UPR (mtUPR) (HSP60) (Figure 5C). Consistent with these findings, mitochondrial DNA content and mitochondrial mass were increased after 48 hr in NR-treated samples (Figures 5D and 5E). The effect of NR was abrogated by EX527, a SIRT1-specific inhibitor, suggesting that SIRT1 is one of the mediators of NR function (Figure S6D). Importantly, NR restored mitochondrial cristae morphology and significantly reduced mtROS production in $gba$-PD neurons (Figures 5F–5H). In parallel, NR
treatment significantly reduced the mitochondrial membrane potential (Figure 5I). Similar results were observed when GBA KO neurons were treated with NR (Figures S6E–S6G). With regard to the observed defects in mitochondrial dynamics, NR altered the levels of mitochondrial shaping proteins, with a nonsignificant increase in DRP1, Mfn1, and OPA1 and a slight decrease in Fis1 (Figures 5J and 5K). The decreased levels of mitochondrial DNA content observed at 24 hr after NR treatment would suggest an increased mitochondrial clearance (Figure 5D). In line with these findings, NR was able to increase the levels of Bnip3L/Nix in GBA-PD neurons (Figure 5L). NR did not enhance mitochondrial respiration (Figures S6H and S6I). On the contrary, a significant decrease in basal respiration was observed in GBA-PD iPSC-derived neurons treated with NR (Figure 5L). NR treatment did not alter protein levels of BiP in GBA-PD neurons (Figure S6J). To confirm that increased NAD+ levels are responsible for the phenotypic rescue, we treated cells with the poly(ADP-ribose)-polymerase (PARP) inhibitor PJ34 that, differently from NAD+ precursors, increases NAD+ levels by inhibiting its consumption. Similar to NR, PJ34 significantly increased NAD+ levels in human-iPSC-derived neurons and rescued mitochondrial defects in GBA-PD neurons (Figures S7A–S7E). Taken together, these results suggest that increasing NAD+ levels rescues mitochondrial dysfunction via increased levels of NAD+ in patient-derived GBA-PD and GBA KO neurons.

**Increasing NAD+ Improves Autophagy in GBA-PD and GBA KO Neurons**

GBA mutations affect autophagy and lysosomal function, leading to autophagic block, defects in autophagosome clearance, and altered lysosomal recycling (Schöndorf et al., 2014; Magalhaes et al., 2016). To examine the effects of NR on autophagy, we treated GBA-PD and GBA KO neurons with NR and assessed parameters of autophagic function. NR treatment did not alter the levels of LC3II at basal conditions; however, it significantly increased LC3II levels in patient neurons treated with leupeptin and ammonium chloride, suggesting an increase of synthesis and clearance of autophagosomes (Figures S7F–S7I). The autophagic flux was significantly increased in GBA KO neurons and showed a nonsignificant increase in GBA-PD neurons after NR treatment (Figures S7F–S7I).

**Figure 4. ER Stress Responses in GBA-PD and GBA KO iPSC Neurons**

Analysis of ER stress responses and UPR was performed in isogenic GBA-PD (N370S), gene-corrected (GC) controls, and isogenic GBA KO (clones 4 and 16) iPSC neurons. (A) Representative western blots showing BiP levels in isogenic GBA-PD, GC, and GBA KO iPSC neurons. BiP intensity bands were normalized to GAPDH and the corresponding isogenic control. Data are expressed as mean ± SEM (n = 5; one-way ANOVA). (B) Analysis of XBP1 splicing (XBP1s). mRNA levels of XBP1s were measured by qPCR. Representative agarose gel electrophoresis of qRT-PCR products is shown. For quantification, GAPDH served as reference gene. Data are expressed as mean ± SEM (n = 5; one-way ANOVA). (C) Representative western blots showing phospho-eIF2α intensity bands were normalized to total eIF2α and the corresponding isogenic control. Data are expressed as mean ± SEM (one-way ANOVA). Gel loading as in (A). (D) Representative western blots showing phospho-PERK/PERK levels in isogenic GBA-PD, GC, and GBA KO iPSC-derived neurons. Data are expressed as mean ± SEM (n = 5; one-way ANOVA).
Figure 5. Nicotinamide Riboside Reverts Mitochondrial Defects in GBA-PD iPSC Neurons

(A) NMNAT1, NMNAT2, NMNAT3, and NAMPT mRNA levels in isogenic GC and GBA-PD (N370S, L444P) iPSC neurons. Data were normalized to the level of the housekeeping genes Rplp0 and OAZ and expressed as fold change over PD. Data are expressed as mean ± SEM (n = 5; two-tailed t test).

(B) The NAD+/NADH redox state was measured in iPSC-derived neurons using the biosensor Peredox. Results are presented as mean ± SEM (n = 5; two-tailed t test).

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lentiviral-mediated short hairpin RNA (shRNA) (Figures 6D and 6E). NRK1 knockdown did not affect basal levels of NAD+ in neurons, as assessed by a NAD+ cycling assay, whereas NR treatment was unable to significantly increase NAD+ levels in NRK1 knockdown neurons (Figure 6F). These data suggest that NRK1 is essential for exogenous NR metabolism in human neurons. To further examine the metabolism of exogenous NR, we treated control and GBA-PD iPSC neurons with 0.5 mM NR and performed LC-MS-based targeted quantitative metabolomics (Figures 6G and S7J). NR treatment significantly increased the levels of NAD+ in control and patient cells. In addition, we found a significant increase of NAM in both groups. On the other hand, iPSC-derived neurons with GBA mutations (N370S and L444P) were treated with NR, and mRNA expression levels of mtUPR and mitochondrial biogenesis markers were measured by qRT-PCR. Data are normalized to untreated and expressed as mean + SEM (n = 3; two-tailed t test).

(B) NRK1 and NRK2 mRNA levels were measured in control unifferentiated human iPSCs and iPSC-derived neurons. Data are normalized to Rplp0 levels and expressed as mean + SEM (n = 3; two-tailed t test).

(C) NRK1 mRNA levels were measured in control iPSC neurons with or without NR treatment. Data are normalized to control and presented as mean + SEM (n = 3).

(D) Knockdown efficiency of NRK1 in iPSC neurons was determined by qRT-PCR and normalized to non-targeting shRNA. Data are presented as mean + SEM (n = 3; two-tailed t test).

(E) Representative western blot for NRK1 showing knockdown efficiency in iPSC neurons.

(F) Scramble (Scrb) or NRK1 knockdown control iPSC neurons were treated with NR, and NAD+ levels were measured using a NAD+ cycling assay. Data are expressed as fold changes over untreated and presented as mean + SEM (n = 3; one-way ANOVA).

(G) Control and GBA-PD (N370S) iPSC neurons were treated with 0.5 mM NR for 24 hr and LC-MS-based targeted NAD+ metabolomics was performed. Levels of NAD, NAM, and NMN are shown. Data are normalized on protein concentration and presented as mean ± SD (n = 7 independent differentiations; two-tailed t test).
hand, levels of NMN, the immediate downstream product of NR, tended to be higher in NR-treated cells, but this difference was not statistically significant (Figure 6G).

NR Rescues Motor Deficits in a Drosophila Model of GBA-PD

To assess the neuroprotective effect of NAD+ precursors in vivo, we employed a Drosophila model of GBA-PD. Flies expressing human N370S GBA show increased ER stress, an age-dependent loss of DA neurons accompanied by progressive defects in climbing activity (Sanchez-Martinez et al., 2016). To explore neuroprotection, flies expressing wild-type (WT) or N370S GBA were first raised on normal food, and then adult flies were aged on food containing NR (500 μM for 30 days). At 30 days, expression of N370S GBA caused loss of DA neurons in the protocerebral posterior lateral 1 (PPL1) cluster. Strikingly, feeding NR significantly prevented DA neuronal loss compared to untreated controls (Figures 7A and 7B). Importantly, NR treatment also significantly prevented the decline in climbing ability in mutant N370S GBA flies (Figure 7C).

DISCUSSION

Mounting evidence suggests that mitochondrial dysfunction plays a key role in PD. Here, we report that neurons from GBA-PD patients and GBA KO neurons exhibit mitochondrial dysfunction characterized by morphological changes, reduced respiration, and increased oxidative stress. As mitochondrial membrane lipid composition regulates many of these functions (Aufsnaiter et al., 2017) and changes in lipid metabolism have been observed in GBA-PD (Schöndorf et al., 2014; Fernandes et al., 2016; García-Sanz et al., 2017), we examined the mitochondrial sphingolipid profile of GBA-PD and GBA KO neurons.

Lipidomic analysis revealed the absence of a significant accumulation of sphingolipids in patient mitochondria. However, we observed a significant increase of C16-GlcCer. On the contrary, the mitochondrial sphingolipid profile of GBA KO neurons was profoundly altered, with significant accumulation of GlcCer and glucosylsphingosine. Given the role of sphingolipids in the regulation of mitochondrial properties, substrate accumulation or even subtle changes in lipid composition of mitochondrial membranes, as observed in patient mitochondria, may interfere with their biophysical properties and signaling pathways. However, we are not able to unambiguously define the exact subcellular localization of these alterations. Even though we have isolated mitochondria to the highest degree of purity, a small amount of contamination with lysosomes and ER was still present. The low degree of non-mitochondrial proteins and the complete absence of additional lysosomal markers (other than LAMP1) suggest the residual presence of organelle contact sites in mitochondrial preparations. Given the importance of contact sites in interorganelle communication (Wong et al., 2018), further studies are needed to investigate the role of distinct sphingolipids in such inter-organelle communication and mitochondrial dysfunction.

Overall, we did not observe a gene dosage effect when comparing mitochondrial function in heterozygous GBA-PD with GBA KO neurons. Thus, distinctly different mechanisms...
likely contribute to mitochondrial dysfunction in these models. One such mechanism could be the alteration of mitochondria sphingolipid composition, which we observed in GBA KO, but not GBA-PD, cells. Interestingly, in other lysosomal storage diseases, the loss of lysosomal enzymatic function leads to substrate accumulation at the ER membranes and subsequent activation of the UPR (Tessitore et al., 2004). However, despite significant substrate accumulation in our model, the complete loss of GCase enzymatic function in GBA KO neurons is not sufficient to trigger ER stress responses that were instead observed in heterozygous GBA-PD neurons. This points toward a key role of gain-of-function mechanisms in ER stress responses in GBA-PD. On the other hand, GCase deficiency results in sphingolipid accumulation and mitochondrial dysfunction including increased mtROS.

GBA-PD neurons showed an imbalance of mitochondrial shaping proteins DRP1, OPA1, Mfn1, and Fis1 and an increased ratio of L-OPA1/S-OPA1. As L-OPA1 processing and impaired DRP1 activity contribute to the dysfunction of mitophagy (MacVicar and Lane, 2014), our results would suggest an impairment of mitochondrial clearance in GBA-PD. This was further supported by the reduced mitochondria-lysosome colocalization and reduced expression of the mitophagy adaptor NIX/BINP3L. Unexpectedly, we did not observe a significant increase of mitochondrial content in patient neurons. The mitochondrial membrane potential was also preserved in GBA-PD neurons. As BINP3L/NIX plays a role in the loss of mitochondrial membrane potential (Sandoval et al., 2008), reduced levels of BINP3L in GBA-PD neurons could explain the lack of decreased mitochondrial membrane potential. Taken together, our data suggest an imbalance of mitochondrial dynamics in GBA-PD neurons that leads to mitochondrial dysfunction in the absence of accumulation of damaged mitochondria. As mitophagy occurs locally in distal neuronal axons, we cannot exclude accumulation of dysfunctional mitochondria within axons (Ashrafi et al., 2014).

Increasing intracellular NAD+ concentrations has been shown to be protective against age-related metabolic decline and disease (Rajman et al., 2018; Katsyuba and Auwerx, 2017). NAD+ is a coenzyme for several enzymes, including SIRT1, which regulates mitochondrial biogenesis, autophagy, and cellular stress responses (Chang and Guarante, 2014; Prola et al., 2017). Several mechanisms lead to NAD+ consumption, including oxidative stress. To examine whether NAD+ decline is involved in GBA-PD, we have first used targeted metabolomics and found that NAD+ levels were maintained in both GBA-PD and GBA KO iPSC-derived neurons, as well as whole brains from gba<sup>−/−</sup> zebrafish. As cellular energy metabolism and the cytosolic NADH/NAD+ redox state differ in various tissues or different cells within the same tissue, we have employed a genetically encoded NADH/NAD+/NAD+ biosensor for live-cell imaging in iPSC-derived neurons. GBA-PD neurons showed a significant reduction of the NAD+/NAD+ redox state. The reduction of NMNAT2 in GBA-PD neurons further supports an alteration of NAD+ metabolism in GBA-PD. Besides its role in NAD+ synthesis, NMNAT2 also acts as a chaperone to reduce proteotoxic stress and its levels decline prior to the onset of neurodegeneration (Ali et al., 2016). Thus, the reduction of NMNAT2 in GBA-PD neurons could also explain the increased proteotoxic stress observed in these cells. The zebrafish metabolomics data further support an alteration of NAD+ metabolism. GBA deficiency led to a significant increase in NMN in zebrafish brains. This strongly suggests reduced NMNAT activity in the zebrafish brain when lacking GBA. We were unable to reliably detect NMN at basal conditions in iPSC-derived neurons, as NMN is a low abundance metabolite in cellular extracts. In conclusion, even though we cannot yet conclude definitively that NAD+ decline occurs in GBA-PD, our data suggest alterations of NAD+ metabolism in these models. Given the existence of different cellular NAD+ pools and the relevance of mitochondrial NAD+ for mitochondrial and cellular function, an important question remains how these NAD+ cellular pools communicate with each other and modulate the aging process and disease risk.

Here, we report that human iPSC-derived neurons rely on NAMPT for maintenance of the NAD+ pool, they are responsive to NAD+ precursors and utilize NRK1 as the main metabolic pathway to synthetize NAD+ from exogenous NR in a NAMPT-independent manner. NR administration caused a significant increase of NAD+. Besides NAD+, levels of NAM were also increased upon NR treatment, suggesting an increase of the activity of NAD+ consuming enzymes that convert NAD+ to NAM. However, we cannot exclude a partial conversion of exogenous NR into NAM before synthesis to NAD+. Levels of the immediate downstream product of NR, NMN, tended to be higher in NR-treated cells, but this difference was not statistically significant. This would suggest a rapid conversion of NMN into NAD+. In our model systems, NR ameliorated mitochondrial function and rescued mitochondrial quality control. We also observed an increased expression of BINP3L/NIX after NR treatment. In line with this, mitochondrial content decreased after 24 hr of NR treatment, which points toward increased mitophagy. However, prolonged NR treatment boosted mitochondrial content and increased TFAM, which underlines the dual role of NAD+ and sirtuins in maintaining mitochondrial biogenesis and quality control. Furthermore, supporting an increase in mitophagy, NR positively regulated autophagic function. Importantly, we found that NAD+ supplementation rescues the age-dependent loss of DA neurons and decline in motor ability in a GBA-PD Drosophila model.

In summary, our study elucidates the mechanisms involved in GBA-PD and reveals mitochondrial dysfunction as a key driver of disease. Our findings show that NAD+ precursors ameliorate GBA-related defects. Among the available NAD+ precursors, NR may be a valuable therapeutic approach due to its high bioavailability, minimal toxicity, and evidence of its ability to cross the blood-brain barrier (Trammell et al., 2016). Future studies will explore the potential therapeutic benefits of combining NAD+ boosters with chaperones and GCase activators (Migdalska-Richards et al., 2016).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

iPSC lines (Table S1) were previously generated and characterized (Schöndorf et al., 2014).

**Generation of GBA Knockout Human iPSCs**

CRISPR-Cas9 constructs were generated as described previously (Ran et al., 2013).
Measurement of Mitochondrial Membrane Potential and mtROS

Neurons were washed once with Hank’s balanced salt solution (HBSS) (Invitrogen) following incubation with 200 nM tetramethylrhodamine methylster perchlorat (TMRM) (Invitrogen). For measurements of mtROS, cells were incubated with 5 μM MitoSOX Red (Invitrogen). Cytosfluorimetric analysis was performed using MACSQuant Analyzer 10 (Miltenyi).

Seahorse XF96 Metabolic Flux Analysis

OCR was analyzed using an XF96 Extracellular Flux Analyzer (Seahorse Biosciences).

NAD/NADH Measurements and Metabolomics

NAD+ levels were measured using the NAD/NADH Assay Kit (Abcam). The NAD+ levels were measured using the NAD/NADH-Glo Assay Kit (Promega). NADH levels were measured using the NAD/NADH Assay Kit (Biosciences).

Drosophila Studies

Transgenic Drosophila lines expressing human WT or N370S GBA were previously generated (Sanchez-Martinez et al., 2016).

Statistical Analysis

The Statistical Package GraphPad Prism version 7.0b (GraphPad Software, San Diego, CA) was used to analyze the data. Statistical testing involved a two-sided Fisher’s exact test, two-tailed Student’s t test, one-way ANOVA with Bonferroni’s multiple comparison test, or Kruskal-Wallis with Dunn’s multiple comparisons test, as appropriate. Data are expressed as mean ± SEM or SD as indicated.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.05.009.

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AUTHOR CONTRIBUTIONS

M.D. conceived, designed, and supervised the study. A.J.W. designed Drosophila studies. D.C.S., D.I., P.B., S.D.C., and L.K.S. analyzed data. S.N. and B.H. performed TEM. A.S.-M. and I.G. performed Drosophila studies and analyzed data. M.K. and O.B. performed zebrafish experiments. T.G. oversaw patient sample collection. M.D. wrote the manuscript with input from all authors. All authors contributed to proofreading of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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