Supporting Information

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Detailed Protocol for Rat Studies

Animals and Housing. The rat studies were performed with adult male Sprague–Dawley rats (Harlan). Animals were individually housed under a 12-h:12-h light-dark cycle with lights on from 0500 hours to 1700 hours. Ambient temperature was maintained at 21 ± 1 °C. Standard laboratory chow food and water were provided ad libitum except where noted.

Experimental Design. In two separate studies, adult male rats were subjected to SR for a 5-d period. The animals were sleep deprived 20 h per day and were allowed to rest during the last 4 h of the light phase. In both study A and B, blood samples were taken for metabolic profiling on the BL day before SR (day 0) and after 1 and 5 d of sleep restriction (SR1 and SR5). In study B, additional samples were taken after 1 and 3 d of unrestricted recovery sleep (R1 and R3). All blood samples were taken at the end of the light phase (i.e., the main resting phase in rats).

Sleep Restriction and Forced Activity Control. SR and control procedures were performed using established procedures adapted from previous studies (e.g., refs. 1 and 2). The rats were subjected to a protocol of SR for 5 d allowing them to sleep 4 h per day at the end of the light phase (1300 hours to 1700 hours) in their home cage. The remainder of the time, the animals were kept awake by placing them in a slowly rotating drum (40 cm in diameter) driven by an engine at constant speed (0.4 m/min). To examine whether effects of the treatment were caused by forced locomotion rather than SR, the second study included a FA control group. Animals of this group were housed in the same type of drums but rotating at double speed for half the time (0.8 m/min for 10 h). These animals therefore walked the same distance as SR animals, but had sufficient time to sleep. The 10-h FA was done in the dark phase, the main activity phase of the rats.

All rats in the two experiments had continuous access to food and water, including in the rotating drums, except for the 6-h period of fasting preceding the blood collection.

SR rats in this model generally show a temporary suppression of growth relative to BL or home-cage controls, but differences with FA controls are small (3, 4). In rat experiment A of the present study, SR rats and FA controls had on average lost 5.1 and 0.3 g of weight after the first day of the protocol (−1.6% and −0.1% of total body weight). After 5 d, the SR rats had lost 5.7 g relative to baseline, whereas FA controls had gained 7.9 g (−1.7% and +2.6%, respectively). Repeated-measures ANOVA did not indicate an overall weight difference between the two groups ($P > 0.10$) but did reveal a significant treatment × time interaction effect [$F_{(2,36)} = 14.9$; $P < 0.001$]. On day 1, there was no significant difference between SR rats and FA controls in body weight or change in body weight relative to BL. On day 5, there was a trend for lower body weight in the SR animals compared with the FA controls [$t_{(18)} = 2.1$; $P = 0.054$].

Previous studies using the same 20-h SR protocol with the same rotating wheel system showed that food intake does not change (3, 4). In rat experiment A of the present study, we measured food intake in the SR animals and FA controls on days 0, 1, and 5 (SR: 19.3 ± 1.0 g, 21.0 ± 1.6 g and 18.7 ± 1.5 g, respectively; FA: 19.5 ± 0.6 g, 21.9 ± 0.9 g, and 15.5 ± 1.0 g, respectively). Repeated-measures ANOVA did not reveal significant differences between the SR and FA rats ($P > 0.10$). Hence, overall changes in food intake cannot account for the differences in metabolic profile between these groups.

Previous studies with this model have shown that plasma levels of the stress hormone corticosterone are only mildly increased during sleep deprivation and rapidly return to BL after sleep deprivation (e.g., refs. 2 and 5). The elevations are in the range of increases seen when animals are feeding or are exposed to a novel cage. Moreover, corticosterone levels in sleep-restricted animals were found to be lower than those in forced activity controls (2). Therefore, it seems unlikely that effects of SR in the present study are explained by elevated levels of stress hormones.

Blood Sampling. Animals were fasted for 6 h before blood sampling by removing all food from their cages. Blood samples (0.5 mL) were taken by incision of the tail and collected within 1–2 min in cold Eppendorf tubes containing EDTA, Samples were centrifuged (4 °C, 2,600 × *g*, 15 min) and the supernatant was stored at −80 °C for later analysis.

Methods and Experimental Design for Human Metabolomics Study

Subjects. Ten healthy subjects, aged 22–50 y (27.5 ± 5.6 y; five females), participated in one of two SR experimental protocols. To be eligible for study participation, subjects met the following inclusionary criteria: age range from 22 to 50 y; physically and psychologically healthy as assessed by physical examination and history; no clinically significant abnormalities in blood chemistry; drug-free urine samples; good habitual sleep, between 6.5–8.5 h daily duration with habitual bedtimes between 2200 hours and 0000 hours, and habitual awakenings between 0600 hours and 0900 hours (verified by sleep logs and wrist actigraphy for at least 1 wk before study entry); absence of extreme morningness or extreme eveningness, as assessed by questionnaire (6); absence of sleep or circadian disorders, as assessed by questionnaire (7) and polysomnography; no history of psychiatric illness and no previous adverse neuropsychiatric reaction to sleep deprivation; no history of alcohol or drug abuse; and no current use of medical or drug treatments (excluding oral contraceptives).

Experimental Design. Subjects participated in one of two protocols in the Sleep and Chronobiology Laboratory at the Hospital of the University of Pennsylvania and were studied for 14 or 18 consecutive days continuously, in a laboratory protocol with daily clinical checks of vital signs and symptoms by nurses (with an independent physician on call). For the present paper, only data from the first seven nights of the protocols—which were procedurally identical between studies—were analyzed. In both protocols, subjects received two BL nights of 10-h or 12-h time-in-bed (TIB) per night (BL1–2; 2200 hours to 0800 hours/1000 hours) followed by five nights of SR of 4-h TIB per night (SR1–5; 0400 hours to 0800 hours) and one night of 12-h TIB recovery sleep (R1; 2200 hours to 1000 hours) (Fig. 3*A*).

Throughout the study, laboratory conditions were highly controlled in terms of environmental conditions and scheduled activities. Ambient light was fixed at <50 lx during scheduled wakefulness, and <1 lx (darkness) during scheduled sleep periods. Ambient temperature was maintained between 22° and 24 °C. Subjects were restricted from exercising or engaging in strenuous activities, although they were allowed to read, play video or board games, watch television, and interact with laboratory staff to help remain awake (no visitors were permitted). Subjects were continuously monitored by trained staff to ensure adherence.

Subjects had ad libitum access to food and drink throughout the protocol. Subjects were allowed to consume food and drink

at any time during the protocol other than when they were completing neurobehavioral tests or sleeping or when they were undergoing a 10–12 h of fasting before each metabolomic blood sample. Full descriptions of ad libitum access can be found in Spaeth et al. (8, 9). Because the fasting was strictly enforced, it is highly unlikely that dietary intake affected the metabolomic measurements.

Metabolomics. Blood samples for metabolomics were collected after a 10- to 12-h overnight fast in the morning following the first night of BL sleep, after the fifth night of SR, and after the night of recovery sleep (Fig. 3*A*). To control for morning-evening effects, sampling occurred during the 2 h following waketime (between 0800 hours and 1000 hours on BL1 and SR5 and between 1000 hours and 1200 hours on R1). Samples were immediately centrifuged and plasma was stored in a −80 °C freezer until analysis.

Detailed Metabolomics Methods

Sample Preparation and MS for Metabolomics Analysis. The serum samples from the studies described above were used in three aliquots for MS. For each sample, GC-MS, HILIC-MS, and CSH-lipidomics were performed in the metabolomics core of University of California, Davis, CA. Sample preparation protocols for three different modes of MS are detailed below.

GC-MS. For GC-MS, 30 μL of serum sample was extracted using 1 mL 3:3:2 acetonitrile/isopropanol/water (vol/vol) solution. Next, 450 μL of the supernatant was evaporated to dryness, resuspended in 450 μL of 50% acetonitrile, centrifuged, and the supernatant was again evaporated to dryness. The residue was used for derivatization by adding 10 μL of 40 mg/mL methoxyamine hydrochloride followed by 1.5 h of shaking and addition of 91 μL of MSTFA [*N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide]- FAME marker mixture (prepared by adding 1 mL MSTFA and 10 mL FAME). This sample was subjected to shaking at 37 °C and transferred to a glass vial and submitted to GC-TOF-MS.

The MS was performed using a Leco Pegasus II with Gerstel MPS II injector system. The column dimension was 30 m × 0.25 mm × 0.25 mm (Restek Rtx-5sil MS with Integra-Guard).

HILIC-MS. Serum samples were extracted using a 3:3:2 acetonitrile/isopropanol/water (vol/vol) mixture using the same protocol as described above and submitted to MS. MS was performed in an Agilent 6530 accurate mass qTOF LC-MS with an Agilent 1290 infinity UHPLC fitted with Water Acquity UPLC BEH HILIC column of dimension 2.1 mm × 150 mm × 1.7 μm.

Lipidomics Using CSH-qTOF-MS. For lipidomics, 20 μL of blood serum was dissolved in 250 μL ice-cold methanol and 750 μL ice-cold methyl tertiary butyl ether (MTBE) followed by vortexing and addition of 188 mL of ice-cold distilled water. The sample was vortexed and centrifuged for 2 min at 14,000 × *g*. Next, 350 mL of the upper organic fraction was dried and reconstituted in 65 mL 9:1 (vol/vol) methanol-toluene with CUDA (12-[[(cyclohexylamino)carbonyl]amino]-dodecanoic acid) as an internal standard. The samples were subjected to UPLC-qTOF-MS using Agilent 6530 Accurate Mass Q-TOF LC/MS with an Agilent 1290 Infinity UHPLC. An Acquity UPLC CSH C18 column was used for this purpose; the column dimension was 1.7 μm × 2.1 mm × 100 mm. The following solvent system was used: A: 60/40 ACN: H20 0.1% formic acid and 10 mM ammonium formate and B: 90/10 IPA/ACN 0.1% formic acid and 10 mM ammonium formate. The solvent gradient started with 15% B that reached to a maximum of 99% at 11.5 min and decreased to 15% at 12 min, and was kept constant at this value until the 15-min mark.

Statistical Analysis of the Data. The data were normalized by the sum of all identified peak heights from the total ion chromatogram of individual samples, followed by normalization by the average of all total ion chromatograms. The data were further used for univariate statistical analysis using MeV 4.9 (10). For each set of experimental groups (for study A: FA and SR animals; for study B: SR animals; and for the human study: SR individuals), the metabolites from BL samples were compared using the SR (or FA) samples by nonparametric unpaired *t* tests using permutation. One-thousand permutations were used in each case; $P < 0.01$ was considered significant for samples across studies, with a threshold of $P < 0.1$ and FDR < 0.2 required within each study to eliminate possible bias from an individual study. Relationships were calculated from the human data using the pvclust package in R, which provides approximately unbiased and bootstrap probability *P* values to ascertain the stability of interactions (11).

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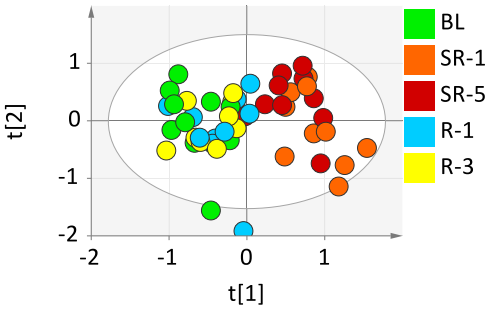

Fig. S1. Principal component analysis scores plot from the rat study including recovery to demonstrate variability. Each point represents a single serum sample from an individual animal, and is colored by the time point, as indicated. The position of each point is determined by the multivariate combination of all measured metabolites for each sample. Although there is some intratime point separation along the second principal component, *t*[2], the major axis of difference lies between the SR and non-SR samples along the first principal component, *t*[1].

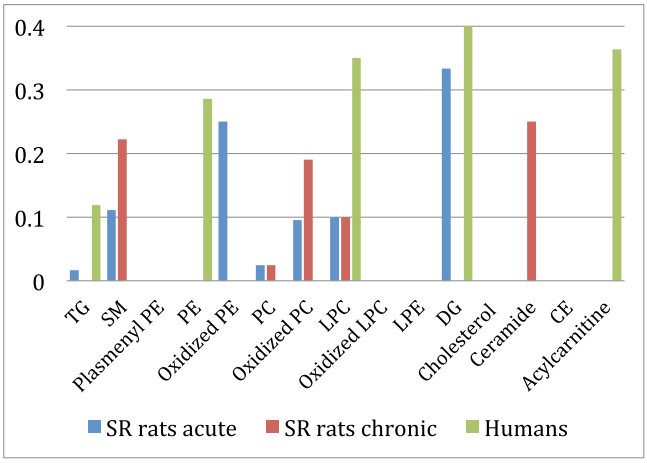

Fig. S2. Fraction of each lipid class measured as significant as a ratio of the total members of that class in human serum.

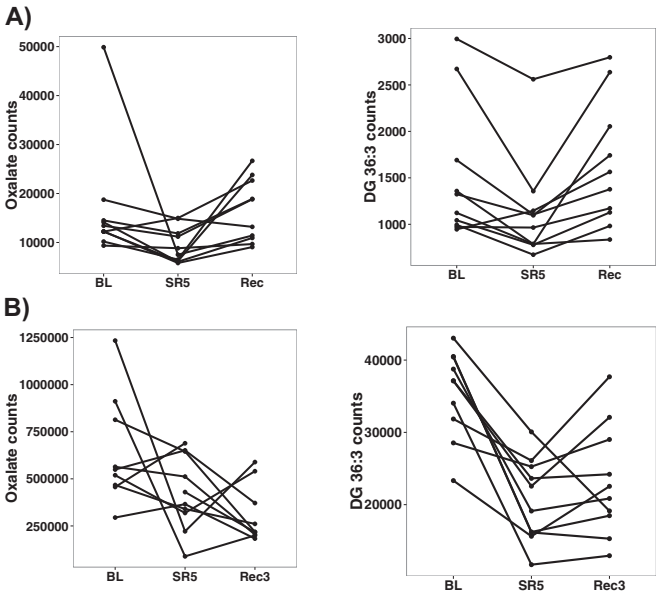

Fig. S3. (*A*) Absolute mass spectral counts for each measured metabolite across the three conditions measured for human serum (BL, baseline; SR5, sleep restriction day 5; Rec, recovery). Note that each subject's measurements are connected by a solid line. (*B*) Absolute mass spectral counts for each measured metabolite across the three conditions measured for rat serum (Rec3, recovery day 3). Note that each animal's measurements are connected by a solid line.

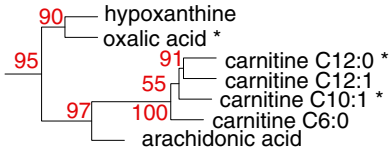

Fig. S4. Bootstrapped hierarchical clustering tree indicating metabolites most correlated to oxalate. Values in red indicate the approximately unbiased probability (%) of branching as computed by multiscale bootstrap resampling. Metabolites that were significantly different in the human study are labeled with an asterisk.

Table S1. Retention index and quantitated *m/z* for GC-MS data

Unknown ID	Retention index	Quantitated *m/z*
201887	1200427	316
211972	473115	98
226844	641635	301
239966	806614	290
267756	795288	156
281172	589910	140
289052	515010	186
294986	884907	390
299159	588963	241
367991	525157	188
438045	735508	174
470909	710744	331
619709	619088	290
624867	357202	241

Table S2. Significant metabolites from rat serum analysis for both acute and chronic sleep restriction pooled from studies A and B

Metabolite	*P* (acute)	FDR (acute)	*P* (chronic)	FDR (chronic)	Up/down in individual studies (significant on day)
2-deoxyerythritol	0.001	0.00156	0.398	0.443486	↓ (1)
DG (36:3)	0.003	0.003656	0.003	0.00468	↓ (1)
Isocitric acid	0.023	0.025629	0.414	0.4485	↓ (1)
Lignoceric acid	0.006	0.007091	0.905	0.905	↓ (1)
LPC (18:1)	0	0	0.001	0.00195	↓ (1)
LPC (22:6)	0.002	0.0026	0	0	↓ (1)
Oxalic acid	0.001	0.00156	0.199	0.235182	↓ (1)
Trihydroxypyrazine	0.001	0.00156	0.017	0.022862	↓ (1)
Urocanic acid	0.023	0.025629	0.352	0.403765	↓ (1)
Leucine	0.003	0.003656	0.446	0.470108	↑ (1)
N-methylalanine	0	0	0.002	0.003545	↑ (1)
Pls-PC (34:1) A	0.001	0.00156	0	0	↑ (1)
Pls-PC (38:5)	0	0	0	0	↑ (1)
PC (40:4)	0	0	0.027	0.033968	↑ (1)
PC (40:6) A	0.001	0.00156	0	0	↑ (1)
Pls-PC (40:5)	0	0	0.025	0.0325	↑ (1)
Pls-PE (36:4)	0.002	0.0026	0.009	0.012536	↑ (1)
SM (33:1)	0	0	0.003	0.00468	↑ (1)
SM (d18:2/23:0)	0	0	0	0	↑ (1)
SM (d41:1)	0	0	0	0	↑ (1)
TG (58:10)	0	0	0.003	0.00468	↑ (1)
Valine	0.001	0.00156	0.823	0.844658	↑ (1)
Glycine	0	0	0.004	0.006	↓ (5)
LPC (16:1)	0	0	0	0	↓ (5)
PC (33:1)	0	0	0	0	↓ (5)
PC (37:6)	0	0	0	0	↓ (5)
Ceramide (d42:1)	0	0	0	0	↑ (5)
LPC (18:0)	0.057	0.060081	0.042	0.051188	↑ (5)
Malic acid	0.757	0.757	0	0	↑ (5)
Pls-PC (38:3)	0	0	0	0	↑ (5)
SM (43:1)	0.009	0.010324	0.008	0.011556	↑ (5)
SM (d18:1/16:0)	0.001	0.00156	0	0	↑ (5)
SM (d18:1/21:0)	0	0	0.002	0.003545	↑ (5)
SM (d42:1)	0	0	0	0	↑ (5)
Sucrose	0.423	0.434132	0	0	↑ (5)
Pls-PC (38:4) A	0	0	0	0	↑ (1, 5)
Pls-PC (40:3)	0	0	0	0	↑ (1, 5)
SM (d16:1/20:0)	0	0	0	0	↑ (1, 5)
SM (d18:1/16:1)	0	0	0	0	↑ (1, 5)

These data were generated by pooling the results from study A and study B and then applying a paired *t*-test between baseline and chronic or acute samples.

Table S3. Demographic and clinical details from human subjects

Subject ID	Age (y)	Sex	Race	BMI (kg/m^2)	Glucose (mg/dL)	Cholesterol (mg/dL)	Triglycerides (mg/dL)	Morningness-eveningness Composite Scale	Actigraphic sleep onset (h)*	Actigraphic sleep offset (h)*	Actigraphic sleep midpoint (h)*	PSG total sleep time SR1 (h)	PSG total sleep time SR5 (h)
A	25	M	AA	27.69	73.0	188.0	112.0	45	23.40	7.17	3.05	3.80	3.93
B	32	M	AA	25.40	76.0	158.0	47.0	55	23.53	6.98	3.03	2.73	3.99
C	32	F	AA	28.16	78.0	165.0	32.0	41	23.33	6.77	3.25	3.83	3.76
D	27	F	AA	25.36	74.0	145.0	42.0	45	23.28	7.05	2.97	3.83	3.97
E	24	F	AA	26.22	83.0	135.0	37.0	37	24.62	8.85	4.73	3.76	3.72
F	22	M	H	25.89	80.0	156.0	36.0	47	24.30	7.83	2.38	3.90	3.92
G	23	F	AA	24.98	81.0	215.0	69.0	45	23.37	6.83	3.10	3.80	3.88
H	23	F	AA	17.25	74.0	209.0	45.0	48	22.88	6.75	2.82	3.73	3.87
I	40	M	AA	27.14	81.0	160.0	295.0	45	23.43	6.70	3.07	3.74	3.78
J	27	M	AA	21.67	92.0	140.0	43.0	43	24.13	8.48	4.30	3.81	3.93

AA, African American; BMI, body mass index; F, female; H, Hispanic; M, male; PSG, polysomnography; SR1, sleep restriction night 1; SR5, sleep restriction night 5.

*One week before study.

Table S4. Metabolite set enrichment analysis

Metabolites	Total	Expected	Hits	Raw *P*	Holm *P*	FDR
Rat sleep restriction metabolites						
Ammonia recycling	18	0.305	2	0.035	1	1
Protein biosynthesis	19	0.322	2	0.0388	1	1
Valine, leucine, and isoleucine degradation	36	0.611	2	0.121	1	1
Malate-aspartate shuttle	8	0.136	1	0.128	1	1
Glutathione metabolism	10	0.17	1	0.158	1	1
Histidine metabolism	11	0.187	1	0.173	1	1
β-Oxidation of very long-chain fatty acids	14	0.238	1	0.215	1	1
Starch and sucrose metabolism	14	0.238	1	0.215	1	1
Sphingolipid metabolism	15	0.255	1	0.228	1	1
Propanoate metabolism	18	0.305	1	0.268	1	1
Phospholipid biosynthesis	19	0.322	1	0.28	1	1
Porphyrin metabolism	22	0.373	1	0.317	1	1
Citric acid cycle	23	0.39	1	0.329	1	1
Methionine metabolism	24	0.407	1	0.341	1	1
Galactose metabolism	25	0.424	1	0.352	1	1
Glycine, serine, and threonine metabolism	26	0.441	1	0.364	1	1
Gluconeogenesis	27	0.458	1	0.375	1	1
Bile acid biosynthesis	49	0.832	1	0.579	1	1
Human sleep restriction metabolites						
Phenylalanine and tyrosine metabolism	13	0.173	1	0.161	1	1
Nicotinate and nicotinamide metabolism	13	0.173	1	0.161	1	1
Protein biosynthesis	19	0.253	1	0.227	1	1
Phospholipid biosynthesis	19	0.253	1	0.227	1	1

Other Supporting Information Files

Dataset S1 (XLSX)
Dataset S2 (PDF)