Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Nicotinamide mononucleotide treatment increases NAD+ levels in an iPSC Model of Parkinson's Disease but does not impact sirtuin activity

Brett Fulleylove-Krause
Medical College of Wisconsin
Samantha Sison
Medical College of Wisconsin
Allison Ebert (aebert@mcw.edu)
Medical College of Wisconsin https://orcid.org/0000-0001-9676-8548

Research note

Keywords: Dopaminergic neurons, LRRK2 G2019S, Sirtuin, Mitochondria

Posted Date: July 22nd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-43558/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Nicotinamide mononucleotide treatment increases NAD+ levels in an iPSC Model of Parkinson's Disease but does not impact sirtuin activity

Brett K. Fulleylove-Krause, Samantha L. Sison, Allison D. Ebert

Department of Cell Biology, Neurobiology and Anatomy
Medical College of Wisconsin
8701 Watertown Plank Rd
Milwaukee, WI 53226

For correspondence:
Allison Ebert
8701 Watertown Plank Rd
Milwaukee, WI 53226
414-955-2979
aebert@mcw.edu

Brett Fulleylove-Krause
bfulleylovek@mcw.edu

Samantha Sison
sammisison@gmail.com

Abstract:

Objectives: Parkinson's disease (PD) is a common neurodegenerative disorder caused by the loss of dopaminergic neurons in the substantia nigra. Although the underlying mechanisms of dopaminergic neuron loss is not fully understood, evidence suggests mitochondrial malfunction as a key contributor to disease pathogenesis. We previously found that human PD patient stem cell-derived dopaminergic neurons exhibit reduced nicotinamide adenine dinucleotide (NAD+) levels and reduce activity of sirtuins, a group of NAD+-dependent deacetylase enzymes that participate in the regulation of mitochondrial function, energy production, and cell survival. Thus, here we tested whether treatment of PD stem cell-derived dopaminergic neurons with nicotinamide mononucleotide (NMN), an NAD+ precursor, could increase NAD+ levels and improve sirtuin activity.

Results: We treated PD iPSC-derived dopaminergic neurons with NMN and found that NAD+ levels did increase. The deacetylase activity of sirtuin (SIRT) 2 was improved with NMN treatment, but NMN had no impact on deacetylase activity of SIRT 1 or 3. These results suggest that NMN can restore NAD+ levels and SIRT 2 activity, but that additional mechanisms are involved SIRT 1 and 3 dysregulation in PD dopaminergic neurons.

Key words:

Dopaminergic neurons

LRRK2 G2019S

Sirtuin

Mitochondria

Introduction:

Parkinson's disease (PD) is a devastating, progressive neurological disorder characterized by the loss of dopaminergic neurons in the substantia nigra and other brain stem nuclei, resulting in decreased dopamine release in the striatum and dysregulated motor output. Although it is known that death of dopaminergic neurons is the underlying cause of motor symptoms in PD, the exact biochemical processes driving this cell death remain elusive. Mounting evidence from human, animal and cell-based model systems have implicated mitochondrial dysfunction and increased oxidative stress as major drivers in the disease process [1-4]. Previous studies within our lab utilizing induced pluripotent stem cells (iPSCs) harboring the familial PD mutation G2019S in the leucine rich repeat kinase (LRRK) 2 gene have found pronounced mitochondrial and sirtuin dysfunction in differentiated dopaminergic neurons [4]. Sirtuins (SIRT) are a class of nicotinamide adenine dinucleotide (NAD+)-dependent protein and histone deacetylases with important regulatory roles in cellular senescence, mitochondrial bioenergetics, DNA repair, and reactive oxygen species production and removal [5]. We previously found that dopaminergic neurons generated from LRRK2 G2019S iPSCs have elevated SIRT protein levels, but decreased SIRT activity compared to healthy dopaminergic neurons [4]. Interestingly, we found that these deficits were more pronounced in LRRK2 G2019S iPSC-derived dopaminergic neurons than in cortical neurons derived from the same LRRK2 G2019S iPSCs [4], suggesting a selective vulnerability of dopaminergic neurons. Moreover, we found that LRRK2 G2019S iPSC-derived dopaminergic neurons had reduced NAD levels compared to control dopaminergic neurons [4], which likely impedes SIRT function in dopaminergic neurons and may be contributing to downstream mitochondrial deficits. Previous studies have found that pharmacologically targeting the NAD+ pathway

improved mitochondrial and SIRT function in both in vitro and in vivo models of PD [3, 6]. Therefore, here we tested if addition of the NAD precursor nicotinamide mononucleotide (NMN) could increase NAD+ levels and improve SIRT deacetylase function in LRRK2 G2019S iPSC-derived dopaminergic neurons.

Main Text:

Methods:

iPSCs and dopaminergic neuron differentiation and treatment

iPSCs were received from Coriell Cell Repository that retains the privacy of the original donors. All iPSC work was approved by the Institutional Biosafety Committee (IBC20120742) and the Institutional Review Board (PRO00025822) at the Medical College of Wisconsin. Human iPSCs were obtained from a commercially available cell repository (Coriell Institute), maintained as undifferentiated colonies on Matrigel in E8 growth medium. The method for dopaminergic neuron differentiation was adapted from previously published protocols [7, 8]. Briefly, embryoid bodies were cultured in dopaminergic neuron base medium (2% B27, 1% N2, 0.1% β-mercaptoethanol, 50µg/ml laminin, 200ng/ml ascorbic acid, 10mM Y27632, and 1% antibiotic/antimycotic in 25% DMEM, 25% F12, and 50% neurobasal medium) over 14 days. From day 0-3, base medium was supplemented with 8.0µM CHIR-99021, 0.2µM LDN193189, and 40µM SB431542. From days 2-10, the medium was supplemented with 300ng/ml sonic hedgehog, 100ng/ml FGF8, and 2µM purmorphamine. From days 9-14, 1µM DAPT was added to the medium. From days 11-14, 10µg/ml BDNF, 10µg/ml GDNF, and 1µM db-cAMP were added to the medium. On day 14, EBs were dissociated with TrypLE and plated onto Matrigel-coated 6-well plates (200,000 cells/well) and coverslips (30,000 cells/coverslip in 24-well plates) in maturation medium (base medium plus 10mM DAPT, 10g/ml BDNF, 10µg/ml GDNF, and 1mM db-cAMP). Medium was

replaced every other day until the end of the experiment. At 42 days of differentiation, control and LRRK2 neurons were treated with 1mM NMN or maturation medium alone for 24hrs.

Immunocytochemistry

Cells were fixed in 4% PFA in PBS. Following blocking of nonspecific labeling and permeabilization in 5% donkey serum and 0.2% TX-100 in PBS, cells were incubated in appropriate primary antibody and then the corresponding secondary antibody. Hoechst dye was used to label nuclei. Primary antibodies used were rabbit anti-tyrosine hydroxylase (Pel-Freez, P40101, 1:1000) and mouse anti-beta III tubulin (Sigma, T8660, 1:2000).

Western Blot

Whole cell lysates were isolated from PD and healthy dopaminergic neurons using 1x Chaps buffer with protease inhibitors. Bradford assay was used to asses protein samples and 20ug of protein per sample was run on 12% precast polyacrylamide gels. Gels were electrophoresed at 105 volts for 90 minutes and transferred to PVDF membrane. Protein content of samples was assessed using REVERT total protein stain (Li-COR, 926-11011) and then probed following standard western blot methods. Primary antibodies used were as follows: rabbit anti-SIRT 1 (Cell Signaling, 9475, 1:1000), rabbit anti-SIRT 2 (Cell Signaling, 12672, 1:1000), rabbit anti-SIRT 3 (Abgent, AP6242a, 1:1000), goat anti-PGC1 alpha (Abcam, ab106814, 1:1000), mouse anti-acetylated tubulin (Sigma, T7451, 1:1000), and rabbit anti-SOD2 (Abcam, ab137037, 1:1000). Appropriate species corresponding red and green tagged secondary antibodies were used. Fluorescence was measured using an Odyssey CLx imager and signal intensity quantified using Image Studio software.

NAD Assay

Quantification of NAD content in samples was carried out using a colorimetric assay kit (Sigma Aldrich). Samples from a single well (200,000 cells) of each experimental group were treated with NADH/NAD extraction buffer and deproteinized using a 10kDa cut-off spin filter to prevent enzymatic degradation of NADH. A standard curve was generated using serial dilutions of 1mM NADH. Samples were then treated with an NAD cycling enzyme mix in duplicate and incubated for 3 hours. Absorbance was measured at 450nm. Following correction for background, NAD content was calculated using the standard curve.

Statistical Analysis

A total of five independent dopaminergic differentiations were carried out for data analysis using one control and one homozygous LRRK2 G2019S iPSC line. Data were analyzed using ANOVA with Bonferroni multiple correction using GraphPad Prism software. Results were considered statistically significant at $p< 0.05$.

Results:

iPSCs from a healthy individual and an unrelated individual with confirmed LRRK2 G2019S PD were differentiated into dopaminergic neurons in vitro using protocols adapted from previously established methods [7, 8]. We have previously found that three independent LRRK2 G2019S iPSC lines all showed consistent dopaminergic neuron differentiation efficiency and a similar reduction in mitochondrial function and SIRT deacetylase activity compared to multiple independent control iPSC lines [4]. Therefore, for this pilot study, we chose to test only a single PD iPSC line and a single control iPSC line. To assess successful differentiation into ventral mesencephalon dopaminergic neurons, we used immunocytochemistry to label cells for Tuj1, a beta-III tubulin specific to neurons, and tyrosine hydroxylase (TH), a dopaminergic neuron specific enzyme responsible for dopamine production.

Consistent with our and others' previous work [4, 7], we observed robust Tuj1 expression with a proportion of the neurons also expressing TH immunofluorescence (Fig. 1).

Next, we tested whether NMN treatment could effectively increase NAD+ levels in G2019S dopaminergic neurons. We first found that untreated G2019S PD neurons had approximately half of the NAD+ concentration compared to control neurons (Fig 2A). Although this reduction did not reach significance, the trend is consistent with our previous data [4]. NMN treatment increased total NAD+ in G2019S PD neurons compared with untreated G2019S PD neurons to a level comparable to untreated healthy control neurons (Fig. 2A). Considering that NAD+ is required for SIRT function, we next examined the effect of NMN treatment on SIRT deacetylase activity. Similar to our previous data [4], western blot analysis showed LRRK2 G2019S dopaminergic neurons exhibited increased expression of SIRT 1, 2, and 3 (Fig. 2B-D). However, NMN treatment did not dramatically alter overall expression levels of any surtuin tested (Fig 2B-D). We next assessed levels of acetylated targets of SIRT 1, 2, and 3 to determine if NMN treatment also impacted SIRT function. We found no change in expression of acetylated targets PGC1α and SOD2 (data not shown) suggesting that the NMN treatment was insufficient to alter SIRT 1 and 3 activity, respectively. However, treatment with NMN induced a significant 2.5-fold reduction in expression of acetylated α-tubulin in NMN-treated G2019S dopaminergic neurons compared to untreated G2019S neurons (Fig. 2E) indicating a partial restoration of SIRT 2 activity despite no overt change in SIRT 2 levels. Together, these data indicate that NMN treatment may preferentially target and restore SIRT 2 function in LRRK2 G2019S dopaminergic neurons. However, these data also indicate that an increase in NAD+ level is not sufficient to impact overall sirtuin function suggesting that other mechanisms are likely contributing to sirtuin malfunction in G2019S iPSC-derived PD dopaminergic neurons.

Discussion:

Sirtuins are integral in protecting the cell during times of oxidative stress or increased metabolic demand due to their role in the regulation of antioxidant defense, mitochondrial function, and mitochondrial trafficking [5]. As dopaminergic neurons are highly active and selectively damaged in PD, chronic alterations in SIRT function may result in accumulating cellular damage and ultimately cell death with aging in PD patients. In the present study, NMN treatment increased NAD levels, which had the most robust effect on SIRT 2 function in human LRRK2 G2019S iPSC-derived dopaminergic neurons. SIRT 2 is involved in mitochondrial trafficking via deacetylation of α-tubulin. Mitochondrial trafficking from the cell body to dendrites and axons is a critical function that neurons undergo constantly for energy maintenance and survival [9], and dysfunction of this process has been found in a number of neurogenerative diseases, including PD. In this regard, decreased SIRT 2 activity in G2019S DA neurons may be playing a role in the mitochondrial trafficking defects observed in these cells [4]. However, whether NMN treatment improves mitochondrial trafficking in G2019S DA neurons has yet to be tested.

A single administration of 1mM NMN was used based on preliminary data and previous studies showing an effect in iPSC derived neurons from PD patients with mutations in beta-glucocerebrosidase (GBA) [3] , but this study did not evaluate the effect on SIRT levels or function. Another study demonstrated that increasing levels of NAMPT, the rate limiting enzyme in the NAD+ salvage pathway, increased SIRT1 activity in 6-hydroxydopamine (6-OHDA) treated PC-12 cells [6]. Although we observed an increase in SIRT2 activity in LRRK2 G2019S iPSC-derived dopaminergic neurons (Fig 2), we did not observe an effect on SIRT1 or SIRT3 expression or function. The discrepancy in results are not entirely clear, but they could be due to differences in model system used (PC-12 cells vs human iPSC-derived dopaminergic neurons), PD stressor (6-OHDA toxicity vs endogenous LRRK2 mutation), or pharmacological treatment (NAMPT vs NMN). Although additional research is needed, the present study shows that NAD+ precursor treatment can improve overall NAD+ levels in dopaminergic neurons, but the overall impact on SIRT deacetylase activity was modest suggesting that additional modulators may

be needed to provide a more robust and therapeutically beneficial effect. As NAD+ supplements are readily available to the general public, it will be important to further evaluate the effect of NAD+ precursor treatment on neuron health and disease to better understand the potential therapeutic benefits and drawbacks to their use.

Limitations:

Limitations of the current study include the small number of iPSC lines used; the mix of differentiated cells in the cultures; the lack of complex interactions between neuronal, glial, immune, and vascular cell types in a 2D environment; and that a single concentration of NMN was tested. We used one PD and one control iPSC line, so it is possible that additional patient lines would show differences in sirtuin function upon NMN treatment. However, our previous studies have shown consistent phenotypes across the PD lines in our hands [4, 10] , so we do not expect that a larger effect would be seen in additional lines. The treatment paradigm for NMN was based on previous studies [3], but future studies should evaluate the efficacy of different NMN dosage on the sirtuin activity and overall mitochondrial energetics and movement to gain a better insight into its potential effects in human dopaminergic neurons. Although TH+ dopaminergic neurons are only a smaller fraction of the differentiated culture, the cells in the culture are patterned toward ventral mesencephalon, so results from these cultures are still relevant for PD. Nevertheless, iPSCs remain a powerful disease modeling method as they allow for in vitro studies of human cells with a known disease genotype, giving a translational advantage over induced disease models or animal studies.

Abbreviations:

PD, Parkinson's disease; LRRK2, leucine-rich repeat kinase 2; NAD+, nicotinamide adenine dinucleotide; iPSC, induced pluripotent stem cells; 6-OHDA, 6-hydroxydopamine; SIRT, sirtuin; NMN, nicotinamide mononucleotide

Declarations:

Ethical Approval and Consent to Participate: iPSCs were received from Coriell Cell Repository that retains the privacy of the original donors. All iPSC work was approved by the Institutional Biosafety Committee (IBC20120742) and the Institutional Review Board (PRO00025822) at the Medical College of Wisconsin.

Consent for Publication: Not applicable

Availability of Supporting Data: Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Competing Interests: The authors declare that they have no competing interests.

Funding: BFK was supported in part by the National Institute on Aging of the National Institutes of Health (NIH) training grant award (2 T35AG29793). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Author contributions: Conception of design: SLS, ADE; Collection of data: BFK, SLS; Analysis of data: BFK, ADE; Supply of resources: ADE; Drafting and editing of manuscript: BFK, SLS, ADE. All authors read and approved the final manuscript.

Acknowledgements: Not applicable.

Authors' information: Not applicable.

Material availability: The iPSC lines used in this study are publicly available from Coriell Cell Repository.

References:

1. Dias, V., E. Junn, and M.M. Mouradian, The role of oxidative stress in Parkinson's disease. J Parkinsons Dis, 2013. 3(4): p. 461-91.

2. Sison, S.L., et al., Using Patient-Derived Induced Pluripotent Stem Cells to Identify Parkinson's Disease-Relevant Phenotypes. Curr Neurol Neurosci Rep, 2018. 18(12): p. 84.

3. Schondorf, D.C., et al., The NAD+ Precursor Nicotinamide Riboside Rescues Mitochondrial Defects and Neuronal Loss in iPSC and Fly Models of Parkinson's Disease. Cell Rep, 2018. 23(10): p. 2976-2988.

4. Schwab, A.J., et al., Decreased Sirtuin Deacetylase Activity in LRRK2 G2019S iPSC-Derived Dopaminergic Neurons. Stem Cell Reports, 2017. 9(6): p. 1839-1852.

5. Singh, C.K., et al., The Role of Sirtuins in Antioxidant and Redox Signaling. Antioxid Redox Signal, 2018. 28(8): p. 643-661.

6. Zou, X.D., et al., NAMPT protects against 6-hydroxydopamine-induced neurotoxicity in PC12 cells through modulating SIRT1 activity. Mol Med Rep, 2016. 13(5): p. 4058-64.

7. Nolbrant, S., et al., Generation of high-purity human ventral midbrain dopaminergic progenitors for in vitro maturation and intracerebral transplantation. Nat Protoc, 2017. 12(9): p. 1962-1979.

8. Maury, Y., et al., Combinatorial analysis of developmental cues efficiently converts human pluripotent stem cells into multiple neuronal subtypes. Nat Biotechnol, 2015. 33(1): p. 89-96.

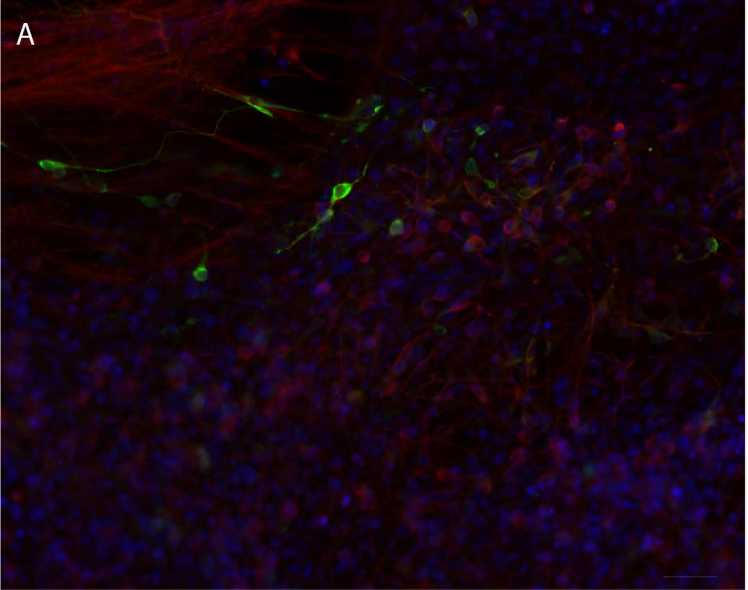
9. Ashrafi, G. and T.L. Schwarz, The pathways of mitophagy for quality control and clearance of mitochondria. Cell Death Differ, 2013. 20(1): p. 31-42.

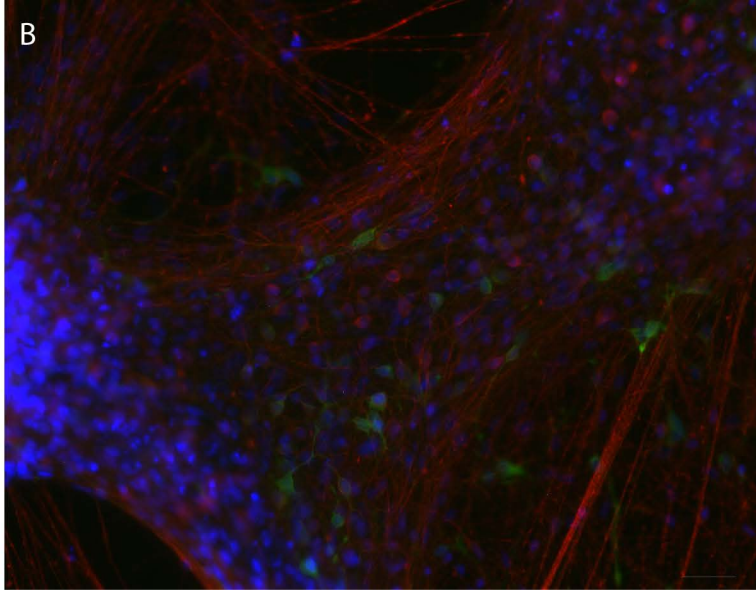
10. Schwab, A.J. and A.D. Ebert, Neurite Aggregation and Calcium Dysfunction in iPSC-Derived Sensory Neurons with Parkinson's Disease-Related LRRK2 G2019S Mutation. Stem Cell Reports, 2015. 5(6): p. 1039-52.

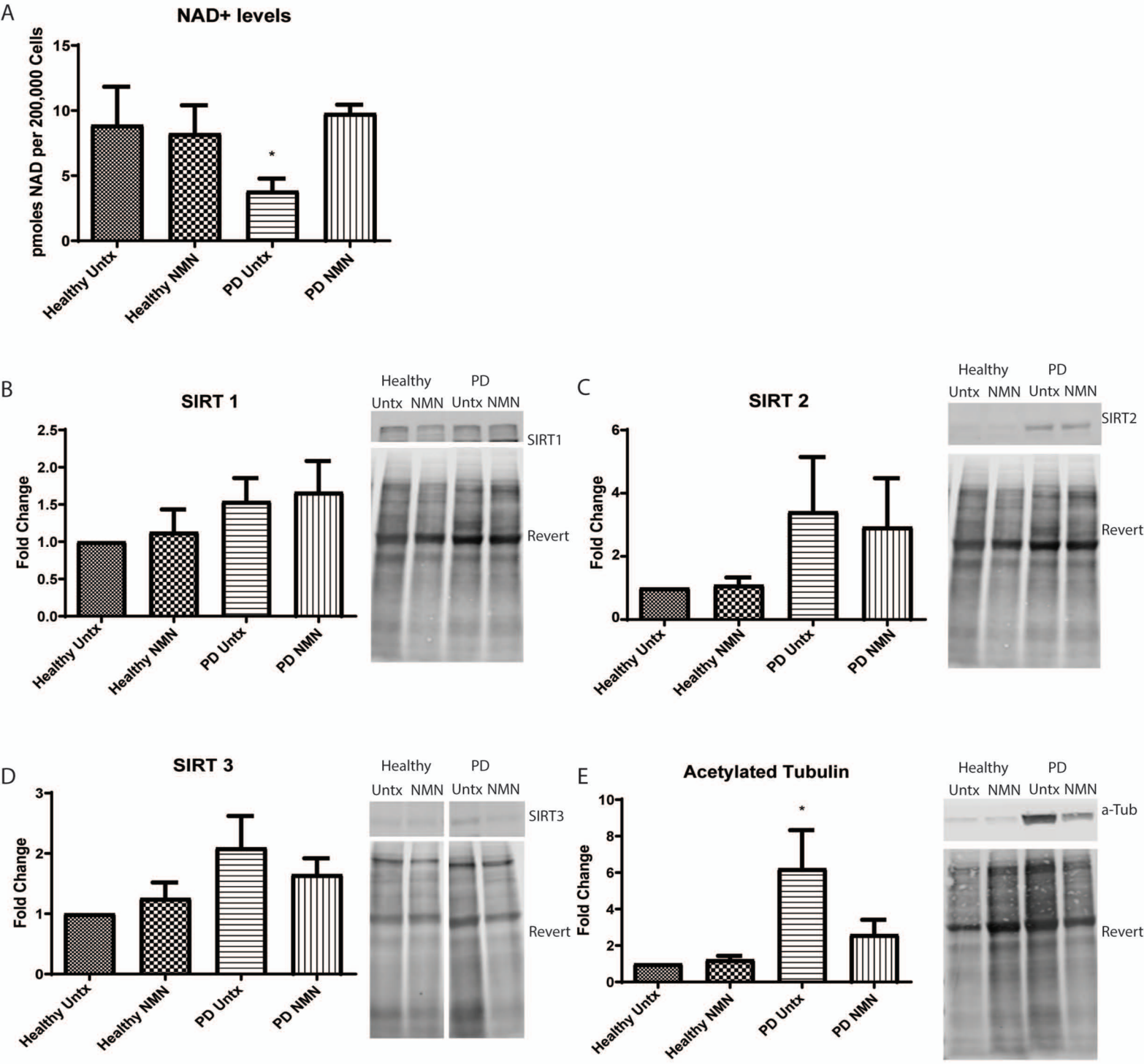
Figure Legends:

Figure 1: Representative images of dopaminergic neurons generated from healthy (A) and LRRK2 G2019S (B) iPSCs as demonstrated by immunocytochemistry for tyrosine hydroxylase (green) and βIII tubulin (red). Nuclei are labeled with Hoechst nuclear dye (blue). Scale bar = 100μm

Figure 2: NMN treatment increased NAD+ levels in LRRK2 G2019S iPSC-derived dopaminergic cultures, but it had little impact on SIRT expression or deacetylase activity. (A) NAD+ levels were significantly increased in NMN treated PD iPSC dopaminergic neurons compared to untreated (untx) PD iPSC dopaminergic neurons. NMN treatment had no significant effect on NAD+ levels in healthy dopaminergic neurons. (B-D) Western blot analysis showed no impact of NMN treatment on SIRT 1 (B), SIRT 2 (C), or SIRT 3 (D) protein expression levels in either healthy or PD dopaminergic neurons compared to untreated (untx) dopaminergic neurons. (E) Acetylated α-tubulin (a-tub) was significantly increased in untreated PD dopaminergic neurons, and NMN treatment induced a 2.5-fold reduction in expression of acetylated α-tubulin in PD dopaminergic neurons (E). Protein was normalized to revert total protein stain. *$p<0.05$ by ANOVA with Bonferroni correction. n=5 independent differentiations. SIRT 1 and SIRT2 were performed on the same membrane, so the revert protein stain is the same in B and C. Uncropped blots included in supplemental data.


A


B


A
NAD+ levels
pmoles NAD per 200,000 Cells
15
10
5
0
*
Healthy Untx
Healthy NMN
PD Untx
PD NMN
B
SIRT 1
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
Healthy Untx
Healthy NMN
PD Untx
PD NMN
Healthy
PD
Untx NMN Untx NMN
SIRT1
Revert
C
SIRT 2
Fold Change
6
4
2
0
Healthy Untx
Healthy NMN
PD Untx
PD NMN
Healthy
PD
Untx NMN Untx NMN
SIRT2
Revert
D
SIRT 3
Fold Change
3
2
1
0
Healthy Untx
Healthy NMN
PD Untx
PD NMN
Healthy
PD
Untx NMN Untx NMN
SIRT3
Revert
E
Acetylated Tubulin
Fold Change
10
8
6
4
2
0
*
Healthy Untx
Healthy NMN
PD Untx
PD NMN
Healthy
PD
Untx NMN Untx NMN
a-Tub
Revert

Figures

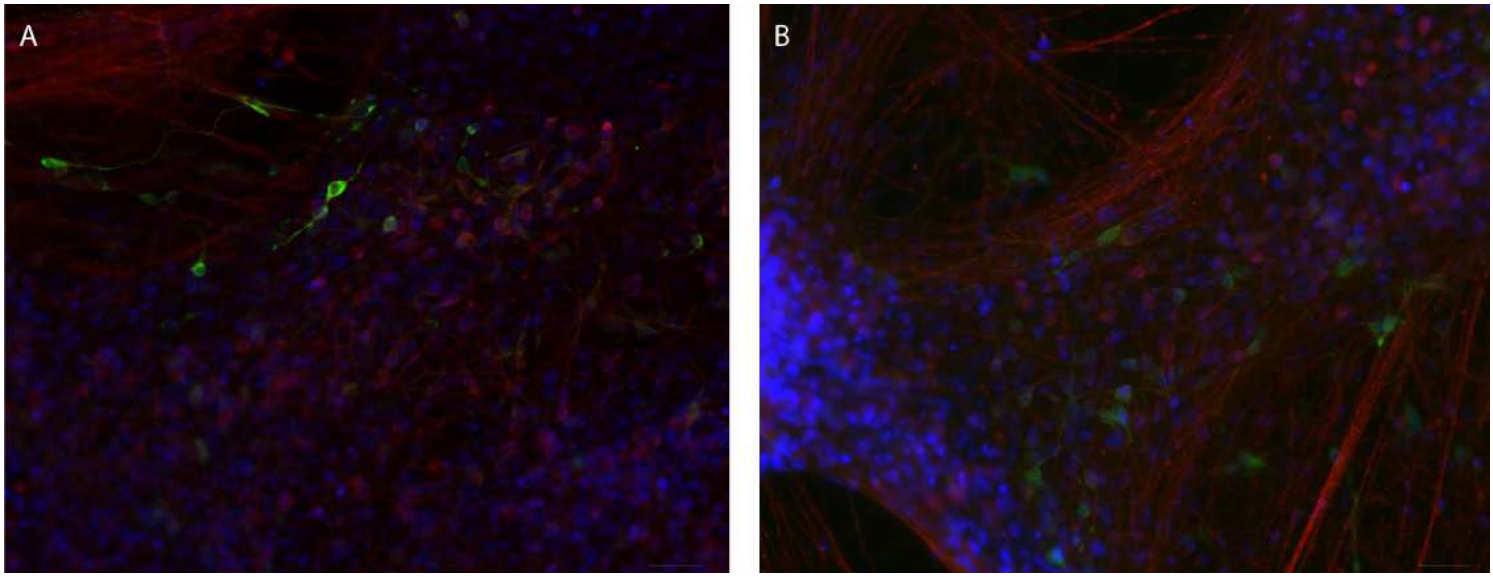


Figure 1

Representative images of dopaminergic neurons generated from healthy (A) and LRRK2 G2019S (B) iPSCs as demonstrated by immunocytochemistry for tyrosine hydroxylase (green) and βIII tubulin (red). Nuclei are labeled with Hoechst nuclear dye (blue). Scale bar = 100μm

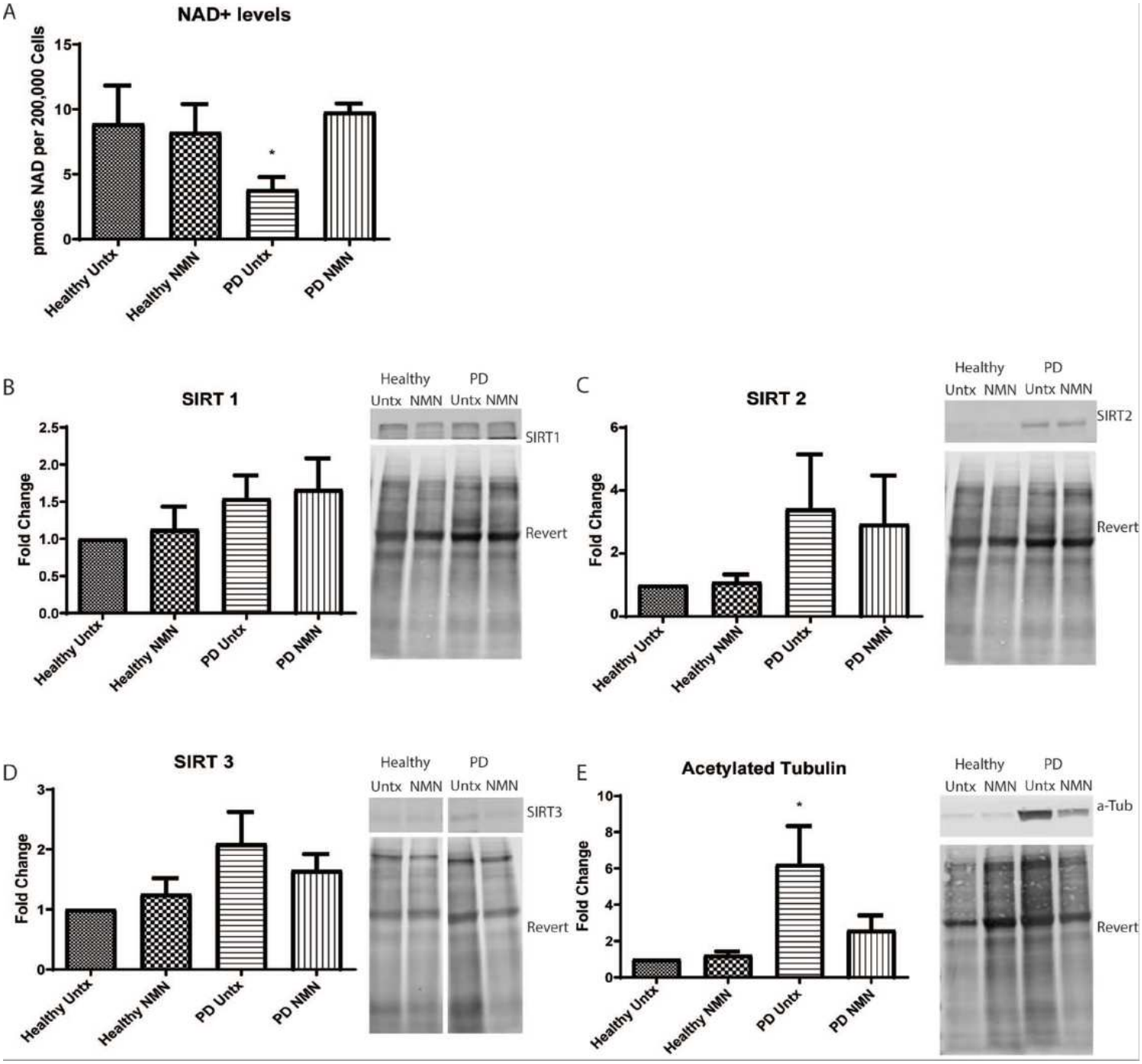


Figure 2

NMN treatment increased NAD+ levels in LRRK2 G2019S iPSC-derived dopaminergic cultures, but it had little impact on SIRT expression or deacetylase activity. (A) NAD+ levels were significantly increased in NMN treated PD iPSC dopaminergic neurons compared to untreated (untx) PD iPSC dopaminergic neurons. NMN treatment had no significant effect on NAD+ levels in healthy dopaminergic neurons. (B-D) Western blot analysis showed no impact of NMN treatment on SIRT 1 (B), SIRT 2 (C), or SIRT 3 (D) protein expression levels in either healthy or PD dopaminergic neurons compared to untreated (untx) dopaminergic neurons. (E) Acetylated α-tubulin (a-tub) was significantly increased in untreated PD dopaminergic neurons, and NMN treatment induced a 2.5-fold reduction in expression of acetylated α-

tubulin in PD dopaminergic neurons (E). Protein was normalized to revert total protein stain. *p<0.05 by ANOVA with Bonferroni correction. n=5 independent differentiations. SIRT 1 and SIRT2 were performed on the same membrane, so the revert protein stain is the same in B and C. Uncropped blots included in supplemental data.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FulleyloveKrauseetal2020supplementalinfo.pdf