Supplementary data

Chronic exposure to low dosesof pharmaceuticals disturbs the hepatic expression of circadian genes in lean and obese mice

Sébastien Anthérieu, Dounia Le Guillou, Cédric Coulouarn, Karima Begriche, Viviane Trak-Smayra, Sophie Martinais, Mathieu Porceddu, Marie-Anne Robin, Bernard Fromenty

SupplementaryMaterials and Methods

Gene expression profiling. For the linear T7-based amplification step, 1 μ g of each total RNA sample was used. To produce Cy3-labeled cRNA, the RNA samples were amplified and labeled using the Agilent Quick Amp Labeling Kit (Agilent Technologies) following the manufacturer's protocol. The hybridization procedure was performed according to the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene Expression Hybridization Kit (Agilent Technologies). Briefly, 1.65 μ g Cy3-labeled fragmented cRNA was hybridized overnight (17 hours, 65 °C) to Agilent Whole Mouse Genome Oligo Microarrays 4x44K using Agilent's recommended hybridization chamber and oven. Microarrays were then washed once with the Agilent Gene Expression Wash Buffer 1 for 1 min at room temperature followed by a second wash with preheated Agilent Gene Expression Wash Buffer 2 (37 °C) for 1 min. The last washing step was performed with acetonitrile. Finally, fluorescence signals of the hybridized Agilent Microarrays were detected using Agilent's Microarray Scanner System (Agilent Technologies).

Real-time quantitative PCR (RT-qPCR) analysis. The PCRconditions were one cycle at 50°C for 2 min and one cycle at 95°C for 10 min followed by40 cycles at 95°C for 15 s and 60°C for 1 min. Amplification of specific transcripts was confirmed by melting curve profiles generated at the end of each run. Moreover, PCR specificitywas further ascertained with a 1.75% agarose gel electrophoresisby checking the length of the PCR products. Expression of cyclophilin B was used as reference, and the2^{$-\Delta\Delta$ Ct} method was used to express the relative expression each selected gene (Massart et al., 2012).Sequences of the primers used in this study are available on request.

Supplementary Table S1.

Features of hepatic steatosis in obese mice treated or not with the cocktail containing 10^5 or 10^6 ng/L of each drug.

Ob/ob mice	Percentage of hepatocytes with steatosis	Pattern of the steatosis micro / medio / macro ^a	
Controls			
mouse 1 ^b	70	50 / 30 / 20	
mouse 2	80	70 / 15 / 15	
mouse 3	70	40 / 35 / 25	
mouse 4	80	50 / 25 / 25	
mouse 5	70	60 / 10 / 30	
mouse 6	70	50 / 30 / 20	
mean for the 6 mice	73	53 / 24 / 23	
Treated (10 ⁵ ng/L)			
mouse 7	85	35 / 30 / 35	
mouse 8	85	40 / 30 / 30	
mouse 9	85	35 / 35 / 30	
mouse 10	80	55 / 25 / 20	
mouse 11	70	60 / 20 / 20	
mouse 12	75	60 / 20 / 20	
mean for the 6 mice	80	47 / 27 / 26	
Treated (10° ng/L)			
mouse 13	90	30 / 40 / 30	
mouse 14 ^b	80	35 / 35 / 30	
mouse 15	80	50 / 30 / 20	
mouse 16	70	40 / 30 / 30	
mouse 17	70	40 / 35 / 25	
mouse 18 ^b	80	35 / 30 / 35	
mouse 19	70	30 / 40 / 30	
mean for the 7 mice	77	37*/34/29	

mean jor the / mice//37*/34/29Note: a Thethree percentages in this column represent the proportion of steatotichepatocytes containing lipid droplets of small, medium and large size, respectively.b These three mice were selected for Figure 1. *Significantly different from controlob/ob mice (P<0.05) with the nonparametric Mann-Withney test.</td>

SupplementaryTable S2.

Gene ontology (GO) analysis of hepatic genes differentially expressed by drugs (10^6 ng/L) in lean and/or ob/ob mice.

N^1	X ²	LOD ³	P ⁴	P_adj⁵	GO ID	Functional category
3	55	1,955	1,29E-05	0,027	GO:0007623	circadian rhythm

A) Genes differentially expressed (up- or down-regulated) by drugs in both lean and ob/ob mice

B) Genes differentially expressed (up- or down-regulated) by drugs in ob/ob mice only

Ν	X	LOD	Р	P_adj	attrib ID	attrib name
14	311	0,681	5,80E-06	0,012	GO:0022403	cell cycle phase
18	437	0,642	9,62E-07	0,002	GO:0007010	cytoskeleton organization
19	508	0,598	2,00E-06	0,003	GO:0022402	cell cycle process
18	501	0,579	6,50E-06	0,013	GO:0051726	regulation of cell cycle
18	524	0,559	1,19E-05	0,026	GO:0008092	cytoskeletal protein binding
36	1351	0,458	2,64E-07	< 0.001	GO:0006996	organelle organization
26	963	0,454	1,09E-05	0,02	GO:0005856	cytoskeleton
27	1006	0,452	8,18E-06	0,018	GO:0044430	cytoskeletal part
42	1936	0,366	5,35E-06	0,011	GO:0043228	non-membrane-bounded organelle
						intracellular non-membrane-bounded
42	1936	0,366	5,35E-06	0,011	GO:0043232	organelle
161	10463	0,347	1,16E-10	< 0.001	GO:0044424	intracellular part
134	8563	0,306	1,25E-08	< 0.001	GO:0043229	intracellular organelle
134	8580	0,304	1,43E-08	< 0.001	GO:0043226	organelle
67	3848	0,282	1,26E-05	0,026	GO:0044422	organelle part
79	4696	0,276	5,58E-06	0,012	GO:0005737	cytoplasm
76	4615	0,261	1,98E-05	0,034	GO:0005634	nucleus
						intracellular membrane-bounded
113	7701	0,234	1,59E-05	0,03	GO:0043231	organelle
113	7706	0,234	1,65E-05	0,03	GO:0043227	membrane-bounded organelle

C) Genes differentially expressed (up- or down-regulated) by drugs in lean mice only

Ν	X	LOD	Р	P_adj	attrib ID	attrib name
5	33	1,274	2,11E-05	0,036	GO:0009267	cellular response to starvation
9	106	0,983	1,59E-06	0,005	GO:0019838	growth factor binding
14	327	0,665	8,64E-06	0,013	GO:0007167	enzyme linked receptor protein signaling pathway
24	730	0,551	6,70E-07	<0.001	GO:0003700	sequence-specific DNA binding transcription factor activity
24	732	0,549	7,04E-07	<0.001	GO:0001071	nucleic acid binding transcription factor activity
38	1646	0,400	2,69E-06	0,006	GO:0006351	transcription, DNA-dependent
38	1656	0,397	3,10E-06	0,006	GO:0032774	RNA biosynthetic process
53	2558	0,363	6,12E-07	< 0.001	GO:0010556	regulation of macromolecule biosynthetic process
51	2494	0,355	1,55E-06	0,005	GO:2000112	regulation of cellular macromolecule biosynthetic process

43	2092	0,349	1,05E-05	0,015	GO:0009059	macromolecule biosynthetic process
48	2363	0,348	4,03E-06	0,007	GO:0051252	regulation of RNA metabolic process
42	2059	0,344	1,62E-05	0,026	GO:0034645	cellular macromolecule biosynthetic process
54	2717	0,344	1,69E-06	0,005	GO:0009889	regulation of biosynthetic process
55	2783	0,342	1,57E-06	0,005	GO:0048518	positive regulation of biological process
66	3425	0,342	2,53E-07	< 0.001	GO:0080090	regulation of primary metabolic process
53	2681	0,340	2,53E-06	0,006	GO:0031326	regulation of cellular biosynthetic process
63	3312	0,332	8,19E-07	0,002	GO:0060255	regulation of macromolecule metabolic process
45	2297	0,328	2,12E-05	0,036	GO:0006355	regulation of transcription, DNA- dependent
52	2693	0,327	6,34E-06	0,012	GO:0010468	regulation of gene expression
53	2756	0,326	5,68E-06	0,012	GO:0051171	regulation of nitrogen compound metabolic process
52	2729	0,321	9,23E-06	0,013	GO:0019219	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process
156	10584	0,320	3,23E-09	< 0.001	GO:0005488	binding
48	2520	0,317	2,22E-05	0,05	GO:0048522	positive regulation of cellular process
55	2954	0,312	9,41E-06	0,013	GO:0032502	developmental process
72	4030	0,309	1,11E-06	0,004	GO:0019222	regulation of metabolic process
62	3463	0,300	7,61E-06	0,013	GO:0031323	regulation of cellular metabolic process
89	5482	0,277	2,29E-06	0,006	GO:0005515	protein binding
79	4904	0,262	1,56E-05	0,026	GO:0043170	macromolecule metabolic process
120	8467	0,232	1,66E-05	0,027	GO:0065007	biological regulation

Note: ¹N, number of entities in the original query that have the row's attribute

² X, total number of entities that have the row's attribute in the entire space of entities under consideration (the "mgi_symbol" genespace was used)

³ LOD, logarithm (base 10) of the odds ratio;

positive and negative values indicate over- and underrepresentation, respectively

⁴ P, single hypothesis one-sided P-value of the association between attribute and query (Fisher's Exact Test)

⁵ P_adj, adjusted P-value: fraction (as a %) of 1000 null-hypothesis simulations having attributes with this single-hypothesis P value or smaller SupplementaryFigure S1. Validation of hepatic gene expression profiles in ob/ob mice.



(A) Hierarchical clustering analysis of 2,434 non-redundant genes differentially expressed between lean and ob/ob mice (P<0.001; FC>2; Suppl. Table 1). (B) Gene set enrichment analysis (GSEA) using independent gene expression profiles of livers from ob/ob and lean mice, uploaded from the GEO database (GSE20878; Sharma et al., 2010). GSEA demonstrated that up- and down-regulated genes in ob/ob livers identified in (A) were significantly enriched in the respective gene profiles of ob/ob and lean mice from the independent GSE20878 dataset. (C) Conversely, GSEA was performed by using the gene expression of the liver from lean and ob/ob mice established in our study and the gene signatures established from the independent dataset. Further validating our gene expression profiles, up- and down-regulated genes in ob/ob livers from the independent GSE20878 dataset are enriched in the respective gene profiles of ob/ob and lean mice from the independent dataset.



SupplementaryFigure S2. Difference of hepatic expression of 8 circadian genes between male and female wild-type and ob/ob mice.

Hepatic mRNA expression of 8 circadian genes in untreated male and female wild-type and obese mice. Results are mean \pm SEM for 4 to 6 mice. Statistical analysis was performed with a two-way ANOVA: G, effect of genotype, S, effect of sex, SxG, interaction between genotype and sex.