UNIVERSITY OF BIRMINGHAM

University of Birmingham Research at Birmingham

Nicotinamide N-methyltransferase increases complex I activity in SH-SY5Y cells via sirtuin 3

Liu, Karolina Y.; Mistry, Rakhee J.; Aguirre, Carlos A.; Fasouli, Eirini S.; Thomas, Martin G.; Klamt, Fábio; Ramsden, David B.; Parsons, Richard B.

DOI:
10.1016/j.bbrc.2015.10.023

License:
Creative Commons: Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)

Document Version
Peer reviewed version

Citation for published version (Harvard):
Liu, KY, Mistry, RJ, Aguirre, CA, Fasouli, ES, Thomas, MG, Klamt, F, Ramsden, DB & Parsons, RB 2015, 'Nicotinamide N-methyltransferase increases complex I activity in SH-SY5Y cells via sirtuin 3', *Biochemical and Biophysical Research Communications*, vol. 467, no. 3, pp. 491-496. https://doi.org/10.1016/j.bbrc.2015.10.023

Link to publication on Research at Birmingham portal

Publisher Rights Statement:
Checked for eligibility: 23/02/2016

General rights
Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

•Users may freely distribute the URL that is used to identify this publication.
•Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
•User may use extracts from the document in line with the concept of ‘fair dealing’ under the Copyright, Designs and Patents Act 1988 (?)
•Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy
While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

Download date: 20. Apr. 2024

Accepted Manuscript

NICOTINAMIDE *N*-METHYLTRANSFERASE increases complex I activity IN SH-SY5Y cells *via* SIRTUIN-3

Karolina Y. Liu, Rakhee J. Mistry, Carlos A. Aguirre, Eirini S. Fasouli, Martin G. Thomas, Fábio Klamt, David B. Ramsden, Richard B. Parsons

PII: S0006-291X(15)30719-1

DOI: 10.1016/j.bbrc.2015.10.023

Reference: YBBRC 34707

To appear in: *Biochemical and Biophysical Research Communications*

Received Date: 30 September 2015

Accepted Date: 3 October 2015

Please cite this article as: K.Y. Liu, R.J. Mistry, C.A. Aguirre, E.S. Fasouli, M.G. Thomas, F. Klamt, D.B. Ramsden, R.B. Parsons, NICOTINAMIDE *N*-METHYLTRANSFERASE increases complex I activity IN SH-SY5Y cells *via* SIRTUIN-3, *Biochemical and Biophysical Research Communications* (2015), doi: 10.1016/j.bbrc.2015.10.023.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

NICOTINAMIDE *N*-METHYLTRANSFERASE INCREASES COMPLEX I ACTIVITY IN SH-SY5Y CELLS *VIA* SIRTUIN-3

Karolina Y. Liu[a], Rakhee J. Mistry[a], Carlos A. Aguirre[a], Eirini S. Fasouli[a], Martin G. Thomas[a], Fábio Klamt[b], David B. Ramsden[c], Richard B. Parsons[a]

[a]King's College London, Institute of Pharmaceutical Science, 150 Stamford Street, London SE1 9NH, UK

[b]Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Departmento de Bioquímica, Rua Ramiro Barcelos, Porto Alegre, 90035 003, Brazil,

[c]University of Birmingham, Department of Medicine, Edgbaston, Birmingham B15 2TH, UK

Corresponding author:

Dr Richard B. Parsons

Tel: 020 7848 4048

Fax: 020 7848 4800

Email: richard.parsons@kcl.ac.uk

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Abstract

Nicotinamide *N*-methyltransferase (NNMT, E.C. 2.1.1.1) *N*-methylates nicotinamide to 1-methylnicotinamide. We have previously shown that NNMT is significantly overexpressed in the brains of patients who have died of Parkinson's disease, and others have shown that NNMT is significantly overexpressed in a variety of diseases ranging from cancer to hepatic cirrhosis. *In vitro* overexpression has revealed many cytoprotective effects of NNMT, in particular increased complex I activity and ATP synthesis. Although this appears to be mediated by an increase in 1-methylnicotinamide production, the molecular mechanisms involved remain unclear. In the present study, we have investigated the role that sirtuins 1, 2 and 3, class III DNA deacetylase enzymes, known to regulate mitochondrial energy production and cell cycle, have in mediating the effects of NNMT upon complex I activity. Expression of NNMT in SH-SY5Y human neuroblastoma cells, which have no endogenous expression of NNMT, significantly increased the expression of all three sirtuins. siRNA-mediated silencing of sirtuin-3 expression decreased complex I activity in NNMT-expressing SH-SY5Y cells to that observed in wild-type SH-SY5Y, and significantly reduced cellular ATP content also. These results demonstrate that sirtuin 3 is a key mediator of NNMT-induced Complex I activity and ATP synthesis. These results further reinforce a central role for NNMT in the regulation of energy homeostasis and provide further mechanistic insight into the consequences of enhanced NNMT expression in cancer.

KEYWORDS: ATP, Bioenergetics, Complex I, Nicotinamide *N*-methyltransferase, Parkinson's disease, Cancer

INTRODUCTION

Nicotinamide *N*-methyltransferase (NNMT, E.C. 2.1.1.1) *N*-methylates nicotinamide to 1-methylnicotinamide (MeN) using *S*-adenosylmethionine as cofactor [1]. In the brain, NNMT is expressed solely in neurons and demonstrates regional variation in expression levels [2]. The expression of NNMT is significantly elevated in the cerebellum and caudate nucleus of patients who have died of Parkinson's disease (PD) compared to non-disease controls [2,3]. NNMT expression is also elevated in a number of non-neurodegenerative diseases such as cirrhosis, chronic obstructive pulmonary disorder and cancer [4-8].

Recently, we have shown that the expression of NNMT in human neuroblastoma cells increased Complex I (CxI) activity, the first enzyme in the mitochondrial respiratory chain of which a significant (~30%) reduction is a cardinal feature of PD [9]. This increase in CxI activity resulted in a subsequent increase in ATP synthesis [10] and protection against a range of mitotoxins [10,11]. The increase in CxI activity observed was mediated *via* a reduction in the degradation of the NDUFS3 30kDa subunit of CxI [10]. As to how this occurs is unclear, but one such possibility is the increased production of MeN, as incubation of SH-SY5Y with MeN replicated the effects of NNMT upon CxI activity and NDUFS3 degradation [10]. Another such possibility is the induction of sirtuin (SirT) activity, class III deacetylase enzymes which regulate many cellular mechanisms such as energy homeostasis, cell cycle, and stress resistance, and whose overexpression extends lifespan in a variety of lower organisms and mammals [12]. NNMT, SirT1 and SirT3 are induced by calorie restriction in mice [13,14], and NNMT expression is required for the pro-longevity effect of the SirT1 homologue Sir-2.1 in *C. elegans*, as well as having the ability, as is MeN, to increase longevity independent of Sir-2.1 [15]. The beneficial effects of SirTs derive in part from their effects upon cellular energy balance, and are thought to act as metabolic sensors *via* their use

of NAD+ as substrate [16,17]. Of particular interest is SirT3, a mitochondrial SirT which is involved in the regulation of ATP production *via* the deacetylation of proteins of CxI [18], and has been implicated in survivorship in elderly humans over the age of 90 [19]. Therefore we investigated whether NNMT expression regulates the expression of SirTs 1, 2, and 3, and whether SirT3 may mediate the effects of NNMT upon CxI activity.

MATERIALS AND METHODS

Unless otherwise stated, all reagents were obtained from Sigma (Poole, Dorset, UK) and were of the highest grade available.

Cell culture

SH-SY5Y human neuroblastoma, which do not endogenously express NNMT, and S.NNMT.LP human neuroblastoma, comprising SH-SY5Y stably expressing NNMT C-terminally tagged to the V5 epitope (NNMT-V5) and produced as part of our ongoing studies [10,11,20], were cultured as previously described. Although SH-SY5Y do not represent a differentiated neurone-like model, they do exhibit pan-neuronal characteristics, express many neuronal markers such as synaptophysin and are readily amenable to genetic manipulation [10,20]. In addition, differentiation of SH-SY5Y using retinoic acid-based protocols significantly increases the rate of oxidative phosphorylation by increasing mitochondrial spare respiratory capacity [21,22]. As such, differentiation may mask many of the cellular effects which we wish to observe. Therefore, for the purposes of our experiments, we chose to use SH-SY5Y in their undifferentiated state. For all experiments, cells of passage 18 – 21 (for SH-SY5Y) and 9 – 13 (for S.NNMT.LP) were used. The lack of endogenous NNMT mRNA expression in SH-SY5Y, and the confirmation of NNMT-V5 mRNA expression in S.NNMT.LP, was confirmed using RT-PCR as previously described [10]. The lack of

endogenous NNMT protein expression in SH-SY5Y, and the presence of NNMT-V5 in S.NNMT.LP, was confirmed using Western blotting using mouse-anti-NNMT (1:1000, Abcam, Cambridge, UK, ab118403) and mouse-anti-V5 (1:2000, Abcam, ab27671) as previously described [10].

Quantitative Western blotting analysis of protein expression

Proteins were subjected to quantitative Western blotting as previously described [10,20] using either rabbit-anti-SirT1 (1:1000, Cell Signalling Technology, Danvers, Massachusetts, USA, #2310), rabbit-anti-SirT2 (1:1000, Cell Signalling Technology, #2313), rabbit-anti-SirT3 (1:1000, Cell Signalling Technology, #2627), mouse-anti-NDUFA6 (0.1 µg/mL, Life Technologies, Paisley, UK, A31856) or anti-acetylated lysine monoclonal antibody (1:1000, Cell Signalling Technology, #9861), combined with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG as appropriate (1:2000, Sigma). To normalise for protein concentration, membranes were stripped using Restore™ Western Stripping Reagent (Fisher Scientific, Loughborough, UK) and re-probed using a combination of mouse-anti-β-tubulin (1:2000, Abcam, ab7792) and rabbit-anti-mouse IgG, horseradish peroxidase conjugate (1:5000, Sigma). Images were digitally captured, band intensities quantified *via* densitometric analysis (with the exception of anti-acetylated lysine) using ImageLab software (BioRad, Hemel Hempstead, UK) and normalised for β-tubulin expression. Results were expressed as percentage expression compared to that observed in SH-SY5Y cells ± S.D (n = 4 for SirTs, n = 3 for NDUFA6).

siRNA-mediated silencing of SIRT3 expression

SIRT3 expression was silenced in S.NNMT.LP cells as previously described [20] using three pooled *SirT3* siRNA sequences (sc-61555, Santa Cruz Biotechnology, Heidelberg, Germany)

ACCEPTED MANUSCRIPT

(S.NNMT.LP^{KD}), with a scrambled sequence siRNA (sc-37007, Santa Cruz Biotechnology) serving as a control (S.NNMT.LP^{Scr}). *SIRT3* silencing was confirmed using RT-PCR [20] using the following primer pair: forward primer, 5'-CTGTACAGCAACCTCCAGCA-3', reverse primer, 5'-GCTCCCCAAAGAACACAATG-3'. *GAPDH* loading control was detected using the following primer pair: forward primer, 5'-AGCCACATCGCTCAGACAC-3', reverse primer, 5'-GCCCAATACGACCAAATCC-3'. Reduction in SirT3 protein expression was confirmed using Western blotting as described above. As a control to ensure that the transfection procedure had no effect upon SirT3 expression and analysis endpoints, S.NNMT.LP cells were also incubated with transfection reagent alone for 72 hours (S.NNMT.LP^{Ctrl}).

Complex I activity analysis

CxI activity in SH-SY5Y, S.NNMT.LP, S.NNMT.LP^{Ctrl}, and S.NNMT.LP^{KD} was assessed as previously described [10,23]. Experiments were performed in triplicate using 6 samples per experiment, and were expressed as percentage of CxI activity observed in the appropriate experimental control ± S.D.

ATP analysis

Cellular ATP content was assessed in S.NNMT.LP^{Ctrl} and S.NNMT.LP^{KD} using the ATP-Glo™ Bioluminetric Cell Viability Assay Kit (Cambridge Bioscience, Cambridge, UK) as previously described [20,23]. Experiments were performed seven times using 11 samples per experiment. Results were calculated and expressed as percentage observed in the appropriate experimental control ± S.D.

Statistical analysis

ACCEPTED MANUSCRIPT

All statistical analyses were performed using the Prism statistical package (GraphPad, La Jolla, USA). Statistical comparisons were performed using student's *t*-test with Welch correction, with the exception of the effect of siRNA silencing of *SIRT3* upon cellular ATP content, which was performed using 1-way ANOVA with Tukey's *post hoc* multiple comparisons test. *P* values of less than 0.05 were taken as significant.

RESULTS

FIG.1

S.NNMT.LP cells expressed recombinant NNMT-V5 and demonstrated increased Complex I activity

The endogenous and recombinant expression of NNMT was assessed in SH-SY5Y and S.NNMT.LP using a combination of RT-PCR and Western blotting (Fig. 1A & 1B). Neither endogenous NNMT mRNA nor protein was detected in SH-SY5Y, whereas recombinant NNMT-V5 was expressed in S.NNMT.LP. In accord with our previous studies [10], CxI activity was significantly increased in S.NNMT.LP cells compared to SH-SY5Y (100 ± 25.9% *vs*. 209.3 ± 44.4%, n = 3, $p = 0.035$) (Fig. 1C).

The stable expression of NNMT-V5 increased SirT protein expression and activity

FIG.2

In order to identify a potential molecular target which mediated the effect of NNMT-V5 upon CxI activity, we focussed upon SirTs 1, 2 and 3 which we compared in SH-SY5Y and S.NNMT.LP cells using quantitative Western blotting (Fig. 2A & 2B). SirT1 expression, detected as a protein of approximately 95 kDa [24], was almost undetectable in SH-SY5Y cells yet was significantly increased in S.NNMT.LP cells (100 ± 11.5 % vs. 1760 ± 138.4 %, n = 4, $p < 0.001$). SirT2, detected as a protein of approximately 39 kDa (corresponding to the

molecular weight of isoform 2) [25], was expressed in both cell-lines, which was significantly increased in S.NNMT.LP cells (100 ± 8.9 vs. 246.1 ± 71.7 %, n = 4, $p = 0.027$). SirT3, detected as a protein of approximately 31 kDa (corresponding to the molecular weight of post-translationally-processed SirT3) [26], was almost undetectable in SH-SY5Y, yet was significantly expressed in S.NNMT.LP cells (100 ± 9.6% vs. 1835.5 ± 129.8%, n = 4, $p < 0.001$).

Protein acetylation was widespread in the SH-SY5Y proteome (Fig. 2C), with the majority of acetylated proteins having a MWt below 200 kDa. Protein acetylation was significantly reduced in the S.NNMT.LP proteome, with proteins greater than 100 kDa exhibiting little or no acetylation, demonstrating an increase in SirT activity in S.NNMT.LP cells.

Silencing SIRT3 expression decreased Complex I activity and cellular ATP content

FIG. 3

Having demonstrated that NNMT-V5 expression increased the expression of SirTs 1, 2 and 3, we focussed upon SirT3, as this SirT is known to regulate oxidative phosphorylation by the direct deacetylation of subunits of the mitochondrial respiratory chain [27], in particular NDUFA9 of CxI [18]. Silencing *SIRT3* expression reduced *SIRT3* mRNA expression in $S.NNMT.LP^{KD}$ (Fig. 3A), with a concomitant reduction in protein expression (100 ± 29% vs. 8.4 ± 1.2%, n = 3, $p = 0.032$) (Fig. 3B). Scrambled siRNA had no effect upon *SIRT3* mRNA expression (Fig. 3A).

CxI activity was not significantly altered in $S.NNMT.LP^{Ctrl}$ cells compared to S.NNMT.LP, demonstrating that the siRNA procedure had no effect upon CxI activity (data not shown). In contrast, CxI activity was significantly reduced in $S.NNMT.LP^{KD}$ compared to

S.NNMT.LP^{Ctrl} (100 ± 21.2% vs. 62.8 ± 2.7%, n = 3, $p = 0.048$) (Fig. 3C), which was not significantly different to that observed in SH-SY5Y cells (131.5 ± 5.6%, n = 3, $p = 0.18$). Likewise, cellular ATP content was not significantly different in S.NNMT.LP^{Ctrl} compared to S.NNMT.LP (88.7% ± 8.1% of that observed in S.NNMT.LP, n = 7, $p > 0.05$), whereas cellular ATP content was significantly reduced in S.NNMT.LP^{KD} compared to S.NNMT.LP^{Ctrl} (100 ± 9.8% vs. 66.3 ± 11.6%, n = 7, $p < 0.001$) (Fig. 3D). ATP content in S.NNMT.LP^{KD} was significantly higher than that observed in SH-SY5Y cells (100 ± 4.6% vs. 275.1 ± 15.8%, n = 7, $p < 0.001$).

Increased Complex I activity and ATP synthesis did not arise from an increase in mitochondrial number

FIG. 4

It is possible that the NNMT-V5-mediated increase in CxI activity, and subsequent ATP synthesis, was due to an increase in mitochondrial number. Although the calculation of CxI activity takes into account mitochondrial number and as such demonstrated an increase in enzyme activity, it does not rule out an increase in mitochondrial number being in some part responsible for the increase in cellular ATP synthesis observed in S.NNMT.LP cells [10]. Therefore, the expression of the CxI subunit NDUFA6 with respect to the cytosolic housekeeping protein β-tubulin was used as a surrogate measure of mitochondrial number (Fig. 4). NDUFA6 was detected as a 15 kDa protein in both cells lines at similar levels in each (100 ± 9.7% vs. 114 ± 10.2%, n = 4, $p = 0.207$) (Fig. 4A).

DISCUSSION

NNMT is central to the regulation of energy balance within the cell

ACCEPTED MANUSCRIPT

Recent studies which provide compelling evidence of a role for NNMT in the regulation of energy metabolism and cell survival *in vitro* [10,11,14], in particular NNMT's ability to induce CxI activity, ATP synthesis and synapse formation [10,20], have transformed NNMT from merely a Phase II enzyme into a key mediator of many fundamental processes essential for the maintenance of cellular health and function. This effect upon CxI is of particular interest, as reduced CxI activity is a cardinal feature of PD [9]. In order to understand more fully how NNMT is able to regulate CxI activity and ATP synthesis, we have investigated whether NNMT-V5 influences the expression of SirTs.

We investigated SirTs for a number of reasons: (1) SirTs 1 & 3 have been shown to regulate mitochondrial functions such as biogenesis, oxidative phosphorylation and ATP synthesis [18,19,28,29], (2) SirT3 has been shown to regulate the activity of CxI *via* the deacetylation of the lysine residues of NDUFA9 [18], and (3) NNMT is induced along with SirT1 and SirT3 during calorie restriction [13,14]. We did not investigate the effects of NNMT-V5 expression upon the remaining SirTs, in particular SirTs 4 & 5, because although these two SirTs are expressed in the mitochondria alongside SirT3, they are involved in the regulation of amino acid metabolism and the urea cycle respectively, with no research to date reporting either a direct or indirect effect upon oxidative phosphorylation [27,30,31].

The demonstration that NNMT-V5 expression induced SirT expression and activity reinforces its pivotal role in regulating energy metabolism and mitochondrial function in the cell. SirT activation most likely arises from the increased metabolism of nicotinamide, the physiological inhibitor of SirTs [32]. This is supported by Schmeisser and colleagues in *C. elegans* [15] who demonstrated that knock-out of the NNMT homologue *anmt-1* inhibited the pro-longevity effects of the SirT1 homologue Sir-2.1, with the overexpression of *anmt-1*

having the opposite effect. As to how NNMT-V5 induced SirT expression is unclear. It is possible that MeN, the metabolic product of NNMT *N*-methylation of nicotinamide, may be responsible, which is supported by our previous studies that demonstrated that MeN increased CxI activity and cellular ATP content in SH-SY5Y cells [10,11]. In accord with this, Xie et al. [33] reported that increased MeN production was responsible for the NNMT-mediated increase in cellular ATP content in human colorectal cancer cells. SirTs are also thought to act as metabolic sensors *via* their use of NAD+ as substrate [16,17], whose availability is regulated by NNMT *via* NAD+ synthesis [3,34]. Both NAD+ and NADH levels are decreased in S.NNMT.LP compared to SH-SY5Y cells [10], demonstrating that pyridine nucleotide synthesis is decreased due to reduced nicotinamide availability, however the NAD+:NADH ratio was also significantly reduced in S.NNMT.LP compared to SH-SY5Y, indicating increased use of NAD+ presumably *via* increased SirT activity. Hence, the ability of NNMT to induce and activate SirT activity places it firmly within the regulation of mitochondrial function and energy regulation.

SirT3 is a key mediator of NNMT's effect upon Complex I activity

The decrease in CxI activity in S.NNMT.LP^{KD}, which was comparable to that observed in SH-SY5Y cells, arising from the silencing of SirT3 expression demonstrates that SirT3 mediates the effects of NNMT-V5 upon CxI activity. This is in accord with SirT3's ability to activate CxI activity *via* the deacetylation of NDUFA9 [18]. What was interesting was that cellular ATP content was not reduced to levels observed in SH-SY5Y cells, indicating that mechanisms other than SirT3 are also involved. One such possibility is an increase in mitochondrial number, however the relative expression of NDUFA6, whose expression relative to the cytosolic housekeeping gene β-tubulin would be expected to increase if mitochondrial number were increased also, did not change, which is in accord with the lack

of change in NDUFS9 expression that we have previously reported [10]. The induction of SirT1 expression, which increases mitochondrial ATP synthesis *via* the induction of other targets not part of oxidative phosphorylation such as PPAR-γ and PGC1α [28], that we observed may contribute to the increase in cellular ATP content in S.NNMT.LP. What is clear, however, is that SirT3 mediates the induction of CxI activity by NNMT-V5, most likely due to increased deacetylation.

Induction of NNMT expression in the Parkinsonian brain may be a stress response of the cell to the underlying disease pathogenesis

The S.NNMT.LP cell-line does not closely mimic the dopaminergic neuronal phenotype of the neurons which degenerate in PD, indeed it was our sole original intention to model NNMT expression using a cell-line which has no endogenous expression of NNMT, to provide the ability to investigate the biochemical effects of NNMT upon the cell in isolation [10,11,20]. However, this originated from our research into the expression of NNMT in human PD brain tissue [2,3], thus our studies provide evidence for what may be occurring biochemically within the dopaminergic neuron in the PD brain. Accumulating evidence from these studies suggest that the increased expression of NNMT that we have observed *in vivo* induces SirT expression and hence increases CxI activity and ATP synthesis, alongside increased synapse formation and protection from PD-relevant mitotoxins, as part of a cytoprotective response of the neuron to the PD pathogenic process. NNMT is also induced in a number of cancers, such as breast and neuroblastoma (from which SH-SY5Y cells are derived). Such induction has several beneficial consequences for the tumour cell, namely reduced radiation sensitivity *via* the metabolism of the radiosensitizer nicotinamide [35], promotion of metastasis *via* the induction of the Akt signalling pathway [36] and protection against a range of cytotoxins [10,11]. It is likely that such induction provides the high energy

demand, namely increased cellular ATP content, required by the tumour cell. In support of this, SirT3 protein levels are elevated in breast cancers [37].

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING

This work was funded by a grant from Parkinson's UK [to RBP, grant ref. G-0505], a King's College London Health Schools Studentship [to MGT] and a scholarship from the NIH UCI-MHIRT Program [to CAA, grant ref. MD-01485].

FOOTNOTES

Abbreviations: CxI: Complex I; MeN: 1-methylnicotinamide; NNMT: nicotinamide *N*-methyltransferase; NNMT-V5: nicotinamide *N*-methyltransferase C-terminally fused to the V5 epitope; PD: Parkinson's disease; PGC1α: peroxisome proliferator-activated receptor γ coactivator-1α; PPAR-γ: peroxisome proliferator-activated receptor γ; SirT: sirtuin

ACCEPTED MANUSCRIPT

REFERENCES

[1] S. Aksoy S, C.L. Szumlanski, R.M. Weinshilboum, Human liver nicotinamide *N*-methyltransferase. cDNA cloning, expression, and biochemical characterisation, J. Biol. Chem. 269 (1994) 14835-14840.

[2] R.B. Parsons, M-L Smith, R.H. Waring, et al., Expression of nicotinamide *N*-methyltransferase (NNMT, E.C. 2.1.1.1) in the Parkinsonian brain, J. Neuropathol. Exp. Neurol. 61 (2002) 111-124.

[3] R.B. Parsons, S.W. Smith, R.H. Waring, et al., High expression of nicotinamide *N*-methyltransferase in patients with idiopathic Parkinson's disease, Neurosci. Lett. 342 (2003) 13-16.

[4] M. Roessler, W. Rollinger, S. Palme, et al., Identification of nicotinamide *N*-methyltransferase as a novel serum tumour marker for colorectal cancer, Clin. Cancer. Res. 11 (2005) 6550-6557.

[5] M. Tomida, H. Ohtake, T. Yokota, et al., Stat3 up-regulates expression of nicotinamide *N*-methyltransferase in human cancer cells. J. Cancer Res. Clin. Oncol. 134 (2008) 551-559.

[6] M. Sternak, T.I. Khomich, A. Jakubowski, et al., Nicotinamide *N*-methyltransferase (NNMT) and 1-methylnicotinamide (MNA) in experimental hepatitis induced by concanavalin A in the mouse, Pharmacol. Rep. 62 (2010) 483-493.

[7] H.C. Kim, M. Mofarrahi, T. Vassilakopoulos, et al., Expression and functional significance of nicotinamide *N*-methyltransferase in skeletal muscle of patients with chronic obstructive pulmonary disease, Am. J. Respir. Care Med. 181 (2010) 797-805.

[8] M. Emanuelli, A. Santarelli, D. Sartini, et al., Nicotinamide *N*-methyltransferase upregulation correlates with tumour differentiation in oral squamous cell carcinoma, Histol. Histopathol. 25 (2010) 15-20.

[9] A.H. Schapira Mitochondrial Complex I deficiency in Parkinson's disease, Adv. Neurol. 60 (1993) 288-291.

[10] R.B. Parsons, S. Aravindan, A. Kadampeswaran, et al., The expression of nicotinamide *N*-methyltransferase increases ATP synthesis and protects SH-SY5Y neuroblastoma cells against the toxicity of Complex I inhibitors, Biochem. J. 436 (2011) 145-155.

[11] Z.B. Milani, D.B. Ramsden, R.B. Parsons, Neuroprotective effects of nicotinamide *N*-methyltransferase and its metabolite 1-methylnicotinamide, J. Biochem. Mol. Toxicol. 27 (2013) 451-456.

[12] M.C. Haigis, L. Guarente, Mammalian sirtuins – emerging roles in physiology, aging and calorie restriction, Genes Dev. 20 (2006) 2913-2921.

[13] T. Shi, F. Wang, E. Stieren, et al., SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes, J. Biol. Chem. 280 (2005) 13560-13567.

[14] P.W. Estep 3rd, J.B. Warner, M.L. Bulyk, Short-term calorie restriction in male mice feminizes gene expression and alters key regulators of conserved aging regulatory pathways, PLoS One 4 (2009) e5242.

[15] K. Schmeisser, J. Mansfeld, D. Kuhlow, et al., Role of sirtuins in lifespan regulation is linked to methylation of nicotinamide, Nat. Chem. Biol. 9 (2013) 693-700.

[16] M. Gertz, C. Steegborn, Function and regulation of the mitochondrial sirtuin isoform Sirt5 in Mammalia, Biochim. Biophys. Acta 1804 (2010) 1658-1665.

[17] S. Michan, D. Sinclair D, Sirtuins in mammals: insights into their biological function, Biochem. J. 404 (2007) 1-13.

[18] B.H Ahn, H.S. Kim, S. Song, et al., A role for the mitochondrial deacetylase Sirt3 in regulating energy homoeostasis, Proc. Natl. Acad. Sci. USA 105 (2008) 14447-14452.

[19] D. Bellizzi, P. Cavalcane, G. Covello, et al., A novel VNTR enhancer within the SIRT3 gene, a human homologue of SIR2, is associated with survival at oldest ages, Genomics 85 (2005) 258-263.

[20] M.G. Thomas, M. Saldanha, R.J. Mistry, et al., Nicotinamide *N*-methyltransferase expression in SH-SY5Y neuroblastoma and N27 mesencephalic neurones induces changes in cell morphology *via* ephrin-B2 and Akt signalling, Cell Death Dis. 4 (2013) e669.

[21] L. Schneider, S. Giordano, B.R. Zelickson, et al., Differentiation of SH-SY5Y cells to a neuronal phenotype changed cellular bioenergetics and the response to oxidative stress, Free Radic. Biol. Med. 51 (2011) 2007-2017.

[22] Z. Xun, D.Y. Lee, J. Lim, et al., Retinoic acid-induced differentiation increases the rate of oxygen consumption and enhances the spare respiratory capacity of mitochondria in SH-SY5Y cells, Mech. Ageing Dev. 133 (2012) 176-185.

[23] D.M. Humphrey, R.B. Parsons, Z.N. Ludlow ZN, et al., *Alternative oxidase* rescues mitochondria-mediated dopaminergic cell loss in *Drosophila*, Hum. Mol. Genet. 21 (2012) 2698-2712.

[24] S. Voelter-Mahlknecht, U. Mahlknecht, Cloning, chromosomal characterization and mapping of the NAD-dependent histone deacetylases gene sirtuin 1, Int. J. Mol. Med. 17 (2006) 59-67.

[25] S. Voelter-Mahlknecht, A.D. Ho, U. Mahlknecht, FISH-mapping and genomic organization of the NAD-dependent histone deacetylase gene, Sirtuin 2 (Sirt2), Int. J. Oncol. 27 (2005) 1187-1196.

[26] M.B. Scher, A. Vaquero, D. Reinberg, SirT3 is a nuclear NAD+-dependent histone deacetylase that translocates to the mitochondria upon cellular stress, Genes Dev. 21 (2007) 920-928.

ACCEPTED MANUSCRIPT

[27] P. Parihar, I. Solanki, M.L. Mansuri, et al., Mitochondrial sirtuins: emerging roles in metabolic regulations, energy homeostasis and diseases, Exp. Gerontol. 61 (2015) 130-141.

[28] M.C. Haigis, D.A. Sinclair, Mammalian sirtuins: biological insights and disease relevance, Ann. Rev. Pathol. 5 (2010) 253-295.

[29] Y. Yang, H. Cimen, M.J. Han, et al., NAD+ dependent deacetylase SIRT3 regulates mitochondrial protein synthesis by deacetylation of the ribosomal protein MRPL10, J. Biol. Chem. 285 (2010) 7417-7429.

[30] M.C. Haigis, R. Mostoslavsky, K.M. SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic beta cells, Cell 126 (2006) 941-954.

[31] J. Yu, S. Sadhukhan, L.G. Noriega, et al., Metabolic characertization of a SirT5 deficient mouse model, Sci. Rep. 3 (2013) 2806.

[32] B.D. Sanders, B. Jackson, R. Marmorstein, Structural basis for sirtuin function: what we know and what we don't, Biochim. Biophys. Acta. 1804 (2010) 1604-1616.

[33] X. Xie, H. Yu, Y. Wang, et al., Nicotinamide N-methyltransferase enhances the capacity of tumourigenesis associated with the promotion of cell cycle progression in human colorectal cancer cells, Arch. Biochem. Biophys. 564 (2014) 52-66.

[34] A.C. Williams, D.B. Ramsden, Autotoxicity, methylation and a road to the prevention of Parkinson's disease, J. Clin. Neurosci. 12 (2005) 6 – 11.

[35] H.S. Kassem, V. Sangar, R. Cowan, et al., A potential role of heat shock proteins and nicotinamide *N*-methyltransferase in predicting response to radiation in bladder cancer, Int. J. Cancer 101 (2002) 454-460.

[36] S.W. Tang, T.C. Yang, W.C. Lin, et al., Nicotinamide *N*-methyltransferase induces cellular invasion through activating matrix metalloprotease-2 expression in clear renal cell carcinoma cells, Carcinogenesis 32 (2011) 138-145.

ACCEPTED MANUSCRIPT

[37] N. Ashraf, S. Zino, A. Macintyre, et al., Altered sirtuin expression is associated with node-positive breast cancer, Br. J. Cancer. 95 (2006) 1056-1061.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Figure legends

Fig. 1. S.NNMT.LP express NNMT-V5 and demonstrate increased Complex I activity. (A) mRNA expression. NNMT and NNMT-V5 mRNA expression was detected using RT-PCR, with equal loading confirmed using *GAPDH*. **(B) Protein expression.** NNMT and NNMT-V5 protein expression was detected using Western blotting, with equal protein loading confirmed using β-tubulin. **(C) Complex I activity.** Complex I activity was assessed using a colourimetric-based assay. Statistical analysis was performed using *t*-test with Welch correction (n = 3).

Fig. 2. Expression of NNMT-V5 in S.NNMT.LP increased the expression of SirTs 1, 2 and 3 and decreased protein acetylation. (A) Sirtuin protein expression. SirTs 1, 2 and 3 protein expression was detected using Western blotting. **(B) Quantitative analysis.** Band intensities were quantified using densitometry using ImageLab, normalised for β-tubulin and expressed as percentage expression compared to SH-SY5Y. Statistical analysis comprised *t*-test with Welch correction (n = 4). **(C) Proteome acetylation.** SH-SY5Y and S.NNMT.LP proteome acetylation was assessed using Western blotting. **For all panels:** SirT1: sirtuin-1; SirT2: sirtuin-2; SirT3: sirtuin-3.

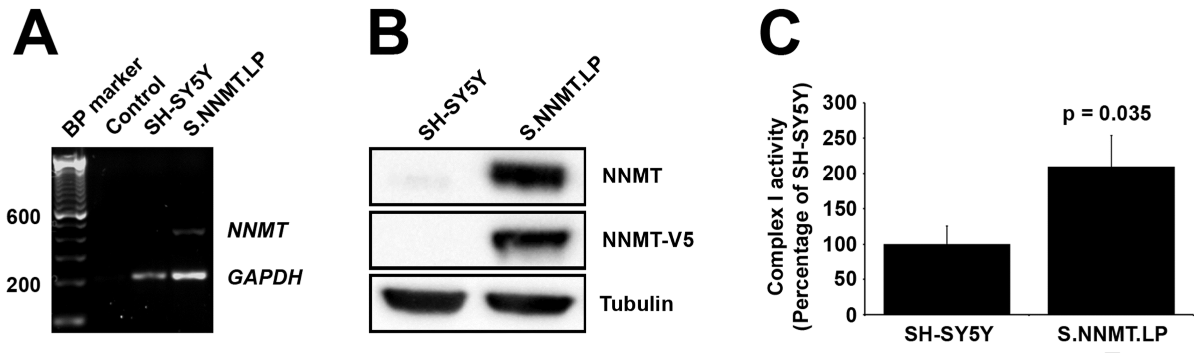
Fig. 3. siRNA silencing of *SIRT3* expression in S.NNMT.LP reversed the effect of NNMT-V5 expression upon Complex I activity, and reduced cellular ATP content. *SIRT3* expression was silenced using transient transfection of S.NNMT.LP with 3 pooled siRNA (S.NNMT.LP^{KD}). As controls, S.NNMT.LP incubated with transfection medium alone (S.NNMT.LP^{Ctrl}) and S.NNMT.LP transfected with scrambled sequence siRNA (S.NNMT.LP^{Scr}) were used. **(A) *SIRT3* mRNA expression.** *SIRT3* expression was assessed using RT-PCR. **(B) SirT3 protein expression.** Sirtuin-3 expression was assessed using

ACCEPTED MANUSCRIPT

Western blotting. **(C) Complex I activity.** Complex I activity was analysed using a colourimetric assay. Statistical analysis comprised *t*-test with Welch correction (n = 3). **(D) Cellular ATP content.** Cellular ATP content was assessed using the ATP-Glo bioluminescence assay. Statistical analysis comprised one-way ANOVA with Tukey's *post hoc* comparisons test (n = 7). **For all panels:** S.NNMT.LP^{Ctrl} = S.NNMT.LP cells incubated with siRNA transfection reagent only; S.NNMT.LP^{Scr} = S.NNMT.LP cells incubated with scrambled siRNA; S.NNMT.LP^{KD} = S.NNMT.LP cells incubated with 3 pooled siRNA sequences targeting *SIRT3*.

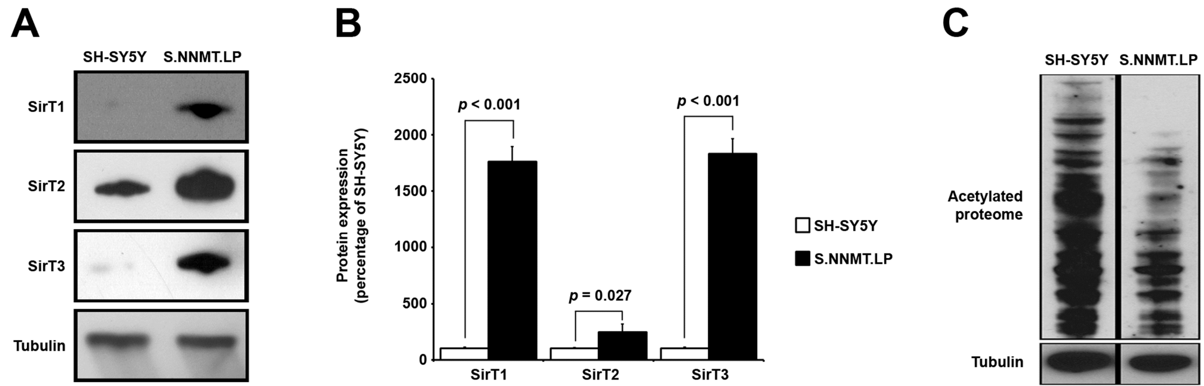
Fig. 4. Expression of NNMT-V5 in S.NNMT.LP did not alter mitochondrial number. As a measure of mitochondrial number, the expression of the Complex I subunit NDUFA6 was measured and compared to the expression of the cytosolic protein β-tubulin. **(A) Western blotting.** NDUFA6 was detected using Western blotting. **(B) Quantitative analysis.** Bands were quantified and analysed using densitometry, normalised for β-tubulin expression and expressed as percentage expression compared to SH-SY5Y cells. Statistical analysis comprised *t*-test with Welch correction (n = 3).

ACCEPTED MANUSCRIPT



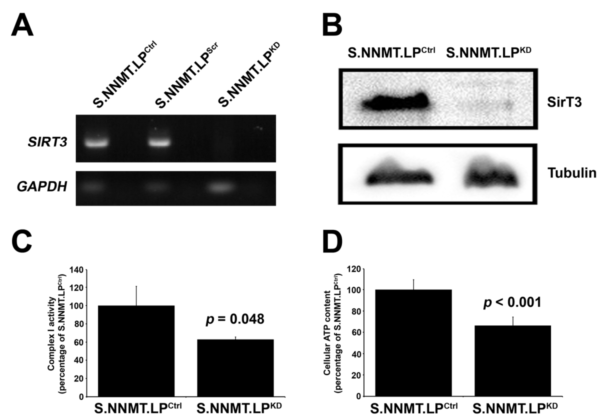
ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT



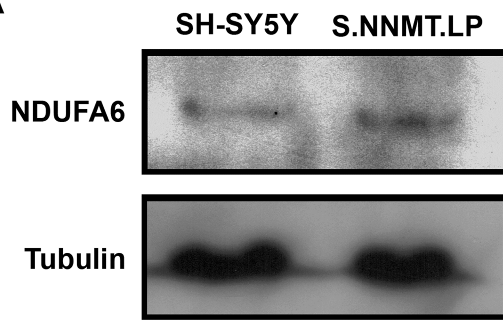
ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

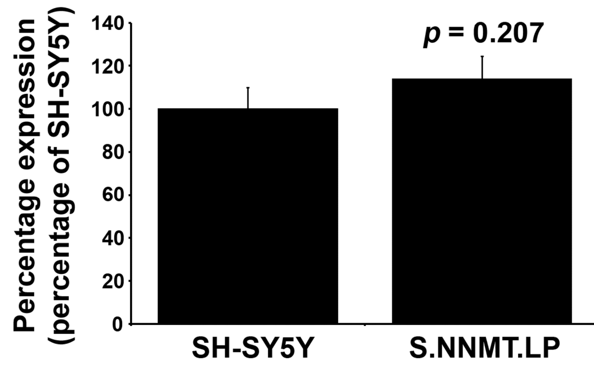


ACCEPTED MANUSCRIPT

A



B



ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

- Nicotinamide *N*-methyltransferase induced Complex I activity
- Nicotinamide *N*-methyltransferase induced expression and activity of sirtuins 1 – 3
- Sirtuin-3 mediated effects of nicotinamide *N*-methyltransferase upon complex 1
- Nicotinamide *N*-methyltransferase has a central role in mitochondrial bioenergetics

ACCEPTED MANUSCRIPT