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Sleep restriction induced energy, methylation and lipogenesis metabolic switches in rat liver

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ABSTRACT

Sleep curtailment is ubiquitous in modern day society. Sleep debt is associated with maladaptive physiological changes that can lead to cardiometabolic and neuropsychiatric pathologies. Recent literature has shown the effects of sleep restriction (SR) on systemic metabolic profiles in biofluids, implying that tissue-specific metabolomes are impacted by SR. To test this hypothesis, we assessed hepatic metabolic profiles of rats after 5 days of SR using UPLC–MS based metabolomics analysis and gene expression analysis. Our data suggests distinctive effects of SR on the liver metabolic profile of rats compared to forced-activity control animals. We observed specific impacts of SR on NAD metabolism through NAD accumulation and upregulation of *Nampt*, the rate determining step of NAD salvage. Additional multi-omic changes were observed in methionine metabolism, with an elevated SAM:SAH ratio under SR. This effect on one carbon metabolism is indicative of increased methylation potential. Changes in TCA cycle intermediates and ATP-citrate lyase (*Acl*y) gene expression were observed that may be related to altered circulatory lipid profiles previously reported documenting the chrono-metabolic connection. Taken together with previous investigations, these observations are consistent with a model of decreased TCA activity with concomitant increase in lipogenesis induced by SR. These tissue-specific mechanistic insights into metabolic effects of SR provide a springboard to future metabolic intervention studies.

1. Introduction

Sleep curtailment is a hallmark of present day society. Chronically restricted or disrupted sleep has been linked to increased risk for cardiovascular diseases (Ayas et al., 2003) and metabolic disorders including obesity (Spiegel et al., 1999).

The link between sleep debt and peripheral metabolism has been demonstrated in humans. For example, sleep-deprived volunteers demonstrate perturbed carbohydrate metabolism and altered endocrine function (Spiegel et al., 1999). We and others have previously demonstrated substantive alterations of the blood metabolome in response to restricted sleep (Weljie et al., 2015) and acute total sleep deprivation (Davies et al., 2014). Sleep is often thought to be a restorative physiological process for body and brain (Nicholas et al., 2017), which may include clearance of physiological waste products accumulated during wakefulness. Of relevance, the role of sleep as a restorative process using so-called ‘glymphatic’ waste metabolite clearance mechanisms has been proposed in the brain (Xie et al., 2013) that may have

implications in incidences of neurodegenerative disorders. Specifically, such mechanism seem to support the drainage of harmful amyloid- β thereby minimizing the possibility of incidental Alzheimer’s disease (Xie et al., 2013). Therefore, it is tempting to speculate that sleep help in equilibrating the anabolic and catabolic activities of organism that facilitates the synthesis of physiologically important precursor molecules and clearance of catabolic waste products. Thus sleep, and lack thereof, can directly affect the metabolic activity of the organism.

Systemic metabolic perturbations from sleep loss are being extensively researched using high throughput metabolomics platforms (Rhoades et al., 2017). This is to some extent driven by recent demonstration of daily rhythms in metabolic regulation and levels of metabolites (Ang et al., 2012; Chua et al., 2013; Dallmann et al., 2012; Kasukawa et al., 2012), which may in part depend on the circadian alternation of sleep and wakefulness. Indeed, a significant number of lipids and other metabolites were shown to be affected by total sleep deprivation protocols in humans (Davies et al., 2014; Giskeødegård et al., 2015). Total sleep deprivation seems to significantly affect the

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metabolic rhythmicity by damping the amplitudes of most plasma metabolites (Davies et al., 2014). 27 metabolites were shown to be increased in plasma during sleep deprivation, majority (~88%) of which are lipids (Davies et al., 2014). Small molecules such as tryptophan, serotonin and taurine were found to be elevated during sleep deprivation and may be associated with the antidepressive effect of sleep (Davies et al., 2014). Similar results were obtained from urine metabolomics profiles of the same study participants (Giskeødegård et al., 2015). Elevation in 3-Indoxylsulfate in the urine of sleep deprived individuals bears a direct link to the elevated plasma tryptophan metabolites and suggest that tryptophan metabolism is one of the most sensitive pathways to sleep loss (Giskeødegård et al., 2015). While information from such studies are invaluable, total sleep deprivation is often not observed in real world and thus sleep restriction (SR), where some degree of sleep opportunity is afforded, is of keen interest. We have shown the existence of two common blood biomarkers of sleep debt in both rats and humans in a SR paradigm allowing for four hours of sleep (Weljie et al., 2015). Specifically, we observed decreased levels of oxalic acid and diacylglycerol 36:3 post sleep restriction irrespective of the species. Our data also showed a strong enrichment in lipid perturbations in rats in response to SR. Specifically, increased sphingolipids and plasmalogens were observed after five days SR leading to the conclusion that chronic SR may lead to induction of peroxisome-proliferator activated receptors and oxidative environment in general.

One limitation of previous studies is the difficulty in discerning mechanistic insights from circulatory metabolic changes. Therefore, here we investigate liver metabolic alterations in rats under the same SR paradigm. SR in this model is achieved by keeping the animals awake for 20 h each day by low level forced locomotion in slowly rotating drums (Novati et al., 2008; Roman et al., 2005). A group of control animals was forced to walk the same distance in half the time, allowing them ample opportunity to sleep (forced activity controls, FA). Liver samples from these animals were compared using UPLC–MS based metabolic profiling. To aid the interpretation of the metabolic findings, we performed preliminary transcriptomic profiling in a subset of these animals.

Our data suggests that hepatic energy metabolism, NAD salvage pathway and transmethylation pathways are significantly impacted by SR. Coupling such observation with gene expression analysis on a subset of samples, we speculate that altered hepatic lipogenesis may be critical to the previously observed changes in circulatory lipid profiles after restricted sleep.

2. Materials and methods

2.1. Study protocol

The rat study protocol was previously described (Weljie et al., 2015). Briefly, 3–4 months old male Sprague-Dawley rats (Harlan) were housed individually under a 12:12 h light-dark cycle. Water and standard chow were provided ad libitum unless otherwise noted. The animals were subjected to SR by forced activity 20 h per day for five days. They were allowed to rest for four hours at the end of the light phase (N = 10). Control animals walked the same distance in the dark phase but with sufficient time for sleep (N = 10). On the last day of the experiment, the animals were fasted for 6 h before collection of the liver tissue. The tissue collection was performed at the end of the light phase (1700 h). After euthanasia, the liver was quickly removed, snap frozen, and stored at –80 °C for later analysis. The experimental protocol was approved by the Ethical Committee of Animal Experiments of the University of Groningen.

2.2. Metabolomics analysis

For metabolomic analysis, 2:1:1 methanol/chloroform/water was added to ~30 mg of liver tissue. The sample was homogenized using

the TissuLyser II bead based tissue homogenizing system (Qiagen, Hilden, Germany). The homogenized sample was centrifuged at 13,300 rpm and the upper layer containing the polar fraction was transferred and evaporated using a vacuum concentrator. The dried sample was reconstituted in 200 µl 1:1 acetonitrile/water for mass spectrometric analysis.

UPLC–MS conditions were adopted from previously reported methods (Rhoades and Weljie, 2016). Briefly, 5 µl of each sample was injected in analytical triplicate on a XBridge BEH amide column (2.5 µm × 100 mm × 2.1 mm) using an Acquity H-class UPLC system (Waters Corporation, Milford, MA, USA). UPLC gradient starts with 15%A (A = 95:5 water/acetonitrile, 20 mM ammonium acetate adjusted to pH 9 using ammonium hydroxide) and 85%B (acetonitrile) at 0.15 ml/min, ramped to 70%A for 10 min of isocratic hold. The column was washed with 98%A and re-equilibrated in starting conditions for 5 min following each injection. Mass spectrometry was performed using a Waters Xevo-TQD MS operating with polarity-switching (positive mode 3 kV, negative mode 2 kV). The desolvation gas was set at 450 °C and gas flow at 900 l/h. The source temperature was set at 150 °C. Data was acquired in multiple reaction monitoring (MRM) mode. The injection order was randomized to remove run order bias. Quality control (QC) samples were injected before, during (once after every 10 injections) and after the run to account for instrumental drift. Data was processed using Waters TargetLynx software (v 4.1).

Integrated data was imported in R (v3.2) and processed further. For every metabolite feature, a locally-weighted scatterplot smoothing (LOESS) function was fitted to the QC data. Metabolite features that appeared in less than 50% of the QC samples were dropped along with features with relative standard deviation ≥ 40%. The data was further normalized with the probabilistic quotient normalization technique using multtest package in R.

The metabolomics data was imported into Simca-P + 14.0 (Umetrics AB, Sweden). The data was analyzed using principal component analysis (PCA) to assess the quality of the data. Further, supervised modeling using orthogonal partial least square discriminant analysis (OPLS-DA) (Bylesjö et al., 2006) was used to identify interclass differences across two experimental conditions (FA and SR). The model was assessed using cross-validation parameter Q²Y and CV-ANOVA p. Further predictive modeling was performed by randomly selecting three samples from each class. These samples were predicted using a training set model built from the leftover samples to assess the predictive power of the multivariate model.

Two sample unpaired student's T-test of 4 metabolite ratios (Glutathione[Ox]:Glutathione[Red], NAD:NADH, NADP:NADPH and SAM:SAH) and the metabolites that were found to be significant by multivariate statistical analysis (VIP > 1.0) were performed using microsoft excel. A significance threshold of 0.05 for p-value was used.

Metabolite set enrichment analysis (MSEA) was performed using Metaboanalyst v3.0 (Xia and Wishart, 2016). Pathways were judged significant based on p-value threshold 0.05 and FDR threshold 0.2.

2.3. Transcriptomics analysis

Transcriptomics analysis was performed on a small subset of the total sample (3 FA and 2 SR animals). RNA was isolated from ~3 mg liver tissue using homogenization in trizol buffer (Life technologies, Carlsbad, CA, USA). 1 µg of total RNA was used for library preparation using Illumina kits and reagents (Illumina, San Diego, CA, USA). Library preparation was performed following manufacturer prescribed protocols. Purity and quantity of input RNA and the final amplified library was checked using a Bioanalyzer 2100 machine (Agilent, Santa Clara, CA, USA). The RNA-Seq library was aligned to the rat genome rn6 using STAR version 2.4.1d (Dobin et al., 2013). Next, normalization and quantification was performed with the PORT v0.8.1 RNA-Seq pipeline (<http://github.com/itmat/Normalization>) which uses a strategy of random read resampling to achieve an equal number of reads across all

samples, before quantification. One library was excluded from the PORT analysis, as its read depth was substantially lower than the other libraries resulting in final set of four libraries (2 FA, 2 SR). The normalized SAM files were then quantified at the gene level by identifying, for each gene, all reads that were consistent with some ENSEMBL annotated splice form of the gene. To reduce variance the resampling and quantification steps were performed 10 times and the average of ten normalized counts was calculated. A p-value based differential expression analysis is generally considered unreliable with just two replicates per condition. However, achieving a meaningful ranking of genes that informs a powerful pathway enrichment analysis is sufficient for our purposes. Ranking by fold change however is unreliable with count data because for example a ratio of two counts to one is as significant as a ratio of 200 counts to 100. Typically a pseudocount of one is added to both numerator and denominator to mitigate this problem, but there's no reason *a priori* to expect that to be optimal. To be systematic with this approach, we performed an evaluation to investigate the sensitivity of the results to the pseudocount. To this end, benchmark data was generated by starting with a data set of real samples which were put into two groups of eight so that no genes were differentially expressed. This was achieved by putting neighbors in a hierarchical clustering into separate groups and then checking the *p*-value distribution was null uniform. Then for 1000 genes, reads were randomly removed to simulate differential expression at ten different fold changes from 1.1 fold to infinity. The genes were then ranked by fold-change using a range of pseudocounts from 1 to 200. For each pseudocount *c* the genes were ranked by $\frac{x+c}{y+c}$ and the number of truly differentially expressed genes in the top 1000 was plotted on the y-axis. This procedure was repeated for several different data sets of various organisms and in all cases a pseudocount of *c* = 20 optimal or near-optimal (see figure Supplementary Fig. S1). We note that a pseudocount of *c* = 1, though widely used in RNA-Seq analyses, was highly sub-optimal in all cases. We proceeded therefore with a pseudocount of *c* = 20. The top 236 upregulated genes with pseudocount-adjusted fold-change of greater than 1.15 were used for pathway enrichment analysis. Pathway analyses were performed with Ingenuity IPA. Using downregulated genes did not reveal any significant pathway enrichments.

3. Results

3.1. Chronic SR alters hepatic metabolism

Corrected and normalized mass spectral profiles from SR and FA animals were compared using multivariate statistical analysis. Initially, principal component analysis (PCA) was performed to investigate the data quality. Proximity of analytical triplicates and centrality of the QC samples verified the analytical quality of the data (Supplementary information S2). The samples were further investigated using orthogonal partial least square discriminant analysis (OPLS-DA). FA and SR samples were significantly clustered (Fig. 1A, $Q^2Y = 0.57$, CV-ANOVA $p < 0.0001$). Metabolites were considered significantly different based on the variable importance on projection metric ($VIP > 1.0$; listed in Table 1). Liver samples of SR animals contained higher levels of valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, hydroxyproline, lysine, glutamine, methionine, methionine sulfoxide, SAH, SAM, histidine, methylhistidine, adenine, adenosine, guanosine, N-methylnicotinamide, carnitine, propionylcarnitine, phosphocholine, GPC, lipamide, nicotinamide riboside, NAD, NADP, AMP, UDP and oxidized glutathione. On the other hand, livers of SR rats contained decreased levels of urea, serine, uracil, creatinine, aspartate, N1-methyl-2-pyridone-5-carboxamide (2-Pyr), N1-methyl-3-pyridone-4-carboxamide (4-Pyr), kynurenine, pantothenate, cytidine, uridine, hexanoylcarnitine, xanthosine, dUMP, disaccharide, riboflavin, ATP, lactate, oxalate, xanthine, aconitate, myo-inositol, phosphoglycerate, citrate, isocitrate and gluconate.

In order to validate the model described above, the samples were randomly divided into a training model (7 FA and SR each) and a test set (3 FA and SR each). The test samples were overlaid into the training model to predict class (Fig. 1C,D). The prediction samples were reasonably well clustered and the classes were also predicted correctly except for one analytical replicate (Fig. 1D). This suggests the metabolic alterations identified by the multivariate modeling are sufficient to identify the FA and SR classes.

In addition, key metabolite ratios such as glutathione [Ox]:glutathione[Red], NAD:NADH, NADP:NADPH and SAM:SAH were also explored. SAM:SAH and glutathione[Ox]:glutathione[Red] were significantly elevated in the SR animals (Fig. 1E, $p < 0.05$). However, it is to be noted that while the ratios are important in relative comparison across groups, they may not reflect the exact physiological situation. For example, Hepatic glutathione[ox]:glutathione[Red] is thought to range between 1:100 and 1:4 (Han et al., 2006), while we observed a much higher ratio (~2:1).

The significantly altered metabolites were analyzed by metabolite set enrichment analysis (MSEA, Table 2). Six pathways were found to be significantly enriched ($p < 0.05$, FDR < 0.2 , Fig. 1F) in SR animals relative to the FA controls. They were – (1) nicotinate and nicotinamide metabolism (elevated NAD, NADP, 1-Methylnicotinamide, nicotinamide riboside and decreased 2-Pyr, 4-Pyr), (2) methionine metabolism (Elevated methionine, SAH, SAM, methionine sulfoxide and decreased serine, ATP), (3) ammonia recycling, (4) urea cycle (elevated histidine, glutamine, NAD, AMP and decreased, serine, aspartate, ATP, urea), (5) purine metabolism (elevated adenine, adenosine, guanosine, AMP, glutamine and decreased xanthine, xanthosine, ATP), and (6) TCA cycle (elevated NAD and decreased aconitate, citrate, isocitrate, ATP). A representative network of closely connected metabolic pathways are represented in Fig. 2 with metabolite level bar charts indicating statistical significance by Student's T-test.

3.2. Gene expression analysis of relevant pathways

Gene expression was analyzed from a subset of the samples. Expression of genes from enriched pathways as identified by MSEA analysis were analyzed from the RNA Seq data (Fig. 3). Four genes showed > 50% change compared to FA. For example, *Nampt* (the rate limiting enzyme of mammalian NAD synthesis) expression was elevated by more than two fold in SR animals. In the methionine metabolic pathway, the gene *Mtr* (which converts homocysteine to methionine) was elevated by three fold and *MsrB2* (which converts methionine to methionine sulfoxide) was decreased by 2.4 fold in the SR liver. Along with this, ATP citrate lyase (*Acly*) was elevated more than 1.5 fold in the liver of SR animals.

3.3. Global gene expression analysis

In addition to analyzing the gene expression related to relevant metabolic pathway, a global gene expression analysis was also carried out using ingenuity pathway analysis on significantly upregulated genes (Fold change > 1.15) identified by pseudocount based adjusted fold change analysis as detailed in methods section. Top canonical pathways and upstream regulators are tabulated in Supplementary Table S3. Using just the upregulated genes (fold change > 1.15), the top canonical pathways included *Eif2* (eucaryotic initiation factor 2) signaling, *Mtor* (mammalian target of rapamycin) signaling and regulation of *Elf4* and *P70s6k* signaling. Among top upstream regulators, we found *Nr3c1* (A nuclear receptor subfamily gene), *Fshr* (follicle stimulating hormone receptor) and *Jun* (A proto-oncogene) to be significant ($p < 0.05$). *Nr3c1* was also found to be a significant causal network ($p < 0.05$).

Fig. 1. Differences in hepatic metabolic profile of animals subjected to sleep restriction and forced activity. (A) Cross-validated OPLS-DA scores plot using all samples. (B) Cross-validated OPLS-DA scores plot from seven samples (21 analytical replicates) from each class; rest of three samples (nine analytical replicates) from each class were used for class prediction (C and D). For A–D, average scores from analytical triplicates of each sample were calculated and the plots are presented as mean scores and standard deviation of scores on each component. Blue – FA, Red – SR (E) Significantly different metabolite ratios ($p < 0.05$). (F) Metabolite set enrichment analysis from metabolites judged significant from the multivariate model (A).

4. Discussion

4.1. SR induces altered hepatic energy metabolism

Metabolic profiling of liver from animals after five days of SR reveals alteration in hepatic energy metabolism. Specifically, TCA cycle metabolites such as citrate, aconitate and isocitrate were reduced post SR. Concomitant elevation in *Acly* gene expression was also observed. *Acly* is known to control lipogenesis and cholesterologenesis by controlling cytosolic acetyl-CoA levels (Chypre et al., 2012). Our observations therefore could indicate that TCA cycle intermediates are utilized for acetyl-CoA regeneration which can further be utilized for lipogenesis. This observation is further strengthened by elevation of NADP that is produced possibly as a result of *de novo* lipid synthesis. We also observed elevated hepatic phosphocholine and GPC which are suggested as possible indicators of tissue lipotoxicity (Saponaro et al., 2015). While we do not have any pathological evidence of lipotoxicity, a global alteration in the plasma lipidome is evident (Weljie et al., 2015). Our gene expression analysis showed changes in certain pathways that are known to regulate lipid metabolism. For example, pathways related to retinoid X receptor (RXR) function and activation are significantly impacted. These pathways were shown to regulate lipid fatty acid metabolism in skeletal muscle and was associated to insulin resistance (Szanto et al., 2004). Interestingly, mTOR has been shown to regulate lipogenesis in liver (Lamming and Sabatini, 2013). Our results demonstrate upregulated mTOR signaling post SR indicating elevated

lipogenesis. In addition, gene expression data suggests changes in LEPR (Leptin receptor) as upstream regulator that regulates leptin reception by cell. Leptin is also known to be a crucial factor to control lipogenesis (Margetic et al., 2002). Previously, lipidomic analysis of plasma in the same SR model revealed that a majority of the circulatory metabolic features affected by SR belongs to lipid and related metabolites (Weljie et al., 2015). Specifically, we observed elevated sphingomyelins and ceramides in the plasma of animals post chronic SR. Sphingolipids are emerging as potential biomarkers of type 2 diabetes (Russo et al., 2013). Although we did not observe significant change in the hepatic/plasma glucose level, previous reports in similar animal models suggested altered glucose tolerance after both acute (Jha et al., 2016) and chronic SR (Barf et al., 2010). Our data from hepatic metabolites suggests that an altered glucose phenotype could be the result of a change in cataplerosis of TCA cycle intermediates that follows acyl-CoA/cholesterol biosynthesis. This chain of events is known to be critical for glucose regulated release of insulin (Flamez et al., 2002).

4.2. Altered NAD biosynthetic pathway post SR

Our data indicate a significant alteration in the NAD biosynthetic pathway. Specifically, the two end products of NAD degradation (structural analogues of *N*-methyl-pyridonecarboxamides) were decreased while *N*-methylnicotinamide, nicotinamide riboside, NAD and NADP were elevated. However, we did not find changes in the NAD:NADH and NADP:NADPH ratio. However, it should be noted that

Table 1

Significantly altered hepatic metabolites post SR. The metabolites were chosen based on VIP metric from multivariate OPLS-DA modeling (VIP > 1.0).

Metabolites increased post SR			Metabolites decreased post SR		
Metabolite	VIP	Loading	Metabolite	VIP	Loading
Adenosine	1.95	0.21	Lactate	1.58	−0.14
Glycerophosphocholine	1.92	0.2	Citrate	1.57	−0.16
AMP	1.88	0.2	Oxalate	1.53	−0.13
Guanosine	1.77	0.19	dUMP	1.53	−0.16
Methionine Sulfoxide	1.71	0.19	2-Pyr	1.48	−0.13
Adenine	1.55	0.15	Xanthine	1.43	−0.13
Histidine	1.52	0.13	Serine	1.4	−0.14
Methionine	1.44	0.15	Gluconate	1.36	−0.12
Tryptophan	1.43	0.14	Xanthosine	1.33	−0.13
SAM	1.41	0.12	Isocitrate	1.32	−0.13
Lipoamide	1.4	0.14	Phosphoglycerates	1.31	−0.13
NADP	1.25	0.13	Riboflavin	1.27	−0.11
Nicotinamide Riboside	1.23	0.08	Aspartate	1.24	−0.12
Phenylalanine	1.19	0.12	Uracil	1.19	−0.09
Valine	1.19	0.1	Creatinine	1.16	−0.04
Oxidized Glutathione	1.19	0.12	Uridine	1.14	−0.09
Glutamine	1.18	0.05	ATP	1.14	−0.13
Methylhistidines	1.17	0.07	Pantothenate	1.12	−0.09
Carnitine	1.17	0.13	Myo-inositol	1.1	−0.12
Propionylcarnitine	1.17	0.1	4-Pyr	1.1	−0.11
SAH	1.16	0.07	Kynurenine	1.07	−0.08
Tyrosine	1.15	0.09	Cytidine	1.05	−0.09
Leucine/Isoleucine	1.13	0.12	Aconitate	1.05	−0.06
Lysine	1.12	0.04	Urea	1.03	−0.04
Hydroxyproline	1.08	0.12	Disaccharide	1.02	−0.08
Phosphocholine	1.07	0.07	Hexanoylcarnitine	1.02	−0.07
N1-Methylnicotinamide	1.05	0.01			
UDP	1.02	0.08			
NAD	1.02	0.11			

our methods are not very well adopted to analyze such ratios. We concomitantly observed a more than 2 fold elevation of *Nampt* gene expression. These observations suggest elevated biosynthesis of hepatic NAD under conditions of restricted sleep. Elevation of mTOR signaling pathway can also be associated to elevation of hepatic NAD salvage (Schuster et al., 2015). It was previously proposed that a circadian oscillation of *Nampt* and hence NAD synthesis could be the mechanistic connection between circadian rhythm, metabolism and sleep (Masri and Sassone-Corsi, 2012). NAD was shown to control SIRT1 dependent deacetylation of PGC-1 α which is a key transcription regulator of glucose production (Rodgers et al., 2005) and SIRT1, in turn is thought to control the negative regulation of hepatic insulin sensitivity (Imai and Kiess, 2009). Thus, NAD positively regulates glucose homeostasis (Kitada and Koya, 2013). Our data certainly suggests that NAD metabolism plays a crucial role in regulation of the physiological response to SR that warrants further investigation.

4.3. Altered methionine metabolism and SAM production

SAM is a key component of many transmethylation reactions in mammals (Lieber and Packer, 2002). We observed an elevation in hepatic methionine, methionine sulfoxide, SAH and SAM post chronic SR,

suggesting elevated production of SAM via the SAM cycle. This could impact the transmethylation process that is known to affect the circadian clock via RNA methylation (Hastings, 2013). The SR animals showed a significantly elevated SAM/SAH ratio, hence elevated methylation potential. Previous studies showed a significantly altered DNA methylation profile in totally sleep deprived human subjects (Nilsson et al., 2016). On the other hand, neurological disorders such as depression are thought to be mediated, at least in part, by histone methylation (Sun et al., 2013) that is thought to be intimately connected to sleep deprivation (Meerlo et al., 2015).

4.4. Relation of hepatic phenotypes of SR to circulatory metabolic profile

We recently reported an altered circulatory metabolic profile in sleep restricted rats (Weljie et al., 2015). Chronic SR was characterized by altered circulatory lipid profile, specifically sphingolipids and plasmalogens. While plasmalogens may reflect altered oxidative status supported by elevation of hepatic oxidized glutathione and altered ratio of oxidized and reduced form of glutathione (Fig. 1E), elevated sphingolipids could indicate altered lipogenesis (Saponaro et al., 2015). Altered hepatic lipogenesis is supported by decreased TCA cycle intermediates (Table 1) and increased expression of *Acly* gene (Fig. 3). We did not observe a significant elevation of blood glucose, although sucrose and branched chain amino acids were elevated acutely (Weljie et al., 2015). Also, a similar animal model showed altered metabolic parameters indicating worsened glucose tolerance (Barf et al., 2010). The notion of altered glucose/energy metabolism is further supported by changes in NAD metabolism as noted above. Overall, the hepatic metabolic phenotype of SR appears to be a complex interplay of effect and organismal response of SR (Fig. 2).

4.5. Limitations of the study

SR in this study was achieved by means of mild forced locomotion. Since forced activity may have effects in itself, we included a control group that walked the same distance but had sufficient time to sleep. However, any effect of extreme exercise/exhaustion is eliminated given the speed of walking was slow for both groups (Weljie et al., 2015). The strategy allowed us to focus largely on effects of sleep loss. Even so, we cannot fully exclude the possibility that some of the effects we observe are due to forced activity. Specifically, exercising is known to elevate NAD levels in skeletal muscle (Cantó et al., 2009). While not much is known about effect of exercise on hepatic NAD production, it may be safe to assume that significant elevation of NAD in spite of equal exercise intensity is related to effects of SR. On the other hand, depleted levels of TCA cycle intermediates is contradictory to what is expected post moderate exercising (Lewis et al., 2010). In general, despite the possibility of some overlapping effects of exercise and SR, our results seem to be significantly SR-specific.

5. Conclusion

The altered circulatory metabolic phenotypes reported earlier (Weljie et al., 2015) are supported by the hepatic metabolic patterns

Table 2

Metabolite set enrichment analysis of metabolites listed in Table 1. The analysis was carried out using metaboanalyst 3.0.

Pathway	Metabolites increased post SR	Metabolites decreased post SR	Raw p	Enrichment
Nicotinate and nicotinamide metabolism	NAD, NADP, N-Methylnicotinamide, nicotinamide riboside	2-Pyr, 4-Pyr	8.06E-05	0.46
Ammonia recycling	histidine, glutamine, NAD	serine, aspartate, ATP, urea	0.00067	0.33
Purine metabolism	adenine, adenosine, AMP, guanosine, glutamine	xanthine, xanthosine, ATP	0.00729	0.18
Urea cycle	AMP, glutamine	aspartate, ATP, urea	0.00791	0.25
TCA cycle	NAD	aconitate, citrate, isocitrate, ATP	0.0147	0.22
Methionine metabolism	methionine, SAH, SAM, methionine sulfoxide	serine, ATP	0.003	0.25

Fig. 2. Proposed hepatic metabolic pathway alteration under SR compared to FA animals. Metabolites found significantly altered by multivariate analysis are plotted using bar charts. * $p < 0.05$ and # $p < 0.1$.

Fig. 3. Significantly different hepatic gene expression profiles across SR and FA animals. Genes from affected pathways (Fig. 1F) were compared and genes with > 1.5 fold expression change were considered.

post chronic SR from metabolic and transcriptomic profiling. Specifically, altered hepatic profiles indicated significant changes in the energy metabolism, NAD salvage pathway and transmethylation pathways. These results could further be pursued for more insightful understandings of clinical complications chronic SR including diabetes, obesity and neurological disorders.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biocel.2017.08.014>.

References

- Ang, J.E., Revell, V., Mann, A., Mäntele, S., Otway, D.T., Johnston, J.D., Thumser, A.E., Skene, D.J., Raynaud, F., 2012. Identification of human plasma metabolites exhibiting time-of-day variation using an untargeted liquid chromatography-mass spectrometry metabolomic approach. *Chronobiol. Int.* 29, 868–881. <http://dx.doi.org/10.3109/07420528.2012.699122>.
- Ayas, N.T., White, D.P., Manson, J.E., Stampfer, M.J., Speizer, F.E., Malhotra, A., Hu, F.B., 2003. A prospective study of sleep duration and coronary heart disease in women. *Arch. Intern. Med.* 163, 205–209.
- Barf, R.P., Meerlo, P., Scheurink, A.J.W., Barf, R.P., Meerlo, P., Scheurink, A.J.W., 2010. Chronic sleep disturbance impairs glucose homeostasis in rats. *Int. J. Endocrinol.* 2010, 81941. <http://dx.doi.org/10.1155/2010/819414>.
- Bylesjö, M., Rantalainen, M., Cloarec, O., Nicholson, J.K., Holmes, E., Trygg, J., 2006. OPLS discriminant analysis: combining the strengths of PLS-DA and SIMCA classification. *J. Chemom.* 20, 341–351. <http://dx.doi.org/10.1002/cem.1006>.
- Cantó, C., Gerhart-Hines, Z., Feige, J.N., Lagouge, M., Noriega, L., Milne, J.C., Elliott, P.J., Puigserver, P., Auwerx, J., 2009. AMPK regulates energy expenditure by modulating NAD⁺ metabolism and SIRT1 activity. *Nature* 458, 1056–1060. <http://dx.doi.org/10.1038/nature07813>.
- Chua, E.C.-P., Shui, G., Lee, I.T.-G., Lau, P., Tan, L.-C., Yeo, S.-C., Lam, B.D., Bulchand, S.,

- Summers, S.A., Puvanendran, K., Rozen, S.G., Wenk, M.R., Gooley, J.J., 2013. Extensive diversity in circadian regulation of plasma lipids and evidence for different circadian metabolic phenotypes in humans. *Proc. Natl. Acad. Sci. U. S. A.* 110, 14468–14473. <http://dx.doi.org/10.1073/pnas.1222647110>.
- Chypre, M., Zaidi, N., Smans, K., 2012. ATP-citrate lyase: a mini-review. *Biochem. Biophys. Res. Commun.* 422, 1–4. <http://dx.doi.org/10.1016/j.bbrc.2012.04.144>.
- Dallmann, R., Viola, A.U., Tarokh, L., Cajochen, C., Brown, S.A., 2012. The human circadian metabolome. *Proc. Natl. Acad. Sci.* 109, 2625–2629. <http://dx.doi.org/10.1073/pnas.1114410109>.
- Davies, S.K., Ang, J.E., Revell, V.L., Holmes, B., Mann, A., Robertson, F.P., Cui, N., Middleton, B., Ackermann, K., Kayser, M., Thumser, A.E., Raynaud, F.I., Skene, D.J., 2014. Effect of sleep deprivation on the human metabolome. *Proc. Natl. Acad. Sci. U. S. A.* 111, 10761–10766. <http://dx.doi.org/10.1073/pnas.1402663111>.
- Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., Gingeras, T.R., 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21. <http://dx.doi.org/10.1093/bioinformatics/bts635>.
- Flamez, D., Berger, V., Kruhoffer, M., Orntoft, T., Pipeleers, D., Schuit, F.C., 2002. Critical role for cataplerosis via citrate in glucose-regulated insulin release. *Diabetes* 51.
- Giskeødegård, G.F., Davies, S.K., Revell, V.L., Keun, H., Skene, D.J., 2015. Diurnal rhythms in the human urine metabolome during sleep and total sleep deprivation. *Sci. Rep.* 5, 14843. <http://dx.doi.org/10.1038/srep14843>.
- Han, D., Hanawa, N., Saberi, B., Kaplowitz, N., 2006. Mechanisms of Liver Injury. III. Role of glutathione redox status in liver injury. *Am. J. Physiol. – Gastrointest. Liver Physiol.* 291.
- Hastings, M.H., 2013. M6A mRNA methylation: a new circadian pacesetter. *Cell* 155, 740–741. <http://dx.doi.org/10.1016/j.cell.2013.10.028>.
- Imai, S.-I., Kiess, W., 2009. Therapeutic potential of SIRT1 and NAMPT-mediated NAD biosynthesis in type 2 diabetes. *Front. Biosci. (Landmark Ed.)* 14, 2983–2995.
- Jha, P.K., Foppen, E., Kalsbeek, A., Challet, E., 2016. Sleep restriction acutely impairs glucose tolerance in rats. *Physiol. Rep.* 4, e12839. <http://dx.doi.org/10.14814/phy2.12839>.
- Kasukawa, T., Sugimoto, M., Hida, A., Minami, Y., Mori, M., Honma, S., Honma, K.-i., Mishima, K., Soga, T., Ueda, H.R., 2012. Human blood metabolite timetable indicates internal body time. *Proc. Natl. Acad. Sci.* 109, 15036–15041. <http://dx.doi.org/10.1073/pnas.1207768109>.
- Kitada, M., Koya, D., 2013. SIRT1 in type 2 diabetes: mechanisms and therapeutic potential. *Diabetes Metab. J.* 37, 315–325. <http://dx.doi.org/10.4093/dmj.2013.37.5.315>.
- Lamming, D.W., Sabatini, D.M., 2013. A central role of mTOR in lipid homeostasis. *Cell Metabol.* 18 (4), 465–469.
- Lewis, G.D., Farrell, L., Wood, M.J., Martinovic, M., Arany, Z., Rowe, G.C., Souza, A., Cheng, S., McCabe, E.L., Yang, E., Shi, X., Deo, R., Roth, F.P., Asnani, A., Rhee, E.P., Systrom, D.M., Semigran, M.J., Carr, S.A., Wang, T.J., Sabatine, M.S., Clish, C.B., Gerszten, R.E., 2010. Metabolic Signatures of Exercise in Human Plasma.
- Lieber, C.S., Packer, L., 2002. S-Adenosylmethionine: molecular, biological, and clinical aspects? an introduction. *Am. J. Clin. Nutr.* 76, 1148S–11150.
- Margetic, S., Gazzola, C., Pegg, G.G., Hill, R.A., 2002. Leptin: a review of its peripheral actions and interactions. *Int. J. Obes.* 26 (11), 1407–1433.
- Masri, S., Sassone-Corsi, P., 2012. The circadian clock: a framework linking metabolism, epigenetics and neuronal function. *Nat. Rev. Neurosci.* 14, 69–75. <http://dx.doi.org/10.1038/nrn3393>.
- Meerlo, P., Havekes, R., Steiger, A., 2015. Chronically restricted or disrupted sleep as a causal factor in the development of depression. *Curr. Top. Behav. Neurosci.* 459–481. http://dx.doi.org/10.1007/7854_2015_367.
- Nicholas, C.L., Jordan, A.S., Trinder, J., 2017. The functions of sleep. *Sleep Medicine.*
- Nilsson, E.K., Boström, A.E., Mwinyi, J., Schiöth, H.B., 2016. Epigenomics of total acute sleep deprivation in relation to genome-Wide DNA methylation profiles and RNA expression. *OMICS* 20, 334–342. <http://dx.doi.org/10.1089/omi.2016.0041>.
- Novati, A., Roman, V., Cetin, T., Hagewoud, R., den Boer, J.A., Luiten, P.G.M., Meerlo, P., 2008. Chronically restricted sleep leads to depression-like changes in neurotransmitter receptor sensitivity and neuroendocrine stress reactivity in rats. *Sleep* 31, 1579–1585.
- Rhoades, S.D., Weljie, A.M., 2016. Comprehensive optimization of LC-MS metabolomics methods using design of experiments (COLMed). *Metabolomics* 12, 183. <http://dx.doi.org/10.1007/s11306-016-1132-4>.
- Rhoades, S.D., Sengupta, A., Weljie, A.M., 2017. Time is ripe: maturation of metabolomics in chronobiology. *Curr. Opin. Biotechnol.* 43, 70–76. <http://dx.doi.org/10.1016/j.copbio.2016.09.007>.
- Rodgers, J.T., Lerin, C., Haas, W., Gygi, S.P., Spiegelman, B.M., Puigserver, P., 2005. Nutrient control of glucose homeostasis through a complex of PGC-1 α and SIRT1. *Nature* 434, 113–118. <http://dx.doi.org/10.1038/nature03354>.
- Roman, V., Walstra, I., Luiten, P.G.M., Meerlo, P., 2005. Too little sleep gradually desensitizes the serotonin 1A receptor system. *Sleep* 28, 1505–1510.
- Russo, S.B., Ross, J.S., Cowart, L.A., 2013. Sphingolipids in obesity, type 2 diabetes, and metabolic disease. *Handb. Exp. Pharmacol.* 373–401. http://dx.doi.org/10.1007/978-3-7091-1511-4_19.
- Saponaro, C., Gaggini, M., Carli, F., Gastaldelli, A., 2015. The subtle balance between lipolysis and lipogenesis: a critical point in metabolic homeostasis. *Nutrients* 7, 9453–9474. <http://dx.doi.org/10.3390/nu7115475>.
- Schuster, S., Penke, M., Gorski, T., et al., 2015. FK866-induced NAMPT inhibition activates AMPK and downregulates mTOR signalling in hepatocarcinoma cell. *Biochem. Biophys. Res. Commun.* 458 (2), 334–340.
- Spiegel, K., Leproult, R., Van Cauter, E., 1999. Impact of sleep debt on metabolic and endocrine function. *Lancet* 354, 1435–1439. [http://dx.doi.org/10.1016/S0140-6736\(99\)01376-8](http://dx.doi.org/10.1016/S0140-6736(99)01376-8).
- Sun, H., Kennedy, P.J., Nestler, E.J., 2013. Epigenetics of the depressed brain: role of histone acetylation and methylation. *Neuropsychopharmacology* 38, 124–137. <http://dx.doi.org/10.1038/npp.2012.73>.
- Szanto, A., Narkar, V., Shen, Q., Uray, I.P., Davies, P.J.A., Nagy, L., 2004. Retinoid X receptors: X-ploring their (patho)physiological functions. *Cell Death Differ.* 11, S126–S143. <http://dx.doi.org/10.1038/sj.cdd.4401533>.
- Weljie, A.M., Meerlo, P., Goel, N., Sengupta, A., Kayser, M.S., Abel, T., Birnbaum, M.J., Dinges, D.F., Sehgal, A., 2015. Oxalic acid and diacylglycerol 36:3 are cross-species markers of sleep debt. *Proc. Natl. Acad. Sci. U. S. A.* 112, 2569–2574. <http://dx.doi.org/10.1073/pnas.1417432112>.
- Xia, J., Wishart, D.S., 2016. Using metaboanalyst 3.0 for comprehensive metabolomics data analysis. *Current Protocols in Bioinformatics*. John Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 14.10. <http://dx.doi.org/10.1002/cpbi.11>. (1–14).
- Xie, L., Kang, H., Xu, Q., Chen, M.J., Liao, Y., Thiyagarajan, M., O'Donnell, J., Christensen, D.J., Nicholson, C., Iliff, J.J., Takano, T., Deane, R., Nedergaard, M., 2013. Sleep drives metabolite clearance from the adult brain. *Science* 342, 80.