The expression of nicotinamide N-methyltransferase increases ATP synthesis and protects SH-SY5Y neuroblastoma cells against the toxicity of Complex I inhibitors

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INTRODUCTION

The correct functioning of the mitochondria is essential for the production of ATP from NADH via oxidative phosphorylation within the inner mitochondrial membrane. CxI (Complex I) deficiency has been implicated in the pathogenesis of PD (Parkinson’s disease) [1]. Nicotinamide, a form of vitamin B3, is the precursor for NAD+ synthesis [2], and plays a crucial role in cell survival and longevity [3]. NNMT (nicotinamide N-methyltransferase, E.C. 2.1.1.1) catalyses the N-methylation of nicotinamide to 1-methylnicotinamide. NNMT expression is significantly elevated in a number of cancers, and we have previously demonstrated that NNMT expression is significantly increased in the brains of patients who have died of Parkinson’s disease. To investigate the cellular effects of NNMT overexpression, we overexpressed NNMT in the SH-SY5Y cell line, a tumour-derived human dopaminergic neuroblastoma cell line with no endogenous expression of NNMT. NNMT expression significantly decreased SH-SY5Y cell death, which correlated with increased intracellular ATP content, ATP/ADP ratio and Complex I activity, and a reduction in the degradation of the NDUFS3 [NADH dehydrogenase (ubiquinone) iron–sulfur protein 3] subunit of Complex I. These effects were replicated by incubation of SH-SY5Y cells with 1-methylnicotinamide, suggesting that 1-methylnicotinamide mediates the cellular effects of NNMT. Both NNMT expression and 1-methylnicotinamide protected SH-SY5Y cells from the toxicity of the Complex I inhibitors MPP+ (1-methyl-4-phenylpyridinium ion) and rotenone by reversing their effects upon ATP synthesis, the ATP/ADP ratio, Complex I activity and the NDUFS3 subunit. The results of the present study raise the possibility that the increase in NNMT expression that we observed in vivo may be a stress response of the cell to the underlying pathogenic process. Furthermore, the results of the present study also raise the possibility of using inhibitors of NNMT for the treatment of cancer.

Key words: cancer, cytoprotection, energy metabolism, NADH dehydrogenase (ubiquinone) iron–sulfur protein 3 (NDUFS3), N-methylation, Parkinson’s disease.
MATERIALS AND METHODS

Unless otherwise stated, all materials were obtained from Sigma and were of the highest purity available.

NNMT plasmid construction and stable mammalian expression

SH-SY5Y human neuroblastoma cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, U.K.) at passage number 16 and cultured as described previously [21]. All experiments were performed using undifferentiated cells of passage number 18–20. The NNMT plasmid construct (pNNMT) was produced by ligating a cDNA encoding NNMT C-terminally fused to a V5 fusion tag into the pcDNA3.1™-TOPO® mammalian expression vector (Invitrogen). The primer pair used was the following: forward primer, 5′-CACCATGGGAATCAGCAGCCTCT-3′ and reverse primer, 5′-TCCTCTTCCCAACAGGGGTCTGCTCAGCTTC-3′. pNNMT plasmid was transfected in SH-SY5Y cells using PolyPlus jetPEI transfection reagent (AutoBiolecular). Stable transfectants were selected using Geneticin (250 μg/ml; Invitrogen), and NNMT-V5 mRNA expression was confirmed using RT (reverse transcription)–PCR using the following primer pair: forward primer, 5′-TGCCGCCCACTATCTCTACG-3′, and reverse primer, 5′-CCCTCTCCAGCAGCCCTCT-3′. β-Actin was used as a loading control, detected using the following primer pair: forward primer, 5′-GGCATCTCACCCTGAAGTA-3′, and reverse primer 5′-GGGTGTTGAAGGCTTCATCA-3′. Recombinant NNMT–V5 expression was confirmed using SDS/PAGE and Western blotting [6,8] using a combination of mouse anti-V5 antibody (1:2000 dilution; Abcam) and anti-mouse IgG conjugated to HRP (horseradish peroxidase) (1:2000 dilution). Protein loading was normalized using mouse anti-β-actin (1:1000 dilution; AutoBiolecular). Detected bands were visualized using ECL (enhanced chemiluminescence) (GE Healthcare). Enzyme activity of NNMT–V5 was confirmed using a radioassay described previously [6]. NNMT activity was calculated and expressed as the specific activity (nmol of MeN produced/hour per mg of protein) ± S.D. SH-SY5Y cells expressing NNMT were named S.NNMT:LP and were used for all subsequent analyses.

Cell death assays

Cell death was measured using the Cytotoxicity Detection Kit® [using LDH (lactate dehydrogenase)] (Roche Diagnostics) [21]. Cell death was calculated as the percentage cell death ± S.D. Cellular apoptosis was measured by analysing caspase activation using the fluorescence-based Caspase-Glo® 3/7 assay kit (Promega) [21]; the assay relies upon the cleavage of a fluorescence resonance energy transfer–quenched substrate by caspases 3 and 7 upon activation of apoptosis. Results were expressed as the percentage change in apoptosis compared with untreated cells ± S.D.

Real-time qPCR (quantitative PCR) analysis of NDUFS3 expression

mRNA expression was quantified using real-time qPCR using the UPL (Universal Probe Library) as internal quantification probes (Roche). NDUFS3 expression was detected using primers and the appropriate UPL (Roche) as outlined in Table 1. qPCR was performed using an ABI Prism 7000 thermal cycler (Applied Biosystems) using FastStart Universal Probe Master Mix (Roche) and the following cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The calculated by dividing the LDH released from cells cultured for 24 h by the baseline LDH value and was expressed as proliferation ratio ± S.D.

Intracellular indices of energy production

ATP and ADP concentrations were measured by bioluminescence using the luciferin/luciferase reaction-based ApoGlow® ATP/ADP assay kit (Lanza), according to the manufacturer’s instructions. Results were expressed as pmol/mg of protein ± S.D. and as the ATP/ADP ratio ± S.D.

Pyridine nucleotide concentrations were measured using the method described by Queval and Noctor [22] with the use of 0.2 M HCl supplemented with 1% (v/v) Tween 20 instead of 0.2 mM HCl for the measurement of NADH, and 0.2 M NaOH supplemented with 1% (v/v) Tween 20 instead of NaOH for the measurement of NAD+. Absorbance was measured at 595 nm every 30 s for 10 min at room temperature (22°C). Results were plotted and calculated as ΔA0−t/Δt and converted into pmol of appropriate pyridine nucleotide. Samples were corrected for volume and finally expressed as both μ M pyridine nucleotide ± S.D. and the NAD+/NADH ratio ± S.D.

Mitochondrial membrane potential and oxygen consumption

Mitochondrial membrane potential was measured using the fluorescence-based JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical Company) in a 96-well plate format, according to the manufacturer’s instructions. Results were calculated and expressed as the red/green fluorescence ratio ± S.D. Oxygen consumption in unstimulated cells and in the presence of oligomycin and 2,4-dinitrophenol was measured using a Clarke oxygen electrode using the method described by Kwok et al. [23]. Results were calculated and expressed as the rate of oxygen consumption/min per mg of protein ± S.D.

Cxi activity analysis

Mitochondria were isolated from cells lysed in an ice-cold homogenization buffer comprising 0.25 mM sucrose, 10 mM Tris/HCl and 1 mM sodium EDTA (pH 7.4). Lysed membranes and nuclei were pelleted by centrifugation at 1500 g for 12 min at 4°C. Mitochondria were isolated from the supernatant by centrifugation at 14000 g for 12 min at 6°C and resuspended in homogenization buffer. Cxi was measured in cell samples using the method described by Janssen et al. [24] in a 96-well plate format. Rotenone-sensitive Cxi activity was calculated and normalized for mitochondrial number using CS (citrate synthase) activity [24]. CS activity was calculated and used to normalize Cxi activity, which is henceforth referred to simply as Cxi activity. Cxi activity was expressed as a percentage of Cxi activity observed in untreated SH-SY5Y cells ± S.D.
Table 1 Primers and universal probes used for the analysis of mRNA expression using real-time qPCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>GenBank® accession number</th>
<th>Forward/reverse</th>
<th>Primer sequence</th>
<th>UPL number</th>
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<td>NDUFS3</td>
<td>NM_004551.2</td>
<td>Forward</td>
<td>5'-ACTTTCCTCTATCTGGCTATGTTGA-3'</td>
<td>53</td>
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<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5'-GAACTTCTTGGCAGACTCC-3'</td>
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<tr>
<td>β-Actin</td>
<td>NM_001101.3</td>
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<td></td>
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<td>Reverse</td>
<td>5'-GGCCATACGCCAGACAC-3'</td>
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<tr>
<td>GAPDH</td>
<td>NM_002046.3</td>
<td>Forward</td>
<td>5'-GCCCAGAATCAGCAATCC-3'</td>
<td>60</td>
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<tr>
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<td></td>
<td>Reverse</td>
<td>5'-AGCCAGACAACTCAGACAC-3'</td>
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Figure 1 The stable expression of NNMT significantly decreased SH-SY5Y cell death

NNMT was directionally inserted into the pcDNA3.1-TOPO® mammalian-only expression vector and stably expressed in SH-SY5Y human neuroblastoma cells, producing the cell line subsequently labelled S.NNMT.LP. (A) Top panel: detection of recombinant NNMT–V5. NNMT–V5 was detected by Western blotting using a combination of mouse anti-V5 antibody (1:2000 dilution) and anti-mouse IgG (HRP conjugated; 1:2000 dilution). Equal protein loading was demonstrated using a combination of mouse anti-β-actin antibody (1:1000 dilution) and anti-mouse IgG (HRP conjugated; 1:2000 dilution). Homogenate samples prepared from cells were loaded as indicated on the Figure. The expression of β-actin and NNMT are as marked. Bottom panel: detection of NNMT mRNA. NNMT mRNA was detected using RT–PCR using gene-specific primers as described in the text. Equal mRNA loading was demonstrated using β-actin. cDNA samples were loaded as indicated on the Figure. The expression of β-actin and NNMT are as marked. 600, 600 bp marker. (B) Top panel: detection of NNMT enzyme activity. NNMT enzyme activity was detected using a radioimmunoassay as described previously [6]. Bottom panel: effect of NNMT expression upon cell death. Cell death was measured using LDH release.

Western blot analysis of NDUFS3 protein expression

Protein samples were prepared, the protein concentration was quantified and samples were subjected to Western blotting. NDUFS3 was detected using a combination of mouse anti-NDUFS3 (1:2000 dilution; Invitrogen) at 4°C and rabbit anti-mouse IgG (conjugated to HRP, 1:5000 dilution). Membranes were stripped using Restore Western Stripping Reagent (Fisher Scientific) and re-probed using anti-β-actin.

Experimental conditions

For all experiments, undifferentiated SH-SY5Y cells were compared with undifferentiated SH-SY5Y cells incubated with various concentrations of MeN, MPP+ (1-methyl-4-phenylpyridinium ion), rotenone and S.NNMT.LP cells, as indicated in the Figure legends, for 24 h at 37°C. The concentrations of MeN investigated are within the range used in other in vitro studies [17,18,25,26] and are below the...
MeN reduced SH-SY5Y cell death

Incubation of SH-SY5Y cells with increasing concentrations of MeN reduced cell death (Figure 2A, \( n = 6 \) for all incubations). In order to discount the possibility that MeN may be interfering with the LDH assay, we also measured cellular apoptosis (Figure 2B). Cellular apoptosis also significantly decreased with increasing MeN concentration (\( n = 6 \) for all incubations).

It was possible that the apparent decrease in cell death observed may be due to increased cell proliferation. To investigate this, we compared the proliferation ratio of SH-SY5Y cells with both S.NNMT.LP cells and SH-SY5Y cells incubated with 1 mM MeN. The proliferation ratios for SH-SY5Y and S.NNMT.LP cells were not significantly different (1.61 ± 0.17, \( P = 0.1446, n = 6 \)). Incubation with 1 mM MeN also did not alter the proliferation ratio, being 1.53 ± 0.016, 1.47 ± 0.007, 1.45 ± 0.018 and 1.51 ± 0.069 for 0.1, 0.25, 0.5 and 1 mM MeN respectively (\( n = 6 \) for all incubations).

Both NNMT expression and incubation with 1 mM MeN increased ATP production, the ATP/ADP ratio and CxI activity, and reduced the NAD+/NADH ratio

In S.NNMT.LP cells the ATP/ADP ratio was approximately 4-fold higher compared with that in SH-SY5Y cells (Figure 3A, \( n = 6 \)), which was due to a significant increase in ATP concentration (Figure 3B) and a significant decrease in ADP concentration (Figure 3C). Likewise, the ATP/ADP ratio in SH-SY5Y cells incubated with 1 mM MeN also significantly increased compared with untreated SH-SY5Y cells (Figure 3A, \( n = 6 \)), as was the intracellular ATP concentration (Figure 3B), although ADP concentration was not significantly different (Figure 3C). There was a corresponding 4-fold increase in CxI activity in S.NNMT.LP cells compared with that observed in SH-SY5Y cells, along with a significant increase observed in SH-SY5Y cells incubated with 1 mM MeN (Figure 3D, \( n = 6 \)). The mitochondrial membrane potential did not significantly alter in either incubation condition, although incubation of SH-SY5Y with 1 mM 2,4-dinitrophenol significantly reduced the mitochondrial membrane potential (Figure 3G, \( n = 3 \)).

It is possible that these changes may have arisen from alterations in NADH availability. To assess this, we measured pyridine nucleotide concentrations and the NAD+/NADH ratio. The NAD+ concentration in S.NNMT.LP cells was significantly reduced compared with SH-SY5Y cells (Figure 4A, \( n = 4 \)), with a smaller, yet still significant, reduction in the NADH concentration in S.NNMT.LP cells (Figure 4B, \( n = 4 \)). Consequently, the NAD+/NADH ratio was significantly reduced in S.NNMT.LP cells compared with SH-SY5Y cells (Figure 4C). Incubating SH-SY5Y cells with 1 mM MeN had no effect upon the NAD+ concentration compared with untreated SH-SY5Y cells (Figure 4A, \( n = 4 \)), although there was a significant increase in the NADH concentration (Figure 4B, \( n = 4 \)). Consequently, the NAD+/NADH ratio was lower in SH-SY5Y cells incubated with 1 mM MeN compared with that in untreated cells (Figure 4C, \( n = 4 \)).

**NNMT expression and incubation with 1 mM MeN increased the expression of NDUFS3-immunoreactive proteins**

We next investigated whether the effects we had observed were due to alterations in CxI subunit expression directly. In particular, we chose NDUFS3 because of the reported interaction...
NNMT expression is cytoprotective via Complex I

Figure 3  NNMT expression and MeN increased the intracellular ATP/ADP ratio and CxI activity

(A) ATP/ADP ratio. SH-SY5Y, S.NNMT.LP and SH-SY5Y cells incubated with 1 mM MeN for 24 h were analysed for their intracellular ATP/ADP ratio. (B) ATP concentration. (C) ADP concentration. (D) Complex I activity. SH-SY5Y, S.NNMT.LP and SH-SY5Y cells incubated with 1 mM MeN for 24 h were analysed for their Complex I activity using a 96-well plate-based assay. (E) Oxygen consumption. Oxygen consumption was measured in whole unstimulated cells using a Clarke electrode. Oxygen consumption in SH-SY5Y cells was compared with SH-SY5Y cells incubated with 1 mM MeN and S.NNMT.LP cells. (F) Typical oxygen consumption traces. Oxygen consumption was measured in resting cells and in the presence of 5 μg/ml oligomycin and 1 mM 2,4-dinitrophenol. (G) Mitochondrial membrane potential. Mitochondrial membrane potential was measured using the JC-1 red/green ratio. Statistical analysis comprised the comparison of untreated SH-SY5Y with both SH-SY5Y incubated with 1 mM MeN and untreated S.NNMT.LP. n/s, not significant; *P < 0.05; **P < 0.01; ***P < 0.001. In (F and G) 2,4-DNP is 2,4-dinitrophenol.

between MeN and NDUFS3 [15,16]. NDUFS3 immunoreactivity was observed in three proteins of 39 kDa, 45 kDa and 50 kDa (Figure 5A). The intensity of the 39 kDa band was significantly higher than both the 45 kDa and 50 kDa bands. The expression of all three bands was increased in S.NNMT.LP cells compared with SH-SY5Y cells. The expression of all three bands also increased with increasing MeN concentration (Figure 5B), with peak expression observed at 0.5 mM MeN (Figure 5C, top panel, n = 4). The increase in expression of the 45 kDa and 50 kDa bands was much more marked, with the maximal increase for both also observed after incubation with 0.5 mM MeN (Figure 5C, bottom panel, n = 4 for both).

NDUFS3-immunoreactive proteins are subcellular localization-specific

Having shown using Western blotting that NDUFS3 immunoreactivity was present in three bands, we decided to determine whether these immunoreactive proteins were subcellular localization-specific using isolated mitochondria and post-mitochondrial supernatant (cytosol) (Figure 6). As previously observed, all three bands were present in SH-SY5Y cell lysate, with the 39 kDa band present solely in mitochondrial lysate, and the 45 and 50 kDa bands present solely in the post-mitochondrial supernatant. No other immunoreactive bands were
observed in any of the subcellular fractions. Omission of primary antibody resulted in the detection of no immunoreactive bands.

NNMT expression and incubation with 1 mM MeN did not alter NDUF3 mRNA expression

To determine whether these increases in NDUF3 protein expression were due to increased protein synthesis arising from increased mRNA expression, we measured NDUF3 mRNA expression in SH-SY5Y cells, S.NNMT.LP cells and SH-SY5Y cells incubated with 1 mM MeN using qPCR. NDUF3 mRNA expression was not significantly different in S.NNMT.LP cells compared with that observed in SH-SY5Y cells (100 ± 2.3% compared with 94.1 ± 6.13%, \( P = 0.7830, n = 3 \)). Likewise, NDUF3 expression was not significantly altered in SH-SY5Y cells following incubation with 1 mM MeN (100 ± 2.8% compared with 79.2 ± 7.93%, \( P = 0.3302, n = 3 \)).

Inhibiting proteolysis increased NDUF3 protein expression

As the increases in NDUF3 expression that we observed did not arise from an increase in mRNA expression, it is possible that this may have arisen from increased protein production or decreased protein catabolism. To investigate this, we inhibited lysosomal protein degradation using leupeptin [27]. All three NDUF3-immunoreactive bands were observed in untreated SH-SY5Y cells, all of which increased significantly in expression with increasing leupeptin concentration (Figure 7A), with a maximal increase observed in cells incubated with 50 μg/ml leupeptin (Figure 7B, bottom panel, \( n = 4 \)).

NNMT expression and incubation with 1 mM MeN reduced the toxicity of the CxI inhibitors MPP+ and rotenone

Having demonstrated that NNMT expression increased cell viability which coincided with the induction of CxI activity, and that this effect appeared to be mediated via a reduction in NDUF3 degradation, we next decided to investigate whether NNMT may protect against the toxicity of the CxI inhibitors MPP+ and rotenone (Figure 8). To account for the lower cell death observed in untreated S.NNMT.LP cells compared with untreated SH-SY5Y (Figure 1B), the comparison of the toxicity of 0.5 mM MPP+ and 0.4 μM rotenone towards SH-SY5Y and S.NNMT.LP cells was expressed as the percentage increase in cell death compared with untreated cells. MPP+ was significantly toxic towards SH-SY5Y cells, but not towards S.NNMT.LP cells. Rotenone was toxic towards both SH-SY5Y cells and S.NNMT.LP cells; however, rotenone was significantly more toxic towards SH-SY5Y cells than S.NNMT.LP cells (\( n = 6 \) for all incubations).

Having demonstrated that NNMT expression protected against the toxicity of MPP+ and rotenone, it was decided to investigate whether MeN replicated these effects. Co-incubation of 0.5 mM MPP+ and 0.4 μM rotenone with 1 mM MeN significantly reduced the toxicity of both CxI inhibitors towards SH-SY5Y cells, both of which were not significantly different to the degree of cell death observed in untreated SH-SY5Y cells (Figure 9A, \( n = 6 \) for all incubations). We next investigated whether the cytoprotective effects of MeN were mediated by changes in ATP and ADP concentrations, the ATP/ADP ratio, CxI activity and NDUF3 expression. Both 0.5 mM MPP+ and 0.4 μM rotenone reduced ATP concentration and increased ADP concentration which resulted in a decrease in the ATP/ADP ratio compared with that observed in untreated cells, which was reversed by co-incubation with 1 mM MeN (\( n = 6 \) for all incubations). Likewise, MPP+ and rotenone decreased CxI in SH-SY5Y cells, which co-incubation with 1 mM MeN reversed back to levels which were not significantly different to those observed in untreated SH-SY5Y cells (Figure 9E, \( n = 6 \) for both). Cellular oxygen consumption decreased significantly in response to MPP+ and rotenone, which was reversed by co-incubation with 1 mM MeN (Figure 9F, \( n = 4 \) for all incubations). Oxyhemoglobin under all conditions decreased and increased in response to oligomycin and 2,4-dinitrophenol respectively (Figure 9G). The mitochondrial membrane potential also altered in a similar manner to that observed for oxygen consumption (Figure 9H, \( n = 3 \)). Both the 45 kDa and 50 kDa NDUF3-immunoreactive proteins were absent in SH-SY5Y cells incubated with MPP+ and rotenone, which was reversed by co-incubation with 1 mM MeN (Figure 9I).

DISCUSSION

NNMT expression reduced cell death by increasing CxI activity

A number of studies have shown that NNMT is overexpressed in a variety of diseases [9–14]. None of these studies have addressed why, nor have they provided any mechanistic insights into why NNMT may have a role in disease progression. In the present study we demonstrate that NNMT expression is involved in maintaining cell viability by increasing CxI activity, and that this appears to be mediated via the protection of NDUF3 from degradation. Furthermore, we have shown that these effects arise due to the increased production of MeN, as incubation of cells with 1 mM MeN replicates all of the effects of NNMT upon...
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Finally, we demonstrate that NNMT and MeN are cytoprotective against the CxI inhibitors MPP+ and rotenone, and that this is mediated via the maintenance of CxI activity arising from the protection of NDUFS3 from inhibitor-mediated damage.

Our previous studies using post mortem brain tissue demonstrated that NNMT levels are significantly higher in PD compared with non-PD patients. This, in tandem with an inverse correlation between disease duration and protein expression level,
suggested a causative role for NNMT in the pathogenesis of PD [6,8]. This was supported by two studies which suggested that MeN is neurotoxic via the induction of free radicals similar in mechanism to that of paraquat, causing the destruction of NDUFS3 [15] and striatal dopamine depletion [16]. However, these studies had used MeN concentrations of 15–20 mM, which are significantly higher than those found endogenously within the brain [28] and which have been shown to be significantly toxic towards macrophages [26]. Other studies have shown that 1 mM is not toxic to primary CGCs, with toxicity only manifesting at concentrations of 100 mM [23]. Additionally, an intracellular MeN concentration of 500 μM did not result in the death of hippocampal neurons in the rat brain in vivo [28].

Our original hypothesis was that overexpression of NNMT leads to a reduction in available nicotinamide for NADH synthesis, and that such a reduction in NADH synthesis may impinge upon the ability of the cell to produce ATP, therefore leading to a reduction in cellular energy levels and thus reducing cell viability and/or reducing the ability of the cell to survive a subsequent cytotoxic challenge. One hurdle to this hypothesis was that NNMT overexpression is a common feature in many cancers, which do not demonstrate reduced cell viability but instead demonstrate increased proliferative capacity. To gain an insight into the potential consequences of NNMT expression that we have previously observed in patient brain samples [6,8], we ectopically expressed NNMT in a commonly used in vitro neuroblastoma cell line which would allow us to investigate the mechanism underlying the effects of NNMT upon the cell. What makes this cell line particularly useful is that it has no endogenous mechanism underlying the effects of NNMT upon the cell. What makes this cell line particularly useful is that it has no endogenous mechanism underlying the effects of NNMT upon the cell. It is also a tumour-derived cell line, therefore it is also relevant to studying the role of NNMT in cancer.

Expression of NNMT in the SH-SY5Y cell line significantly reduced cell death, which was replicated by incubation with MeN. The decreases in cell death that we observed correlated with increases in the ATP/ADP ratio and CxiI activity. Various ATP/ADP ratios have been quoted in the literature for in vitro cell systems. The ATP/ADP ratio that we observed is similar to those reported for SH-SY5Y cells and other in vitro human cell lines [29–31]. The increases in ATP concentration and ATP/ADP ratio coincided with similar increases in CxiI activity, therefore we concluded that it was likely that NNMT and MeN increased cell viability via increased CxiI activity, resulting in an increased ATP concentration. Increases in ATP levels have been shown to rescue neurons from toxicity in several dopaminergic model systems [32–34], and is likely to be responsible for the decrease in cell death that we observed.

The effects of NNMT expression and MeN are mediated via CxiI

Having identified that NNMT expression increased CxiI activity, the next question we addressed was the mechanism by which this occurred. Alterations in NAD+/NADH ratio could conceivably alter electron flow through the mitochondrial respiratory chain, therefore significant increases in NADH availability may increase ATP synthesis. However, as nicotinamide is a precursor for pyridine nucleotide synthesis, we expected NADH and NAD+ concentrations to be significantly lower in the S.NNMT.LP cell line than in the parental SH-SY5Y cells. As expected, we observed a decrease in the NAD+/NADH ratio which arose from a small decrease in NADH concentration and a very significant decrease in NAD+ concentration. Although global pyridine nucleotide synthesis is significantly reduced, as evidenced by the significantly lower NAD+ levels in S.NNMT.LP cells, the regeneration of NADH via the tricarboxylic acid cycle is not compromised, as NADH concentrations were not sufficiently reduced to have a negative impact upon ATP synthesis.

Another mechanism that we investigated was an interaction with the NDUFS3 subunit of CxiI. CxiI is a supercomplex of 45 subunits derived from both nuclear and mitochondrial DNA [35]. NDUFS3 is one of three nuclear DNA-encoded iron–sulfur protein subunits of CxiI, the incorporation of which occurs early in CxiI assembly by the sequential association of NDUFS3 with other subunit proteins [35,36]. We investigated NDUFS3 because studies using rat brain have shown that 15–20 mM MeN destroyed NDUFS3 via the production of free radicals in a mechanism similar to that of paraquat [15]. There are a number of problems with the abovementioned study. The concentrations of MeN used in the study were 10–100-fold greater than the 1 mM used in our present study, which the majority of studies investigating the cellular effects of MeN have also used [17,18,25,26]. Also, their analysis of CxiI subunit abundance was made using Coomassie Brilliant Blue staining of the mitochondrial fraction separated using SDS/PAGE, which, because of the number of proteins present in the sample and their inability to conclusively identify subunits without the use of specific antibodies, makes it difficult to identify the NDUFS3 subunit. Finally, the lack of a protein loading control such as β-actin makes it very difficult to quantify any changes that the authors observed. Using an antibody specific to NDUFS3, we observed that NDUFS3 immunoreactivity was present in a major protein of 39 kDa plus two other bands of 45 kDa and 50 kDa. It is likely that this 39 kDa protein is monomeric NDUFS3 and that the 45 kDa and 50 kDa proteins are NDUFS3 having either undergone post-translational modification or becoming tightly associated with other proteins as part of an assembly complex. One such candidate is prohibitin, a chaperonin shown to protect CxiI subunits before assembly [37], which binds directly to NDUFS3 [38]. The reason for the subcellular fraction specificity of these proteins is unclear, but it may be related to the localization of the NDUFS3 gene to the nuclear DNA, resulting in the production of a cytosolic protein which undergoes translocation to the mitochondria.

Our use of a specific anti-NDUFS3 antibody also showed that the abundance of all three of these proteins increased with increasing MeN concentration. Although we have not
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Figure 9 MeN reduced the toxicity of the CxI inhibitors MPP+ and rotenone towards SH-SY5Y cells

SH-SY5Y cells were incubated with 0.5 mM MPP+ and 0.4 μM rotenone both in the presence and absence of 1 mM MeN for 24 h. (A) Cell death. Cell death was measured using LDH release. (B) ATP/ADP ratio. The ATP/ADP ratio was measured using a bioluminescence-based 96-well plate assay. (C) ATP concentration. (D) ADP concentration. (E) Complex I activity. (F) Oxygen consumption. (G) Typical oxygen consumption traces. Oxygen consumption was measured in untreated SH-SY5Y cells (black continuous line) and in SH-SY5Y cells incubated with 1 mM MeN (dashed and double-dotted line), 0.5 mM MPP+ (long-dashed line), 0.5 mM MPP+ plus 1 mM MeN (dashed and single-dotted line), 0.4 μM rotenone (grey continuous line) and 0.2 μM rotenone plus 1 mM MeN (small-dashed line). (H) Mitochondrial membrane potential. Statistical analysis was used to compare (i) cells treated with 1 mM MeN, 0.5 mM MPP+ and 0.4 μM rotenone both in the presence and absence of 1 mM MeN with untreated cells (P values above error bars) and (ii) the effect of co-incubation with 1 mM MeN compared with that observed in the absence of MeN for each incubation condition (P values above linking bars). n/s, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; 2,4-DNP, 2,4-dinitrophenol. (I) NDUFS3 immunoreactivity. NDUFS3 was detected using Western blotting. 39, 39 kDa protein; 45, 45 kDa protein; 50, 50 kDa protein; 1, untreated SH-SY5Y cells; 2, 0.5 mM MPP+; 3, 0.5 mM MPP+ plus 1 mM MeN; 4, 0.4 μM rotenone; 5, 0.4 μM rotenone plus 1 mM MeN.

NNMT expression and MeN fully characterized the identity of each of the NDUFS3-immunoreactive proteins, it is likely that they have functional relevance upon CxI activity, as their increase in expression correlated with an increase in CxI activity and ATP synthesis. Furthermore, we were able to remove the 50 and 45 kDa proteins by incubating SH-SY5Y cells with MPP+ and rotenone, and subsequently reverse this by co-incubating with MeN. Most significantly, these changes correlated with alterations in ATP synthesis, the ATP/ADP ratio and the CxI ratio; in the absence of the 45 kDa and 50 kDa proteins, the ADP concentration increased, whereas ATP concentration, the ATP/ADP ratio and CxI decreased, which was reversed in the presence of both proteins. These changes suggest that these immunoreactive proteins are not an artefact of non-specific binding of the anti-NDUFS3 antibody, but represent true proteins which contain NDUFS3 whose expression is integral to the activity of CxI. Other reports have shown that NDUFS3 immunoreactivity is present in more than one protein [37–40]. Therefore it is likely that the increase in the abundance of these proteins has functional relevance for CxI activity. This increase in protein expression arising from NNMT expression and MeN did not result from increased mRNA synthesis, but instead arose from a decrease in NDUFS3 degradation. This is evidenced by similar changes in NDUFS3 immunoreactive protein abundance in the presence of the lysosomal protease inhibitor leupeptin. Therefore it is likely that NNMT increases CxI activity by reducing the degradation of the CxI subunit NDUFS3. As yet, it is unclear how NNMT and MeN reduce the degradation of NDUFS3. Possibilities include the direct inhibition of protein degradation itself, or the regulation of the expression of genes involved in protein transport or proteolysis. If the mechanism could be elucidated, it may serve as a possible target for drug development studies in PD.

NNMT expression and MeN protect against the toxicity of inhibitors of CxI

Having identified that NNMT expression increases the ATP/ADP ratio by maintaining CxI activity, mediated by a decrease in the degradation of NDUFS3, we wondered whether NNMT may protect against the toxicity of CxI inhibitors. When we exposed S.NNMT.LP cells to the CxI inhibitors MPP+ and rotenone, which were both significantly toxic towards SH-SY5Y cells, we
observed that MPP+ was not significantly toxic, and rotenone was significantly less toxic towards S.NNMT.LP cells. This demonstrates that the reduction in pyridine nucleotide synthesis did not leave S.NNMT.LP cells susceptible to further neurotoxic challenge. Further experiments using MeN showed that this neuroprotection occurred via the protection of NDUF3 from damage by MPP+ and rotenone, resulting in the maintenance of CxI activity and the ATP/ADP ratio. This suggests that MeN is a naturally occurring cytoprotective compound which, if replicated in vivo, raises the possibility of using MeN-based compounds as potential neuroprotective therapies for the treatment of PD.

Our in vitro data provide an insight into the potential cellular consequences of such an increase in NNMT expression in vivo and the mechanism by which it may occur. If our in vitro data were to be replicated in vivo, it raises the intriguing possibility that the significant increase in NNMT expression that we observed in PD patient brains [6,8] is not contributing to the pathogenic process of PD, but is instead a stress response of the cell. Although no study has been performed investigating MeN levels in PD patient brains, it is conceivable that the significant increase in NNMT expression observed may be an attempt to raise intracellular levels of MeN in order to increase CxI activity. Such a cytoprotective effect for NNMT is not without precedent. A recent study has demonstrated that the reduction in pyridine nucleotide synthesis observed in SH-SY5Y cells from the toxicity of MPP+ and rotenone by maintaining CxI activity.

AUTHOR CONTRIBUTION

Richard Parsons, Brian Austen and David Ramsden were involved in study conception and design. Richard Parsons, Stylios Aravind, Anusha Kadampeswaran, Emily Evans, Kanwaljeet Sandhu, Elizabeth Levy and Martin Thomas were involved in study implementation. Richard Parsons and David Ramsden analysed results and prepared the manuscript.

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