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NNT is a key regulator of adrenal redox homeostasis and steroidogenesis in male mice

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Abstract

Nicotinamide nucleotide transhydrogenase, NNT, is a ubiquitous protein of the inner mitochondrial membrane with a key role in mitochondrial redox balance. NNT produces high concentrations of NADPH for detoxification of reactive oxygen species by glutathione and thioredoxin pathways. In humans, NNT dysfunction leads to an adrenal-specific disorder, glucocorticoid deficiency. Certain substrains of C57BL/6 mice contain a spontaneously occurring inactivating Nnt mutation and display glucocorticoid deficiency along with glucose intolerance and reduced insulin secretion. To understand the underlying mechanism(s) behind the glucocorticoid deficiency, we performed comprehensive RNA-seq on adrenals from wild-type (C57BL/6N), mutant (C57BL/6J) and BAC transgenic mice overexpressing Nnt (C57BL/6J\(^{BA5}\)). The following results were obtained. Our data suggest that Nnt deletion (or overexpression) reduces adrenal steroidogenic output by decreasing the expression of crucial, mitochondrial antioxidant (Prdx3 and Txnrd2) and steroidogenic (Cyp11a1) enzymes. Pathway analysis also revealed upregulation of heat shock protein machinery and haemoglobins possibly in response to the oxidative stress initiated by NNT ablation. In conclusion, using transcriptomic profiling in adrenals from three mouse models, we showed that disturbances in adrenal redox homeostasis are mediated not only by under expression of NNT but also by its overexpression. Further, we demonstrated that both under expression or overexpression of NNT reduced corticosterone output implying a central role for it in the control of steroidogenesis. This is likely due to a reduction in the expression of a key steroidogenic enzyme, Cyp11a1, which mirrored the reduction in corticosterone output.

Background

Adrenal insufficiency is a rare, potentially fatal, endocrine disorder resulting from a failure of the adrenal cortex to respond to hormonal stimuli. In familial (or isolated) glucocorticoid deficiency, adrenal hormone output is preserved apart from a specific deficit of glucocorticoids. Normally, under the control of hypothalamic corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), the pituitary releases adrenocorticotropic hormone (ACTH), which acts on the adrenal via the ACTH receptor (otherwise known as MC2R) to produce glucocorticoids, mainly cortisol. This in turn acts on the hypothalamus and pituitary to suppress further production...
of ACTH in a negative feedback loop (Fig. 1A) (Keller-Wood & Dalman 1984). The human adult adrenal is characterised by three distinctive cortical zones surrounding the medulla; the zona glomerulosa (ZG) where mineralocorticoids are produced, the zona fasciculata (ZF), which synthesises glucocorticoids (mostly cortisol; in mice, the major glucocorticoid is corticosterone) and the zona reticularis (ZR) where androgen synthesis occurs (Vinson 2003). The first step of steroidogenesis occurs once cholesterol is transported from the outer to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (STAR) and is converted to pregnenolone by the cholesterol side chain cleavage enzyme (CYP11A1). To make cortisol, pregnenolone then undergoes a series of intermediate reactions catalysed by microsomal enzymes (CYP17A1, HSD3B2 and CYP21A2) before the final step of cortisol production, catalysed by 11beta-hydroxylase (CYP11B1) or aldosterone production, catalysed by CYP11B2, both occurring in mitochondria (Fig. 1B) (Miller & Auchus 2011). The activities of steroidogenic cytochrome P450 (CYP) enzymes are reliant upon electron-donating reduct partners; for mitochondrial (or type 1) enzymes, electrons are transferred from the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) by ferredoxin reductase (FDXR) and ferredoxin (FDX1), whereas the microsomal enzymes (Type 2) use P450 oxidoreductase (POR) as their reduct partner. Hence, the first and last steps of cortisol production occur in the mitochondria and require a constant supply of reductant NADPH. This NADPH is regenerated from NADP by a few pathways including the thioredoxin and glutathione pathways, which are ultimately enabled by NNT (Fig. 2).

Perturbations in this pathway cause a number of steroidogenic defects affecting adrenal and gonadal steroidogenesis. STAR mutations give rise to lipoid congenital adrenal hyperplasia (OMIM 201710), a severe syndrome of adrenal and gonadal insufficiency resulting in XY sex reversal; CYP11A1 defects give a similar clinical picture but without the lipid build up in steroidogenic tissues seen with STAR mutations. HSD3B2, CYP17A1, CYP21A2, CYP11B1 and POR mutations give rise to four variants of congenital adrenal hyperplasia (OMIM 201810, 202110, 201910, 202010, and 201750 respectively) and CYP11B2 mutations give rise to hypoaldosteronism (OMIM 203400) (Fig. 1B) (Miller & Auchus 2011). No mutations have yet been described in humans in FDXR/FDX1 – perhaps due to embryonic lethality. Partial loss-of-function changes in STAR and CYP11A1 can present with a less severe phenotype akin to our disease of interest, familial or isolated glucocorticoid deficiency (FGD) (Baker et al. 2006, Metherell et al. 2009, Rubtsov et al. 2009, Sahakitrungruang et al. 2010, 2011, Parajes et al. 2011). In FGD, the two most common gene defects are mutations in the melanocortin 2 receptor and its accessory protein (MC2R and MRAP), but recently, we have described defects in NADPH supply to and/or antioxidant defence in mitochondria, with defects in two
genes, NNT and thioredoxin reductase 2 (TXNRD2), giving disorders of adrenal insufficiency primarily compromising glucocorticoid production (Meimaridou et al. 2012, 2013, Prasad et al. 2014). MC2R and MRAP are adrenal zone and ACTH pathway specific, so it is unsurprising that they give rise to isolated glucocorticoid deficiency, whereas NNT and TXNRD2 are ubiquitously expressed.

NNT is the major mitochondrial enzymatic source of NADPH contributing 45% of the total NADPH supply (Nickel et al. 2015). It exists as a dimer and spans the inner mitochondrial membrane modulating H+ movement and supplying the high concentrations of NADPH required for the detoxification of ROS by glutathione and thioredoxin pathways (Fig. 2). Even though the gene is ubiquitously expressed, the organ specific physiological roles of NNT are only gradually being revealed by the study of a C57BL/6J mouse substrain that has a spontaneous mutation in Nnt (an in-frame 5-exon deletion), resulting in the truncation of the message and absence of the protein (Nickel et al. 2015). The first consequence of this murine Nnt deletion, described by Toye and coworkers in 2005, was glucose intolerance and reduced insulin secretion (Toye et al. 2005). Subsequent to the finding of human mutations causing FGD, we showed that 3-month-old mice had 50% lower basal and stimulated levels of corticosterone than their wild-type counterparts. Histological examination of their adrenals revealed a slightly disorganized ZF with higher levels of apoptosis than the wild-type C57BL/6N strain (Meimaridou et al. 2012). More recently, it was reported that liver mitochondria from C57BL/6J mice have major redox impairments resulting in an inability to maintain NADP and glutathione in their reduced states (Ronchi et al. 2013).

Previously, we have shown that H295R cells where Nnt has been stably knocked down undergo oxidative stress as demonstrated by low glutathione levels, and increased mitochondrial superoxide production (Meimaridou et al. 2012). Similar defects in energy metabolism due to Nnt ablation have also been demonstrated in other mouse tissues (heart, liver, pancreas) emphasising the importance of NNT for cellular bioenergetics (Sauer et al. 2004, Ronchi et al. 2013, Nickel et al. 2015). However, the mechanism by which loss of Nnt causes the adrenal-specific pathology we observe is unclear.

Here, we aim to investigate the effect of NNT loss and overexpression in the adrenal cortex by performing RNA-seq on adrenals from mice, which are wild-type (C57BL/6N, Nnt+/+), null (C57BL/6J, Nnt−/−) or 2-fold overexpressors (BAC transgenic, NntBAC) of Nnt (Freeman et al. 2006).

Materials and methods

Mouse strains

All mice were bred, housed and culled at MRC Harwell and therefore the husbandry was identical for all 3 substrains. The mouse strains used were C57BL/6NHisd originally

![Figure 2](Image)

Detoxification of free radicals in the mitochondria. NNT encodes a protein, integral to the inner mitochondrial membrane, which under normal physiological conditions uses energy from the mitochondrial proton gradient to generate high concentrations of NADPH. This is required for many processes in the cell including the supply of reductive power to a network of antioxidant enzymes, specifically the glutathione (GSH/GSSG) and thioredoxin (Trx(SH)/TrxS2) systems, to allow the detoxification of H2O2. Manganese superoxide dismutase (MnSOD) converts O2− into H2O2 and protects ROS-sensitive proteins from oxidative damage. H2O2 is then removed by glutathione peroxidases (e.g. GPX1) or peroxiredoxins (e.g. PRDX3) using GSH and Trx(SH), as co-factors. GSH and Trx(SH)2 can be regenerated by glutathione reductase (GR) and thioredoxin reductase-2 (TXNRD2), respectively, using the reducing power from NADPH. Without NNT, the production of NADPH is compromised, causing the mitochondria to become more sensitive to oxidative stress. Enzymes underlined in red are affected by one or more mutations in FGD patients.
from Harlan (Harlan Laboratories UK), which is wild type for Nnt (Nnt+/+), C57BL/6J originally from Charles River (Charles River UK), which has an in-frame deletion of 5 Nnt exons (Nnt−/−) and C57BL/6J mice carrying a BAC transgene to restore murine Nnt (NntRAC; Freeman et al. 2006), which we show are 2-fold overexpressors. For RNA-seq, 18-month-old male mice of the three different substrains were utilised, 5 mice per group. Mice were culled with an overdose of Euthatal (to allow for the collection of blood) and tissues were then removed quickly and either fixed or flash frozen in liquid nitrogen. All mice were culled between 10:00 and 11:30h and adrenals were removed. The animal protocols used in this study were approved by United Kingdom Home Office.

Genotyping

Genomic DNA was extracted from the mouse tail tissue using a Qiagen DNeasy tissue kit. Mice were genotyped for Nnt status using previously published primers (Huang et al. 2006).

Mouse histology

Mouse adrenals from Nnt+/+, Nnt−/− and NntRAC were fixed in 4% paraformaldehyde (Sigma) and embedded in paraffin. Sections were obtained using a microtome (Microm HM 325, Thermo Fisher) at 6-μm thickness, and hematoxylin & eosin (H&E) staining was performed using standard procedures (Guasti et al. 2011).

To assess changes in lipid content between the three mice strains, we performed Oil Red O staining as described previously (www.ihcworld.com/_protocols/special_stains/oil_red_o.htm). Briefly sections of fresh frozen adrenal tissues were obtained at 5 μm thickness and fixed in ice cold 10% formalin for 5 min. Sections were air dried and placed in absolute propylene glycol for 5 min to avoid carrying water into Oil Red O. Sections were then stained with pre-warmed Oil Red O solution for 8–10 min at 60°C and then washed twice with distilled water. Images were acquired using a Leica DMR microscope (Leica), and digital images were captured using a Leica DC200 camera (Leica) and DCViewer software (Leica).

Steroid profile

Serum steroids were quantified using liquid chromatography–tandem mass spectrometry (LC–MS/MS) as previously described (O’Reilly et al. 2014). Steroids were extracted from 200 μL of serum (after addition of internal standard) using 1 mL tert-butyl methyl ether (MTBE). After freezing at −20°C for 1 h, the MTBE layer was transferred into a 96-well plate and evaporated under nitrogen at 55°C. Samples were reconstituted in 125 μL of a 50:50 solution of methanol (Sigma) and H2O (Sigma). Steroids were analysed on a Waters Xevo with Acquity uPLC, steroids were eluted from a HSS T3 1.8μm, 1.2×50 mm column with a methanol/water 0.1% formic acid gradient system. Two mass transitions were used to identify and quantify each steroid (corticosterone: 347.2>121.2 and 347.2>97; deoxycorticosterone: 331>97 and 331>109).

Generation of stable NNT knockdown (KD) and scrambled (SCR) H295R cell lines

Lentiviral plasmids (RHS4430-98851990; RHS4430-98913600; RHS4430-98524425; RHS4430-10103169 RHS4430-101025114) were obtained from OpenBiosystems in a pGIPZ backbone and contained shRNA specific for human NNT (NM 012343) under the control of the CMV promoter, plus the puromycin resistance and green fluorescence protein (GFP) genes. HEK293T cells (packaging cells) were transiently transfected with the shRNA plasmids, two days after transfection virus containing media was collected, filtered using a 0.22 μm filter and used to transduce H295R cells. Four days after infection GFP-positive cells were selected in 4μg/mL puromycin. Transduction efficiency was determined by fluorescence microscopy. A scrambled (control) cell line was generated in a similar fashion using a non-specific shRNA.

NADP/NADPH assay

To measure total and reduced nicotinamide adenine dinucleotide phosphates (NADP+ and NADPH respectively) stably transfected H295R cells were plated onto white-walled and white bottomed 96-well culture dishes (Corning Costar). After 24h, NADP+ and NADPH were measured using NADP/NADPH-Glo assay (Promega), a luminescence-based system and according to the manufacturer’s protocol. Luminescence was recorded after 15 min using Omega Luminometer (BMGLabTech) and with integration time of 0.5 s.

Oxygen consumption rate-XF extracellular flux analyser

Scrambled (SCR) and stable knockdown H295R cells (NNT-KD) were cultured on Seahorse XF-96 microplates
and allowed to grow overnight. On the day of metabolic flux analysis, cells were changed to unbuffered DMEM (DMEM base medium supplemented with 10 mM glucose, 1 mM sodium pyruvate, 2 mM l-glutamine, pH 7.4) and incubated at 37°C in a non-CO₂ incubator for 1 h. All medium and injection reagents were adjusted to pH 7.4 on the day of assay. Baseline measurements of oxygen consumption rate (OCAR, measured by oxygen concentration change) and extracellular acidification rate (ECAR, measured by pH change) were taken before sequential injection of treatments/inhibitors: oligomycin (ATP synthase inhibitor, 4 µM), FCCP (mitochondrial respiration uncoupler, 1 µM) and rotenone (Complex I inhibitor, 1 µM).

**RNA-seq**

RNA from mouse adrenal tissues (Nnt+/+, Nnt−/−, NntBAC) was extracted using the RNeasy Mini kit (Qiagen). Once RNA quality and concentration were tested, samples were processed by Oxford Gene Technology (www.ogt.co.uk). Enrichment and library preparation were performed using Illumina TrueSeq RNA Sample Prep Kit, v2; total RNA was captured with oligo-dT-coated magnetic beads. The mRNA was fragmented and then first-strand cDNA synthesis was initiated from random primers, followed by second-strand synthesis. After end-repair, phosphorylation and A-tailing, adapter ligation and PCR amplification were performed to prepare the library for sequencing. Paired-end sequencing was performed over 100 cycles on the Illumina HiSeq2000 platform using TruSeq v3 chemistry. Fastq files were generated from the sequencing platform via the manufacturer's proprietary software. For mapping and alignment, reads were processed through Tuxedo suite (Trapnell et al. 2013). Reads were mapped to their location on the appropriate Illumina iGenomes build using Bowtie, and splice junctions were identified using Tophat. Cufflinks was used to perform transcript assembly, abundance estimation and differential expression and regulation for the samples. RNA-Seq alignment metrics were generated using Picard.

**Lipid peroxidation assay**

Lipid peroxidation in mouse adrenal lysates was assessed by using a lipid peroxidation assay kit (Abcam) based on the detection of malondialdehyde (MDA) in the samples. Adrenal tissues from Nnt+/+, Nnt−/− and NntBAC mice were excised and homogenised in MDA lysis buffer provided in the kit. Lysates were then centrifuged at 13,000g for 10 min and the supernatant was collected for lipid peroxidation measurements. Samples were incubated with thiobarbituric acid (TBA), which interacts with MDA present in the samples to generate MDA-TBA adducts. These adducts were quantified colorimetrically at 532 nm.

**Immunoblotting analysis**

Immunoblotting was used to assess protein expression. Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (SIGMA) and then left on ice for 30 min. Samples were centrifuged for 15 min at 17,000g. Supernatant was collected and an equal volume of Laemmli buffer was added. Samples were heated at 95–100°C for 5 min and then loaded on 4–12% SDS gels. Protein separation was performed by using the Invitrogen electrophoresis system. Proteins were then transferred to nitrocellulose membrane (Sigma Aldrich) using semi-dry transfer blot (Biorad) at 15V for 1h. Membranes were probed with one of mouse anti-Nnt (1:1000; SIGMA, HPA004829), mouse anti-actin (1:5000; SIGMA, A5441), rabbit anti-TXNRD2 (1:1000;SIGMA, SAB2702064), rabbit anti-PRDX3 (1:500; ProteinTech, 55087-1-AP), rabbit anti-GPX1 (1:500; Abcam ab108429), mouse anti-STAR (1:1000; Abcam, ab58013), rabbit anti-CYP11A1 (1:1000; Cell Signalling, 14217), rabbit anti-HSD3B2 (1:500; Aviva Biosystems, OAGA02009) or rabbit anti-CYP21A2 (1:500; SIGMA HPA053371). Visualisation of the proteins was performed by using Alexa-Fluor 680 and 800 secondary antibodies (1:5000; Invitrogen) and the Li-CoR Odyssey system.

**Statistics**

Statistical analyses were performed using a combination of one-way ANOVA using Tukey HSD (honest significant difference) test and a two-tailed Student’s t-tests assuming unequal variance in 5 mice per group. All values were expressed as a mean ± s.e.m., and P values <0.05 were considered significant.

**Results**

**Mouse phenotyping**

We have previously reported that 3-month-old mice carrying a spontaneous Nnt 5-exon deletion (C57BL/6J, Nnt−/−) have significantly lower levels of corticosterone (50%) than their wild-type counterparts (C57BL/6N, Nnt+/+) (Meimaridou et al. 2012). To identify the
NNT is key for adrenal redox and steroid control

E Meimaridou et al.

Mechanism by which loss of NNT affects steroidogenesis

We employed the same mouse models but also included an additional mouse line where the Nnt loss had been rescued in C57BL/6J mice by transgenic expression of the entire murine Nnt gene contained within a bacterial artificial chromosome (BAC transgenic, NntBAC) (Freeman et al. 2006).

Glucocorticoid levels in the three mouse strains

The adrenal steroid output of the three mouse strains was measured at 18 months in murine serum samples employing liquid chromatography–tandem mass spectrometry (LC–MS/MS). Changes in steroid production were observed predominantly for corticosterone, with reduction to 14% of wild-type levels in Nnt−/− mice (Fig. 3A). Corticosterone levels in the NntBAC mice restored serum corticosterone in part, but only to 40% of wild-type (Nnt+/+) levels, suggesting that overexpression of Nnt also perturbs steroidogenesis. 11-Deoxycorticosterone levels were not significantly different among the mice and therefore the ratio of 11-deoxycorticosterone/corticosterone was significantly higher in Nnt−/− and NntBAC mice indicating lower enzyme activity of Cyp11b1 (Fig. 3B). We also noted that, over time, the deficit in glucocorticoid output worsened for the Nnt−/− mice, between 3 and 18 months corticosterone levels in Nnt+/+ were unaltered, whereas there is a 60% decrease for Nnt−/− mice, suggesting progressive loss of function (Fig. 3C).

No changes to adrenal histology

In humans with FGD, there is a specific loss of glucocorticoid output from the ZF and relative preservation of steroid output from the other zones suggesting loss of this specific zone. Consistent with

**Figure 3**

Biochemical and phenotypic characterisation of Nnt+/+, Nnt−/− and NntBAC mice. (A) Serum corticosterone in Nnt+/+, Nnt−/− and NntBAC mice was measured by LC-MS/MS and showed 80% and 50% reduction in Nnt−/− and NntBAC mice, respectively. (B) The 11-deoxycorticosterone (DOC)/corticosterone ratio (CYP11B1 inhibition) was significantly higher in Nnt−/− and NntBAC than Nnt+/+ mice. (C) Corticosterone synthesis deteriorates in 18 months Nnt−/− mice whereas there is no significant difference in the levels between 3 and 18 month Nnt+/+ mice. (D) H&E staining of mouse adrenals showed no major histological differences in architecture or zonation (left panel) and (E) Oil Red O staining revealed no difference in lipid content of the adrenals among the three mouse strains (right panels). Results are means±standard deviation (s.d.); n=5 mice per group, *P<0.05, **P<0.01, ***P<0.001.
these findings, Mc2r−/− mice have smaller adrenals with preservation of ZG and medulla but atrophied ZF (Chida et al. 2007). Interestingly all Mc2r−/− mice on a pure C57BL/6j background die within two days of birth whereas those on a mixed B6/Balb/c have much higher survival rates, with half making it to adulthood perhaps due to the restoration of Nnt levels (Chida et al. 2009), making Nnt a genetic modifier of their adrenal phenotype akin to the situation in another mouse model where NNT has a protective role in superoxide dismutase deficient mice (Huang et al. 2006). To determine whether loss of ZF occurs in Nnt−/− mice we performed H&E staining in adrenal sections from the three mouse substrains. This showed no morphological differences among the strains in adrenal zonation (Fig. 3D). This was supported by the finding that genes differentially expressed between zones were not altered (see below). This suggested no major remodelling of the adrenals due to NNT loss and gave us confidence that the RNA-seq variations were not due to changes in zonation. Furthermore, on Oil Red O staining, we observed no differences in lipid levels between the mouse strains suggesting there is neither a dearth of cholesterol supply for steroidogenesis nor a surplus due to a cholesterol transport defect as seen with Star mutations (Sasaki et al. 2008) (Fig. 3E).

**Nnt deletion causes oxidative stress in mouse adrenals**

We measured lipid peroxidation (LPO) by a malondialdehyde (MDA) assay as a measure of oxidative stress in these mice (5 mice per group). There was a significant increase in LPO in the adrenals of Nnt−/− mice which returned towards wild-type levels in NntBAC mice indicating lipid damage by free radicals in adrenals upon Nnt deletion (Fig. 4A). We saw the same trend in H2O2 levels (increased in Nnt−/− and reduced again in NntBAC) (data not shown).

Impaired redox homeostasis caused by lack of NNT was further supported by in vitro studies in human adrenocortical H295R cells. The total cellular NADP/NADPH ratio was significantly higher in H295R with lentiviral knockdown of NNT (NNT-KD) compared to scrambled control cells (SCR), suggesting that NNT is required to maintain the redox state of the intracellular NADPH/NADP+ pools (Fig. 4B).

The perturbation in NADP/NADPH balance affected mitochondrial respiration resulting in significantly lower oxygen consumption rates (OCAR) in cells where NNT is knocked down (Fig. 4C).
Transcriptome profiling by RNA-seq in adrenals from the C57BL/6 mouse substrains

To investigate the cause of the reduced steroid production upon Nnt deletion, RNA was extracted from Nnt+/+, Nnt−/−, and NntBAC mouse adrenals and the transcriptome profiled by RNA-seq. Using an Illumina HiSeq 2000 sequencer we obtained an average of 30 million reads per sample, with ~98% of these reads mapped to the mouse reference genome. In total, 27,622 genes were analysed in Nnt+/+, Nnt−/− and NntBAC adrenals (Fig. 5A and Supplementary Table 1, see section on supplementary data given at the end of this article). The most highly expressed categories of genes were mitochondrial electron transport chain genes (mt-Co1, mt-Co2, mt-Atp6, mt-Co3, mt-Cytb, mt-Nd4, mt-Nd2, mt-Nd1, mt-Atp8, mt-Nd6, mt-Nd5, mt-Nd4l, mt-Nd3, mt-Rnr1), followed by steroid pathway genes Star, Cyp21a1 and Hsd3b1, which are high up in the steriodogenic cascade (Supplementary Table 1) perhaps reflecting the high mitochondrial content of and high demand for steriodogenesis in the adrenal.

Initial data analysis was performed between paired samples comparing Nnt+/+ and Nnt−/−, Nnt+/+ and NntBAC and Nnt−/− and NntBAC adrenals. To identify differentially expressed genes from each group we used the following criteria; (1) gene expression level greater than or equal to 1 read per kilobase of exon per million fragments mapped (RPKM) in all samples; (2) change in expression level greater than or equal to 1.5-fold; and (3) significance P value <0.05. This revealed differential expression (fold change ≥1.5; P < 0.05) of 400 genes in total in the pairwise comparisons (Fig. 5B, C and Supplementary Table 2). Only 1 gene varied between all three pairwise analyses and that was Nnt itself (see below and Fig. 5C). We hypothesized that genes that were up- or downregulated in Nnt−/− and their levels restored in NntBAC would be genes that were modulated by NNT.

NNT levels in the three mouse strains

Nnt expression levels in the three mouse strains were determined and while we observed very low mRNA levels in Nnt−/− mice (3.7-fold downregulation P = 0.012), there was a 2.7-fold (P = 1.8 × 10−5) increase in Nnt expression in NntBAC mice over that for Nnt+/+ (Fig. 6A). To investigate whether mRNA levels corresponded to protein expression we performed a Western blot on adrenal lysates from the same mice. NNT was undetectable in the Nnt−/− mice whereas a two-fold increase in NNT was observed in the adrenals of NntBAC mice, in keeping with the RNA-seq data (Fig. 6B). These results suggest that the NntBAC represents a modest Nnt overexpressor.
No differential expression of other genes with polymorphisms identified between substrains

Recently, comparative genomics between C57BL/6J and C57BL/6N strains has identified many SNPs and structural variants that may contribute to the phenotypic differences between the two strains (Simon et al. 2013). To check whether these SNPs altered expression of these genes in the adrenal we specifically looked at their mRNA expression levels. Except for Cilp, which was up in NntBAC (1.6 fold over Nnt+/+ (P 0.013)) but unaltered between Nnt+/+ and Nnt−/−, we found no alterations in expression levels of the genes with interstrain variations, suggesting that the effects we describe are largely due to differential Nnt levels.

No differential expression of zone specific genes between substrains

Many genes show differential expression between the zones of the adrenal, classically for example tyrosine hydroxylase (Th), aldosterone synthase (Cyp11b2), 3-beta-hydroxysteroid dehydrogenase (Hsd3b2), cytochrome b5 (Cyb5) and aldo-keto reductase family 1 member C3 (Akr1c3). More recently transcriptomic analyses have identified hundreds of other genes with differential expression between ZF and ZG (Nishimoto et al. 2012, Rege et al. 2014). None of these genes showed differential expression in our study suggesting no major remodelling of adrenals glands in Nnt−/− or NntBAC mice.

Nnt deletion does not alter the levels of ACTH receptor pathway genes or other genes associated with adrenal insufficiency

Mc2r expression was unaltered between Nnt+/+ vs Nnt−/− but up in NntBAC vs Nnt−/− (1.6-fold P=0.023) whereas Mrap levels were the same across the 3 groups (data not shown). No significant expression level changes were observed at RNA level for other genes causing adrenal insufficiencies in humans (Aaas, Abcd1, Aire, Cdkn1c, Cyp11a1, Cyp11b1, Cyp17a1, Cyp21a1, Mcm4, Nr5a1, Por and Txnrd2).

Nnt deletion alters antioxidant gene levels

Nnt provides a constant supply of NADPH required for ROS detoxification by the thioredoxin and glutathione systems. Malic enzyme 3 (Me3) and isocitrate dehydrogenase 2 (Idh2) are alternate NADPH suppliers although NNT is the major contributor (14). To investigate whether Nnt deletion leads to perturbation of other antioxidant enzymes perhaps to compensate for its loss, we looked at the mRNA and protein levels of antioxidant enzymes in these pathways. There was a modest reduction in gene expression levels of the antioxidants Prdx3 and Txnrd2 which did not reach statistical significance, however at the protein level they were significantly reduced in Nnt−/− mice vs Nnt+/+ and the levels remained significantly low in NntBAC when compared to Nnt+/+ (Fig. 6C and D). This is likely because NntBAC overexpress NNT which may also cause redox imbalance, implying fine tuning.

Figure 6
Effect of NNT loss on redox homeostasis.
(A) mRNA Nnt levels in the three mouse strains. (B) No NNT protein expression was observed in Nnt−/− (Western blot) however there was a two-fold upregulation in NntBAC when compared to Nnt−/−. (C) Protein levels of TXNRD2, PRDX3 and GPX1 in the Nnt+/+, Nnt−/− and NntBAC mouse adrenals were normalised to actin with representative Western blots shown to the right.
of NNT is required for redox homeostasis. We observed no compensatory increase in the alternative NADPH suppliers Me3 or Idh2.

**Nnt deletion alters mitochondrial cytochrome P450scC levels**

STAR is a protein involved in the transport of cholesterol from the outer to the inner mitochondrial membrane and specific partial loss-of-function mutations in STAR account for 10% of FGD cases (Meimaridou et al. 2013). Similarly, “mild” mutations in the cholesterol side chain cleavage enzyme (CYP11A1, the first enzyme in the steroid pathway), can give rise to FGD (Rubtsov et al. 2009, Parajes et al. 2011, Sahakirunguang et al. 2011). Mutations in 11β-hydroxylase (CYP11B1, the last enzyme in the glucocorticoid pathway) cause congenital adrenal hyperplasia. Previous work revealed that oxidative stress, resulting from the application of exogenous ROS, lead to inhibition of STAR protein expression and steroidogenesis in MA-10 Leydig cells, with no effect on CYP11A1 (Diemer et al. 2003). We hypothesized that a similar phenomenon might occur with endogenous mitochondrial oxidative stress resulting from NNT deficit affecting Star and/or the mitochondrial CYP450 enzymes, Cyp11a1 and Cyp11b1/b2. RNA-seq and Western blot analysis showed no significant changes in the expression of Star at mRNA or protein level, indicating that a defect in cholesterol

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**Figure 7**

Effect of NNT loss on steroidogenesis. (A) mRNA and protein fold change of enzymes involved in glucocorticoid synthesis, starting from cholesterol transport (STAR), to the first step of steroid synthesis (mitochondrial CYP11A1), and subsequent reactions catalysed by microsomal enzymes (HSD3B2, CYP21A1) to the final synthesis of corticosterone. (B) The conversion of cholesterol to corticosterone in mice is achieved by a series of catalytic reactions catalysed by mitochondrial (in blue) and microsomal (in pink) enzymes (right panel). (C) Panel of Western blots representing changes in expression of protein involved in steroidogenesis. Results are means ± standard deviation (s.d.); n=5 mice per group, *P<0.05, **P<0.01, ***P<0.001.
transport due to oxidative insult is an unlikely mechanism (Fig. 7A and C). However, a suggestive 25% decrease in Cyp11a1, Cyp11b1 and Cyp11b2 mRNA levels in Nnt−/− mice with incomplete recovery in NntBAC suggested a possible mechanism for steroid depletion. In agreement with this we observed a 65% reduction in CYP11A1 at protein level in Nnt−/− and partial restoration (to approx. 50% of Nnt+/+ levels) in NntBAC (Fig. 7A and C). We were unable to assess CYP11B1/B2 levels since no specific murine antibody exists. The failure to completely recover CYP11A1 levels in the NntBAC mice may be due to redox imbalance in these overexpressing mice and, significantly, protein abundance mirrors the levels of corticosterone in the three mouse substrains (Figs 3A and 7A). This is analogous to partial loss-of-function mutations in CYP11A1, which give rise to adrenal insufficiency in humans; the proteins may retain 30–40% of wild-type activity this is insufficient to maintain normal cortisol production (Parages et al. 2011).

In contrast the intermediate steps of steriodogenesis occur in the ER, once pregnenolone is synthesized it undergoes reactions catalysed by 3β-hydroxysteroid dehydrogenase (Hsd3b2) and 21-hydroxylase (Cyp21a1) to produce progesterone and deoxycorticosterone respectively (to produce progesterone and deoxycorticosterone underpins reactions catalysed by 3β-hydroxysteroid dehydrogenase (Hsd3b2) and 21-hydroxylase (Cyp21a1) to produce progesterone and deoxycorticosterone respectively). In total 400 genes were differentially expressed in pairwise analyses between the mouse substrains (Supplementary Table 2). Differentially expressed genes between Nnt+/+ vs Nnt−/− numbered 187 (89 up and 98 downregulated), between Nnt−/− vs NntBAC numbered 157 (130 up- and 37 downregulated) and between Nnt+/+ vs NntBAC numbered 141 (119 up- and 22 downregulated) (Supplementary Tables 3, 4, 5, 6, 7 and 8).

We hypothesized that genes with altered expression in Nnt−/− that reverted to wild-type levels in the NntBAC would be genes that were modulated by Nnt levels. 40 genes including Nnt were altered in Nnt−/− with their expression levels rescued in NntBAC. 23 of these were downregulated in Nnt−/− and back up in NntBAC, while 17 were upregulated in Nnt−/− and back down in NntBAC (Table 1). Two groups of genes were enriched in this list; chaperones and haemoglobins. Specifically, there was a 25-fold increase in Hspa1a and Hspa1b in Nnt−/− mice,

Table 1. Forty genes modulated by NNT levels.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1500015O10Rik</td>
<td>RIKEN cDNA 1500015O10 gene</td>
</tr>
<tr>
<td>9330151L19Rik</td>
<td>ENSMUSG00000097061 – uncharacterized protein</td>
</tr>
<tr>
<td>A530020G20Rik</td>
<td>RIKEN cDNA A530020G20 gene</td>
</tr>
<tr>
<td>C1qtnf6</td>
<td>C1q and tumour necrosis factor related protein 6</td>
</tr>
<tr>
<td>Ccdc160</td>
<td>Coiled-coil domain containing 160</td>
</tr>
<tr>
<td>Ctxn3</td>
<td>Cortexin 3</td>
</tr>
<tr>
<td>Cyp21a2-ps</td>
<td>Cyp21a2 pseudogene</td>
</tr>
<tr>
<td>E330017L17Rik</td>
<td>RIKEN cDNA E330017L17 gene</td>
</tr>
<tr>
<td>Epb4.14aos</td>
<td>Erythrocyte membrane protein band 4.1</td>
</tr>
<tr>
<td>Gnat2</td>
<td>Guanine nucleotide binding protein, alpha transducing 2</td>
</tr>
<tr>
<td>Ism1</td>
<td>Isthmin 1 homolog (zebrafish)</td>
</tr>
<tr>
<td>Kcnn2</td>
<td>Potassium intermediate/small conductance calcium-activated channel, subfamily N,</td>
</tr>
<tr>
<td></td>
<td>member 2</td>
</tr>
<tr>
<td>Lilr4b</td>
<td>Leukocyte immunoglobulin-like receptor, subfamily B, member 48</td>
</tr>
<tr>
<td>Ly96</td>
<td>Lymphocyte antigen 96</td>
</tr>
<tr>
<td>Nnt</td>
<td>Nicotinamide nucleotide transhydrogenase</td>
</tr>
<tr>
<td>Pascin3</td>
<td>Protein kinase C and casein kinase substrate in neurons 3</td>
</tr>
<tr>
<td>Sox12</td>
<td>SRY-box containing gene 12</td>
</tr>
<tr>
<td>Steap1</td>
<td>Six transmembrane epithelial antigen of the prostate</td>
</tr>
<tr>
<td>Trim12c</td>
<td>Tripartite motif-containing 12C</td>
</tr>
<tr>
<td>Trim21</td>
<td>Tripartite motif-containing 21</td>
</tr>
<tr>
<td>Trim30d</td>
<td>Tripartite motif-containing 30D</td>
</tr>
<tr>
<td>Tuft1</td>
<td>Tuftelin 1</td>
</tr>
<tr>
<td>Vsnl1</td>
<td>Visinin-like 1</td>
</tr>
<tr>
<td>Arl4d</td>
<td>ADP-ribosylation factor-like 4D</td>
</tr>
<tr>
<td>Cwc25</td>
<td>CWC25 spliceosome-associated protein homolog (S. cerevisiae)</td>
</tr>
<tr>
<td>Cyr61</td>
<td>Cysteine rich protein 61</td>
</tr>
<tr>
<td>Dnajb1</td>
<td>Dnaj (Hsp40) homolog, subfamily B, member 1</td>
</tr>
<tr>
<td>Egr1</td>
<td>Early growth response 1</td>
</tr>
<tr>
<td>Fam46a</td>
<td>Family with sequence similarity 46, member A</td>
</tr>
<tr>
<td>Gadd45g</td>
<td>Growth arrest and DNA-damage-inducible 45 gamma</td>
</tr>
<tr>
<td>Hba-a</td>
<td>Hemoglobin alpha, adult chain 1</td>
</tr>
<tr>
<td>Hba-a2</td>
<td>Hemoglobin alpha, adult chain 2</td>
</tr>
<tr>
<td>Hbb-bs</td>
<td>Hemoglobin, beta adult s chain</td>
</tr>
<tr>
<td>Hbb-bt</td>
<td>Hemoglobin, beta adult t chain</td>
</tr>
<tr>
<td>Ier2</td>
<td>Immediate early response 2</td>
</tr>
<tr>
<td>Ier3</td>
<td>Immediate early response 3</td>
</tr>
<tr>
<td>Irs2</td>
<td>Insulin receptor substrate 2</td>
</tr>
<tr>
<td>Klf4</td>
<td>Kruppel-like factor 4 (gut)</td>
</tr>
<tr>
<td>Nr4a2</td>
<td>Nuclear receptor subfamily 4, group A, member 2</td>
</tr>
<tr>
<td>Zfp36l2</td>
<td>Zinc finger protein 36, C3H type-like 2</td>
</tr>
</tbody>
</table>

Transcriptomics-differentially expressed genes

In total 400 genes were differentially expressed in pairwise analyses between the mouse substrains (Supplementary Table 2). Differentially expressed genes between Nnt+/+ vs Nnt−/− numbered 187 (89 up and 98 downregulated), between Nnt−/− vs NntBAC numbered 157 (130 up- and 37 downregulated) and between Nnt+/+ vs NntBAC numbered 141 (119 up- and 22 downregulated) (Supplementary Tables 3, 4, 5, 6, 7 and 8).

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and these levels were restored to Nnt+/− levels in the NntBAC mice. A smaller, but still significant, increase was observed in another heat shock (Hspb1) and a co-chaperone (Dnajb1) in Nnt−/− mice (Fig. 8A).

The upregulation of heat shock proteins suggests that proteins are undergoing damage due to increased ROS and the molecular chaperone machinery is activated to correct or degrade such damaged or misfolded proteins. Interestingly, alpha- and beta-haemoglobins (Hba-a1, Hba-a2, Hbb-b1 and Hbb-b2 (aka Hbb-bs and Hbb-bt)) were 4 to 5-fold upregulated in Nnt−/− mice (P<0.025) and their levels returned to normal in NntBAC (Fig. 8B). Erythroid contamination of the tissues from Nnt−/− mice was considered but ruled out as other genes highly expressed in the development of erythroid lineages were not significantly upregulated (51 genes from An et al. 2014). Recently the expression of haemoglobins in tissues other than erythrocytes has been reported suggesting their role in other basic cellular functions apart from O2 transport (Fordel et al. 2006, Vinogradov & Moens 2008).

Discussion

We have previously shown that mutations in NNT cause adrenal dysfunction in humans primarily affecting theZF cells of the adrenal cortex responsible for cortisol production, and observed a 50% reduction in corticosterone levels in 3-month-old Nnt null mice (Meimaridou et al. 2012). Further, we showed mitochondrial perturbations and limited antioxidant capacity in human adrenocortical carcinoma cells where NNT expression was stably knocked down (Meimaridou et al. 2012). In this study, we investigated the mechanism by which NNT affects steroidogenesis in older mice by utilising three models with differing expression levels of Nnt; wild-type Nnt+/−, null Nnt−/− and two-fold overexpressing NntBAC mice. Gene expression and Western blotting analysis revealed restricted levels of key mitochondrial antioxidant and steroidogenic proteins in Nnt−/− mice leading to glucocorticoid deficiency, which was partially rescued in the overexpressing mice. Interestingly, we demonstrate for the first time that overexpression of Nnt also negatively impacts steroidogenesis; this may be due to a persistent redox imbalance initiated by the oversupply of NADPH by NNT.

The mouse inbred C57BL/6 strain is widely used for genetic and functional studies. There are two substrains of these mice depending on their site of origin; C57BL/6J established in Jackson laboratory and C57BL/6N line from the National Institutes of Health (NIH). In 2005, a spontaneous loss-of-function Nnt mutation in C57BL/6J was characterised, which was associated with impaired glucose tolerance (Ronchi et al. 2016). Since then, these mice have been used to clarify the roles of NNT in mammalian biology.

More recently, comparative genomics between C57BL/6J and C57BL/6N strains has identified many SNPs and structural variants that may contribute to the phenotypic differences between the two strains (24). We compared expression levels of the genes noted in this publication but revealed no differences in mRNA levels exception for Nnt. This suggests that the transcriptome changes and the endocrine phenotype observed in Nnt−/− mice is largely due to differential NNT levels.
In this study, we have employed, in addition, a mouse strain with transgenic rescue of 
Nnt expression (NntBAC) (Freeman et al. 2006). The Nnt replacement has previously
been shown to induce improvements in glucose tolerance and insulin secretion rescuing
the phenotype seen in Nnt−/− mice. Corticosterone levels recovered somewhat
in the NntBAC; however, they remained significantly lower than wild-type levels.

In this in vivo model, we also showed that the antioxidant capacity of the
Nnt−/− adrenals is significantly compromised when compared to Nnt+/− counterparts.
The protein levels of key mitochondrial antioxidant enzymes PRDX3 and TXNRD2 are significantly reduced
in Nnt−/− mice and fail to recover in NntBAC. This strongly suggests that a set level of Nnt expression is required
to maintain mitochondrial redox homeostasis. Furthermore, since mitochondrial NADPH can be regenerated not
only by Nnt, but also by isocitrate dehydrogenase 2 (Idh2) and malic enzyme 3 (Me3), we excluded a possible
compensatory mechanism, as expression levels of these enzymes remain unchanged between Nnt+/− and Nnt−/− mice.
Studies by other groups have similarly demonstrated that
liver, heart, brain and skeletal muscle mitochondria from
Nnt−/− mice have unaltered Idh2 and Me3 enzymatic
activities meaning they cannot compensate for the loss of NNT to restore NADPH levels (Nickel et al. 2015,
Ronchi et al. 2016). When NNT is ablating in human adrenocortical
cells, we also see a disturbance of redox balance, but here this does not affect cortisol output, perhaps due to
adaptive alterations in sulfiredoxin and peroxiredoxin III levels, which are known to occur in H295R cells, and
even primary adrenocortical cells, when grown in culture (Kil et al. 2013).

NNT dysregulation not only affects the antioxidant
capacity of the adrenal but also its steroidogenic
capacity. We demonstrated that, in Nnt−/− mice, adrenal
steroidogenesis is severely affected (86% reduction in corticosterone in 18 months old mice), due to the low
protein levels of a crucial steroidogenic enzyme, CYP11A1. Interestingly, NntBAC mice also exhibit glucocorticoid
deficiency as indicated by 60% reduction in levels of corticosterone suggesting that Nnt overexpression also
impacts on the steroidogenic output of these mice. It is increasingly recognised that redox balance is key to
physiological health. Where one might assume that underexpression of antioxidants would lead to oxidative
stress and overexpression would give reductive stress; this is not necessarily what occurs in vivo (reviewed in
Lei et al. 2016), with clear examples of antioxidant gene
knockdown inducing reductive stress (Yan et al. 2017) and
antioxidant gene overexpression also causing reductive
stress (Zhang et al. 2010). In addition, paradoxically, both reductive and oxidative insult can lead to overproduction
of ROS (Barrett et al. 2004, Arrigo et al. 2005, Filomeni et al.
which can cause protein damage. We suggest
that this may be the explanation for the failure to rescue
glucocorticoid secretion in the overexpressing mice with
the demonstration of higher levels of lipid peroxidation
in the NntBAC mice compared to Nnt+/− mice lending
support to this.

Differential gene expression studies between
Nnt+/− and Nnt−/− revealed a significant upregulation
of heat shock proteins in Nnt−/− mice. The failure of
ROS detoxification may lead to oxidative damage of
proteins and the canonical chaperone machinery will be
upregulated to cope with the resultant protein misfolding
and degradation. The increased ROS will also render cells
more susceptible to apoptosis but heat shock proteins 27
and 70 (Hsp27 and Hsp70 respectively) are activated by
mitochondrial ROS and are protective of cells preventing
apoptosis by replenishing reduced glutathione and
reducing intracellular iron levels (Barrett et al. 2004,

In addition to heat shock protein machinery, we
show haemoglobins are regulated by NNT levels. There is
a significant increase in the mRNA levels of haemoglobins
in Nnt−/− mice possibly as a compensatory mechanism
to combat oxidative stress. Haemoglobins are composed
of alpha and beta HbA chains and their accepted
main function is to transport O2 to cells thorough the
vascular network. However, their involvement in other
fundamental cellular functions and in non-erythroid
cells is increasingly being recognised. Detection of
haemoglobin chains in macrophages, alveolar cells,
kidney, brain and vaginal epithelial cells has been reported
and their function has been linked with antioxidant
defence and the regulation of mitochondrial activity (Liu
et al. 2008, Saha et al. 2017). In adrenal, overexpression of
α-Hb in rat phaeochromocytoma (PC12) cells resulted in
downregulation of Gpx1 and Sod1 mRNAs suggesting that
it may have a role in the scavenging of ROS (Biagioli et al.
2009, Maria et al. 2012). Whether a similar mechanism
explains the upregulation we see in intact adrenals
requires further investigation.

In this study, we showed that the reduced steroidogenic
capacity of the adrenals in Nnt−/− and NntBAC mice is due to
the inability of other antioxidant enzymes to compensate
for redox imbalance resulting from altered Nnt levels.
This leads to limited availability of mitochondrial CYP11A1 and a reduction in corticosterone output. Recently a similar mechanism was demonstrated to underlie the adrenal dysfunction seen in Triple A, a disorder of adrenal insufficiency, alacrima and achalasia due to mutations in AAAS encoding the protein ALADIN. A deficiency of ALADIN results in cytosolic, as opposed to mitochondrial, oxidative stress and a deficit of microsomal, rather than mitochondrial, CYP450 enzymes thereby retarding adrenal steroidogenesis (Juhlen et al. 2015).

Conclusions

Using transcriptomic profiling in adrenals from three mouse substrains, we showed that disturbances in adrenal redox homeostasis are mediated not only by under expression of NNT but also by its overexpression. Further, we demonstrated that both underexpression or overexpression of NNT reduces corticosterone output implying a central role for it in the control of steroidogenesis. Reduced expression of CYP11A1, a key mitochondrial steroidogenic enzyme, mirrored the reduction in corticosterone output. Our data also suggest that oxidative stress and/or ROS damage to proteins is activating mito- and cytoprotective proteins (haemoglobins and heat shock proteins respectively) that may help maintain cell viability but do not rescue the steroidogenic phenotype.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-16-0638.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

E M, L A M designed the study and analysed the RNA-seq data. E M and F E performed the immunoblotting and mouse histology. M G and R C generated and validated mouse substrains. V C, P A F and W A performed the steroidogenic profiling of mice and analysed the mass spectrometric data. E M and L A M prepared the draft manuscript. All authors contributed to the discussion of results and edited and approved the final manuscript.

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NNT is key for adrenal redox and steroid control

E Meimaridou et al.

C57BL/6J mice. *Diabetologia* **48** 675–686. (https://doi.org/10.1007/s00125-005-1680-2)


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