

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

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

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23 *Abbreviations:* Alas1, aminolevulinic acid synthase 1; ALT, alanine aminotransferase; APAP,  
24 acetaminophen; Arntl, aryl hydrocarbon receptor nuclear translocator-like; Arrdc3, arrestin domain  
25 containing 3; AST, aspartate aminotransferase; Atp2b2, ATPase calcium transporting, plasma  
26 membrane 2; Bmal1; brain and muscle ARNT-like 1; Clock, circadian locomotor output cycles kaput;  
27 Cry1, Cryptochrome 1; CYP, cytochromes P450; Dbp, D site albumin promoter binding protein; GEO,  
28 gene expression omnibus; GSEA, gene set enrichment analysis; H&E, hematoxylin-eosin; Mt,  
29 metallothionein; Npas2, neuronal PAS domain protein 2; Per, period circadian clock; RT-qPCR, Real-  
30 time quantitative PCR; Usp2, ubiquitin specific peptidase 2.

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34

35 ABSTRACT

36

37 Drinking water can be contaminated with pharmaceuticals. However, it is uncertain whether this  
38 contamination can be harmful for the liver, especially during obesity. Hence, the goal of our study  
39 was to determine whether chronic exposure to low doses of pharmaceuticals could have deleterious  
40 effects in livers of lean and obese mice. To this end, lean and ob/ob male mice were treated for 4  
41 months with a mixture of 11 drugs provided in drinking water at concentrations ranging from 10 to  
42  $10^6$  ng/l. At the end of the treatment, some liver and plasma abnormalities were observed in ob/ob  
43 mice treated with the cocktail containing  $10^6$  ng/l of each drug. For this dosage, a gene expression  
44 analysis by microarray showed altered expression of circadian genes (e.g. Bmal1, Dbp, Cry1) in  
45 lean and obese mice. RT-qPCR analyses carried out in all groups of animals confirmed that  
46 expression of 8 different circadian genes was modified in a dose-dependent manner. For some  
47 genes, a significant modification was observed for dosages as low as  $10^2$ - $10^3$  ng/l. Drug mixture and  
48 obesity presented an additive effect on circadian gene expression. These data were validated in an  
49 independent study performed in female mice. Thus, our study showed that chronic exposure to trace  
50 pharmaceuticals disturbed hepatic expression of circadian genes, particularly in obese mice.  
51 Because some of the 11 drugs can be found in drinking water at such concentrations (e.g.  
52 acetaminophen, carbamazepine, ibuprofen) our data could be relevant in environmental toxicology,  
53 especially for obese individuals exposed to these contaminants.

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57 *Key words:* microarray; drug; liver; mouse; obesity; circadian rhythm

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69 **Introduction**

70

71 Contamination of air, soil and water with pharmaceuticals and personal care products is currently  
72 a major concern for many countries. Indeed, such contamination could endanger the health of  
73 millions of individuals, especially in the case of chronic and multiple exposures in sensitive  
74 populations (Sanderson, 2011). Regarding pharmaceuticals, it is noteworthy that the parent drugs  
75 and their metabolites are excreted by patients as waste and some people also get rid of unused  
76 pharmaceuticals in the toilets. Hence, all these pharmaceuticals can thereafter be found in water  
77 (Kasprzyk-Hordern et al., 2008; Mompelat et al., 2009). For instance, the following drugs were  
78 detected in tap water in several independent studies: acetaminophen (APAP), bezafibrate and other  
79 fibrates, caffeine, carbamazepine, cotinine (a nicotine metabolite), diazepam, diclofenac,  
80 erythromycin, flumequine, ibuprofen, phenazone (antipyrine), roxithromycin, salicylic acid and  
81 sulfamethoxazole (Daughton and Ternes, 1999; Halling-Sorensen et al., 1998; Heberer, 2002;  
82 Mompelat et al., 2009; Stackelberg et al., 2004; Webb et al., 2003; Ye and Weinberg, 2007; Yu et  
83 al., 2007; Zuccato et al., 2000). As reported in most of these investigations, these drugs were found  
84 in the drinking water at concentrations ranging generally between 1 and 20 ng/l. However, for some  
85 pharmaceuticals such as APAP, caffeine, carbamazepine, fibrates, ibuprofen and phenazone, several  
86 hundreds of ng/l were sometimes found in tap water (Daughton and Ternes, 1999; Mompelat et al.,  
87 2009).

88 Long-term exposure to drug contaminants could have deleterious consequence for some  
89 sensitive tissues, especially the liver. Indeed, liver expresses high levels of cytochromes P450  
90 (CYPs) that can transform drugs into toxic metabolites, which are able to induce oxidative stress,  
91 mitochondrial dysfunction and cell death (Aubert et al., 2012; Leung et al., 2012). It is also worthy  
92 to mention that recent investigations indicated that the liver could be particularly sensitive to drug-  
93 induced toxicity in the context of obesity (Aubert et al., 2012; Fromenty, 2013).

94 Taken all these data into consideration, the aim of the present study was to determine the long-  
95 term hepatic effects of pharmaceutical contaminants in lean and obese ob/ob mice. These mice were  
96 treated for 4 months with a mixture of 11 drugs provided in drinking water at concentrations ranging  
97 from 10 to 10<sup>6</sup> ng/l. These drugs included APAP, caffeine, carbamazepine, cotinine, diclofenac,  
98 erythromycin, ibuprofen, phenazone, roxithromycin, salicylic acid and sulfamethoxazole.  
99 Importantly, all these molecules were previously detected in drinking water, as mentioned  
100 previously.

## 101 **Materials and methods**

102

103 *Animals and exposure to drugs.* Five-week-old male C57BL/6J-+/+ mice (wild-type, also referred to  
104 as lean mice) weighing 19 to 20g and C57BL/6J-ob/ob mice, weighing 28 to 32 g, were purchased  
105 from Janvier (Le-Genest-St-Isle, France) and housed in the animal house facility of Rennes 1  
106 University under a 12 h light-dark cycle. All mice were fed ad libitum on a normal diet bringing 2820  
107 kcal per kg of food (A04 biscuits; UAR, Villemoisson-sur-Orge, France). After 1 week of  
108 acclimatization, wild-type and ob/ob mice were further split into 7 different groups that were treated  
109 or not with a drug cocktail containing 11 molecules at the following concentrations: 10, 10<sup>2</sup>, 10<sup>3</sup>,  
110 10<sup>4</sup>, 10<sup>5</sup> and 10<sup>6</sup> ng/l. All these drugs (APAP, caffeine, carbamazepine, cotinine, diclofenac,  
111 erythromycin, ibuprofen, phenazone, roxithromycin, salicylic acid and sulfamethoxazole) were  
112 purchased from Sigma-Aldrich (St. Quentin-Fallavier, France). Considering the molecular weight of  
113 the molecules, 10<sup>6</sup> ng/l corresponded to concentrations ranging from 1.2 μM for roxithromycin to  
114 7.2 μM for salicylic acid. In a second independent series of investigations, five-week-old female  
115 C57BL/6J-+/+ and C57BL/6J-ob/ob mice were purchased from Janvier (Le-Genest-St-Isle, France)  
116 and housed in the animal house facility of the Robert Debré Hospital. In this study, mice were  
117 treated or not with the same cocktail containing the 11 drugs at the following concentrations: 10<sup>4</sup>,  
118 10<sup>5</sup> and 10<sup>6</sup> ng/l. For both studies conducted in Rennes and Paris, mice were exposed during 4  
119 months to these drugs by way of the drinking water, which was renewed every week. Because ob/ob  
120 mice are drinking more than lean mice (Fromenty et al. 2009; Massart et al. 2012), drug  
121 concentrations in the drinking water was adapted in the group of obese mice to keep constant the  
122 daily intake of the drugs between lean and obese mice. Consumption of water was monitored every  
123 week and it was not reduced by the presence of the mixture, whatever the drug concentrations. The  
124 last day of the treatment, blood was drawn in the morning between 10 a.m. and 2 p.m. Blood  
125 withdrawal was carried out from the retro orbital sinus with heparinized capillary Pasteur pipettes  
126 for biochemistry analyses. Mice were then sacrificed by cervical dislocation and liver was quickly  
127 removed. While a majority of the liver fragments were immediately frozen in liquid nitrogen some  
128 of them were rapidly processed for appropriate histological staining. Collected tissues frozen in  
129 liquid nitrogen were subsequently stored at -80°C until use. All experiments were performed  
130 according to national guidelines for the use of animals in biomedical research and approved by the  
131 local Ethics Committee in Animal Experiment of Rennes 1 University and Robert Debré Hospital.

132

133 *Plasma analyses.* Immediately after collection, blood was centrifuged for 10 min at 1000g and  
134 plasma was stored at -20°C until assay. Plasma activity of alanine aminotransferase (ALT) and

135 aspartate aminotransferase (AST), total cholesterol, triglycerides and glucose levels were measured  
136 on an automatic analyzer AU2700 (Olympus Diagnostics, Rungis, France) with Olympus  
137 commercial Kits OSR6107, OSR6109, OSR6116, OSR6133 and OSR6121, respectively.

138

139 *Liver histology.* To evaluate necrosis, inflammation and steatosis, liver fragments were fixed in 10%  
140 neutral formalin and embedded in paraffin. Then, 4- $\mu$ m thick sections were cut and stained with  
141 hematoxylin-eosin (H&E). All these sections were thoroughly examined by an experienced  
142 pathologist (V.T.-S.). The amount of hepatic steatosis in ob/ob and the classification of this lesion  
143 into 3 different categories (i.e. microvesicular, mediovesicular and macrovacuolar) were determined  
144 as recently described (Trak-Smayra et al., 2011).

145

146 *RNA extraction and gene expression profiling.* For the microarrays, total RNA extraction, linear T7-  
147 based amplification step, hybridization procedure, detection and read out of the fluorescence signals  
148 were carried out by Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Total RNA was  
149 extracted from liver samples using a standard RNA extraction protocol (Trizol) and quality-checked  
150 with an Agilent 2100 Bioanalyzer platform. All RNA samples revealed RNA Integrity Number  
151 (RIN) values between 7.2 and 8. Further information on T7-based amplification, Cy3-labeling,  
152 hybridization of the Agilent microarray and detection of the fluorescent signals is given in the  
153 Supplementary Data.

154

155 *Analysis and data mining of microarray data.* Gene expression data were analyzed using Feature  
156 Extraction and GeneSpring softwares (Agilent Technologies) and further analyzed using R-based  
157 array tools, as previously described (Sulpice et al., 2013). Agilent Feature Extraction Software was  
158 used to read out and process the microarray image files. This software determines feature intensities  
159 (including background subtraction), rejects outliers and calculates statistical confidences. Features  
160 that were not positive and significant or below the background signal were filtered out. Microarray  
161 data were normalized by using the 75<sup>th</sup> percentile shift normalization algorithm. Differentially  
162 expressed genes were identified by a two-sample univariate *t*-test and a random variance model as  
163 previously described (Coulouarn et al., 2012). Clustering analysis was done using Cluster 3.0 and  
164 TreeView 1.6 with uncentered correlation and average linkage options. Enrichment for specific  
165 biological functions or canonical pathways was evaluated by using gene ontology annotations, as  
166 described previously (Coulouarn et al., 2012). Gene set enrichment analysis (GSEA) was performed  
167 using the Java-tool developed at the Broad Institute (Cambridge, MA, USA). Microarray data were  
168 submitted to the gene expression omnibus (GEO) database ([www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo);  
169 GSE49195).

170

171 *Real-time quantitative PCR (RT-qPCR) analysis.* In order to study the hepatic mRNA expression of  
172 selected genes, cDNAs were prepared by reverse transcription of 1 µg of total RNA using the High  
173 Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Courtaboeuf, France). cDNAs were  
174 thus amplified with specific primers using the Power SYBRGreen PCR Master Mix (Applied  
175 Biosystems), in an ABI Prism 7900 instrument (Applied Biosystems). Further information on PCR  
176 analysis is provided in the Supplementary Data.

177

178 *Statistical analysis.* Data are presented as means ± SEM (standard error of mean). In order to assess  
179 statistical significances for body weight, plasma parameters and hepatic mRNA expression of  
180 selected genes, a two-way Analysis of variance (ANOVA) was performed with the factors of  
181 genotype (G) and drug mixture treatment (T). Individual means were compared with the post hoc  
182 Bonferroni test.

183

184 **Results**

185

186 *Body weight, plasma parameters and liver histology*

187 Body weight was measured on a regular basis for each mouse so that the gain of body weight  
188 could be calculated over the 4-month period of treatment. Body weight was  $31.2 \pm 1.0$  and  $60.6 \pm$   
189  $1.0$  g, respectively in untreated lean and obese mice at the end of the treatment. The gain of body  
190 weight was higher in obese mice compared to lean mice and the treatment significantly modified  
191 this parameter (Table 1). However, it was either decreased, unchanged or increased depending in the  
192 different groups with no dose-dependent effect (Table 1).

193 Some plasma parameters were measured at the end of the treatment. When compared to lean  
194 mice, untreated ob/ob mice presented higher levels of plasma ALT, AST and total cholesterol but  
195 normal levels of plasma triglycerides (Table 1), as previously reported (Fromenty et al., 2009;  
196 Lindström, 2007; Massart et al., 2012). In addition, the obese mice did not present hyperglycemia  
197 (Table 1), which was consistent with previous data showing that the diabetic state of ob/ob mice  
198 ameliorated with age (Fromenty et al., 2009; Lindström, 2007). Interestingly, treatment with the  
199 drug mixtures significantly modified the plasma levels of total cholesterol and ALT (Table 1). This  
200 was particularly evident in ob/ob mice treated with the cocktail containing  $10^6$  ng/l of each drug  
201 since this group of animals presented the highest plasma levels of total cholesterol and ALT (Table  
202 1).

203 Liver histology was also studied at the end of the treatment in mice treated or not with the  
204 cocktails containing  $10^5$  and  $10^6$  ng/l of each drug. There were no histological alterations in lean  
205 mice whatever the groups of animals (data not shown). Slight necroinflammation was observed in  
206 untreated obese mice (data not shown), which could explain higher levels of plasma AST and ALT  
207 (Begrache et al., 2008; Trak-Smayra et al., 2011). However, treatment with the drug mixture did not  
208 aggravate the histological score of necroinflammation (data not shown).

209 Hepatic steatosis was observed in all obese mice (Supplementary Table 1) but this lesion was  
210 absent in lean mice, treated or not with the cocktails (data not shown). Although the percentage of  
211 hepatocytes with steatosis was unchanged by the treatment in ob/ob mice, a lower proportion of  
212 microvesicular steatosis was observed in obese mice treated with the cocktail containing  $10^6$  ng/l of  
213 each drug (Supplementary Table 1). Accordingly, the fat droplets were larger in treated ob/ob mice  
214 when compared to the controls (Fig. 1).

215

216 *Microarray analysis*



217 Gene expression profiling was carried out in order to determine whether the cocktail containing  
218  $10^6$  ng/l of each drug induced transcriptional changes in the livers of lean and obese mice.  
219 Unsupervised microarray analysis demonstrated that the cocktail significantly modified the hepatic  
220 expression of 280 and 284 genes ( $P < 0.05$ ; fold-change  $> 1.5$ ), respectively in lean and obese mice.  
221 Among these genes, only 21 were dysregulated in both genotypes thus suggesting that drug-induced  
222 transcriptional changes were tightly dependent of the genetic background. Table 2 provides the top  
223 10 well-annotated genes that were up or down-regulated by the cocktail in lean and obese mice.  
224 Interestingly, when a gene ontology analysis was performed, circadian rhythm was found as the  
225 only functional category significantly enriched for the hepatic genes differentially expressed by the  
226 cocktail in both lean and obese mice (Supplementary Table 2). In addition, this analysis indicated  
227 that the functional categories were linked to biosynthetic processes and transcription factor activity  
228 in lean mice and to cell cycle and cytoskeleton for ob/ob mice (Supplementary Table 2).  
229 Although this was not the main goal of this study, our unsupervised microarray analysis allowed us  
230 to determine the differential hepatic gene expression between lean and ob/ob mice. Our analysis  
231 identified 2,434 non-redundant genes that were differentially expressed between lean and obese  
232 mice ( $P < 0.001$ ; fold-change  $> 2$ ; data not shown). As previously reported (Fromenty et al., 2009;  
233 Yang et al., 2010), ob/ob mice were characterized by an increased expression of many genes  
234 involved in lipid synthesis, such as Elov17 (x15.9), Scd2 (x12.7), Agpat9 (x8.3), Elov16 (x7.7),  
235 Gpam (x5.3), Fasn (x4.6), Acaca (x4.6), Acly (x4.2), Pparg (x4.0) and Thrsp (x3.1). Moreover,  
236 there was in ob/ob mice a profound up- or down-regulation of the hepatic expression of several  
237 genes involved in the circadian rhythm such as Dbp (x14.3), Per3 (x7.1), Clock (x0.49), Arntl (also  
238 referred to as Bmal1) (x0.42) and Cry1 (x0.28). Interestingly, a recent study carried out in ob/ob  
239 mice reported lower hepatic expression of the last three genes compared to wild-type mice when the  
240 animals were investigated at zeitgeber times similar to our investigations (i.e. zeitgeber times 2 to 6)  
241 (Ando et al., 2011). In order to validate the whole data set obtained from our microarray analysis, a  
242 GSEA was performed using independent hepatic gene expression profiles from ob/ob and lean mice  
243 (Sharma et al. 2010), uploaded from the GEO database (GSE20878). Importantly, GSEA  
244 demonstrated that up- and down-regulated genes in ob/ob livers identified in our study were  
245 significantly enriched in the respective gene profiles of ob/ob and lean mice from the independent  
246 GSE20878 dataset (Supplementary Fig. 1).

247

#### 248 *mRNA expression of specific genes*

249 Some genes of interest were selected to determine by RT-qPCR their hepatic expression in mice  
250 treated or not with the cocktail containing the 11 drugs at concentrations ranging from 10 to  $10^6$   
251 ng/l. Since the gene ontology analysis pointed a specific dysregulation of genes involved in the

252 circadian rhythm in lean and obese mice, the expression of *Bmal1*, *Clock*, *Dbp*, *Npas2*, *Per1*, *Per2*,  
253 *Per3*, and *Cry1* was studied in all groups of mice. As shown in Fig. 2, the drug cocktail presented a  
254 significant effect on gene expression, which was either decreased (*Bmal1*, *Clock*, *Npas2*, *Cry1*) or  
255 increased (*Dbp*, *Per1*, *Per2*, *Per3*). A clear dose-effect response was observed in lean and/or obese  
256 mice for the expression of all these genes. Moreover, drug mixture and obesity presented an additive  
257 effect on circadian gene expression. Importantly, the cocktail presented a significant effect on  
258 *Bmal1*, *Dbp*, *Npas2*, *Per2*, *Per3*, and *Cry1* expression when it contained as low as  $10^2$  and  $10^3$  ng/l  
259 of each drug in the mixture (Fig. 2). For these concentrations, it could be estimated that each drug  
260 was administered to mice at the respective doses of 20 and 200 ng/kg/d, when considering the daily  
261 intake of water and the body weight of the animals.

262 Because our gene expression profiling was performed on male mice solely, we next validated  
263 our results on female mice. To this end, an independent series of lean and *ob/ob* female mice was  
264 treated or not for 4 months with mixtures containing  $10^4$ ,  $10^5$  and  $10^6$  ng/l of each drug, as described  
265 in the Methods section. Interestingly, the profiles of drug-induced modification of circadian liver  
266 gene expression were about similar to those observed in male mice, except for *Per1* (Fig. 3). It was  
267 noteworthy, however, that the effect of obesity on the hepatic expression of these genes was less  
268 pronounced in female mice (Fig. 3) when compared to males (Fig. 2). We also noticed that the liver  
269 expression of the circadian genes was in general higher in female mice, especially in wild-type  
270 animals (Supplementary Fig. 2), which was consistent with a recent study (Xu et al., 2012).

271 Finally, we determined the hepatic expression of several genes not directly related to circadian  
272 rhythm such as *Alas1*, *Arrdc3*, *Mt1*, *Mt2*, *Usp2* and *Atp2b2*, the expression of which was found  
273 dysregulated in the microarray analysis performed in male mice treated with the highest doses of the  
274 drugs (Table 2). Interestingly, a dose-effect response was in general observed for the expression of  
275 these 6 genes (Fig. 4). However, in female mice, only the expression of *Arrdc3* and *Usp2* was found  
276 to be comparable when compared to male mice (data not shown).

277

## 278 **Discussion**

279

280 This study showed for the first time that chronic oral exposure to low doses of pharmaceuticals  
281 disturbed the hepatic expression of genes involved in circadian rhythm in mice. These  
282 transcriptional effects were dose-dependent and particularly robust since they were found in lean  
283 and obese male mice but also in female animals of both genotypes. In male mice, and to a lesser  
284 extent in female mice, drug mixture and obesity presented an additive effect on the expression of  
285 circadian genes such as *Dbp*, *Per2* and *Per3*. Moreover, the expression of some circadian genes was  
286 dysregulated when the drinking water contained the 11 drugs at concentrations as low as  $10^2$  and  $10^3$   
287 ng/l. Such exposure corresponded to a daily intake of about 20 to 200 ng/kg/d. Hence, our data  
288 indicated that a mixture of pharmaceutical contaminants can alter the expression of hepatic genes at  
289 concentrations that can be found in drinking water. Indeed, for some drugs such as APAP, caffeine,  
290 carbamazepine, ibuprofen and phenazone, several hundreds of ng/l were found in tap water in  
291 previous studies (Daughton and Ternes, 1999; Mompelat et al., 2009).

292 A “whole mixture approach” was used in this study, and thus our investigations were not  
293 designed to determine which drug(s) could be involved in these hepatic effects, in particular on the  
294 expression of circadian genes. Nevertheless, it is noteworthy that some drugs such as prednisolone  
295 (Koyanagi et al., 2006), 5-fluorouracil (Terazono et al., 2008), interferon- $\alpha$  (Shinohara et al., 2008)  
296 and caffeine (Oike et al., 2011; Sherman et al., 2011) can disturb in liver the expression of genes  
297 involved in circadian rhythm. However, apart from caffeine, no information was found in the  
298 literature regarding this issue for the other drugs contained in the mixture. Although caffeine could  
299 have induced the genomic effects reported in our study, it is noteworthy that studies reporting  
300 caffeine-induced altered hepatic expression of clock genes were performed with much higher doses  
301 (from 3.5 to ca. 75 mg/kg/d) (Oike et al., 2011; Sherman et al., 2011). Thus, further investigations  
302 by treating mice with each drug separately would be needed to determine which component(s) of  
303 the mixture could alter the liver expression of clock genes when administered at very low doses.  
304 However, it is possible that the deleterious effects of the mixture uncovered in the present study  
305 might have been less significant with each individual drug (or might not have been observed), as  
306 suggested by some investigations in other experimental models (Christiansen et al., 2009;  
307 Kortenkamp et al., 2007). Interestingly, some of the drugs included in the cocktails belong to the  
308 same pharmacological classes (mainly nonsteroidal anti-inflammatory drugs and antibiotics).  
309 Therefore, it would be worth finding out whether the genomic effects detected in this study could be  
310 attributed to a particular drug class.

311 Although the expression of hepatic genes was disturbed in mice treated for 4 months with the  
312 cocktails containing  $10^2$  and  $10^3$  ng/l of each drug, these concentrations did not change the plasma  
313 parameters assessed in this study, namely ALT, AST, glucose, triglycerides and total cholesterol.  
314 Thus, the effects on gene expression observed in treated mice with these doses were not associated  
315 with significant hepatic cytolysis and systemic alterations of lipid homeostasis. Therefore, changes  
316 in circadian gene expression observed for these doses might not be functionally significant. It would  
317 be interesting to determine whether a much longer exposure to these low concentrations of  
318 pharmaceuticals could have harmful effects on liver biology and integrity beyond the alterations of  
319 gene expression. Furthermore, some selected circadian genes (e.g. *Bmal1*, *Per1*, *Per2*) could be  
320 useful markers for further investigations specifically designed to look for potential genomic effects  
321 of individual drug (or subset of drugs) included in the mixture.

322 It is now acknowledged that disturbances of circadian rhythmicity can promote different  
323 metabolic disorders and chronic diseases such as dyslipidemia, insulin resistance, obesity and  
324 cardiovascular diseases (Duez and Staels, 2008; Maury et al., 2010; Prasai et al., 2008). Moreover,  
325 some studies showed that liver-specific deletion of genes involved in the liver clock could disturb  
326 glucose and lipid homeostasis (Lamia et al., 2008; Tao et al., 2011). In this study, however, the  
327 genomic response to drug exposure was investigated only in the liver. Hence, further investigations  
328 would be required to determine whether an altered expression of clock genes could also be observed  
329 in other tissues such as the CNS and in tissues playing a key role in carbohydrate and lipid  
330 homeostasis. Different time points of the light-dark cycle should also be studied during these  
331 investigations.

332 In this study, the 4-month treatment with the cocktail containing  $10^6$  ng/l of each drug  
333 significantly increased plasma ALT and total cholesterol and changed the pattern of hepatic  
334 steatosis in *ob/ob* mice, whereas these effects were not observed in wild-type mice. For this dosage,  
335 the hepatic expression of most circadian genes in *ob/ob* mice was the highest, or the lowest, among  
336 the different groups of animals. Interestingly, the daily intake corresponding to this dose (ca. 0.2  
337 mg/kg/d) was well below the pharmacological dosages for all drugs included in the mixture. Indeed,  
338 the daily dosage of these drugs is comprised between a few mg/kg/d for diclofenac and  
339 roxithromycin to 40 and 60 mg/kg/d for salicylic acid and APAP, respectively. Importantly, some of  
340 these drugs such as APAP, diclofenac, ibuprofen and salicylate can be taken over the long-term (i.e.  
341 several months) for the treatment of inflammatory diseases or chronic pain (Kuffner et al., 2006;  
342 Schaffer et al., 2006), which are often observed in obese patients (Guh et al. 2009; Wright et al.  
343 2010). Thus, our work could also be relevant for obese patients with multiple drug prescriptions,  
344 since obesity appears to increase the risk of hepatotoxicity, at least for some drugs (Fromenty, 2013;  
345 Massart et al., 2012). Further investigations will be needed to determine whether the genomics

346 changes observed in this study could be involved in higher plasma ALT and total cholesterol  
347 observed in ob/ob mice.

348 In conclusion, our study showed for the first time that chronic exposure to trace  
349 pharmaceuticals was able to disturb the hepatic expression of genes involved in circadian rhythm in  
350 lean and obese mice. This effect was dose-dependent and the expression of some circadian genes  
351 was already dysregulated when the drinking water contained the 11 drugs at concentrations as low  
352 as  $10^2$  and  $10^3$  ng/l. Moreover, drug mixture and obesity presented an additive effect on this gene  
353 expression. Because some of the 11 drugs can be found in the drinking water at such concentrations  
354 (e.g. APAP, caffeine, carbamazepine, ibuprofen, phenazone) further investigations would be needed  
355 to determine whether our data could be extrapolated to human populations exposed to  
356 pharmaceutical contaminants, in particular in countries with high prevalence of obesity.

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## 361 **Acknowledgments**

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364 PHARMECO 09-CESA-014-01).

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## 367 **Supplementary data**

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369 Supplemental data were provided with this manuscript. The transcriptomic dataset (GSE49195)  
370 presented in this study is available in the GEO database at the following URL address:  
371 <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=jvqblqimewcyaju&acc=GSE49195>.

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## 375 **Conflict of interest**

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377 Sébastien Anthérieu, Dounia Menouer, Cédric Coulouarn, Karima Begriche, Viviane Trak-  
378 Smayra, Sophie Martinais and Marie-Anne Robin have nothing to disclose. Mathieu Porceddu  
379 reports co-founding of Mitologics SAS, outside the submitted work. Bernard Fromenty reports

380 personal fees from Medicines for Malaria Venture and from Medicen Paris Region, and a grant from  
381 Société Francophone du Diabète, outside the submitted work.

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- 499

500 **Table 1.**

501 Body weight gain and plasma parameters in the different groups of mice at the end of the treatment.

<b>Animals</b>	<b>Treatment</b>	<b>Body weight gain (g)</b>	<b>Glucose (mM)</b>	<b>Total cholesterol (mM)</b>	<b>Triglycerides (mM)</b>	<b>ALAT (UI/L)</b>	<b>ASAT (UI/L)</b>
<b>Lean</b>	0	11.1 ± 0.8	9.6 ± 0.9	2.49 ± 0.12	1.42 ± 0.17	86 ± 15	194 ± 50
	10 ng/l	9.8 ± 2.1	9.2 ± 0.6	2.21 ± 0.09	1.00 ± 0.09	90 ± 13	147 ± 25
	10 <sup>2</sup> ng/l	11.4 ± 0.7	8.8 ± 0.4	2.28 ± 0.08	1.21 ± 0.16	76 ± 9	164 ± 30
	10 <sup>3</sup> ng/l	10.5 ± 0.4	9.4 ± 0.4	2.31 ± 0.09	1.15 ± 0.08	113 ± 26	187 ± 24
	10 <sup>4</sup> ng/l	11.9 ± 0.5	10.0 ± 0.4	2.43 ± 0.05	1.29 ± 0.10	71 ± 5	145 ± 23
	10 <sup>5</sup> ng/l	12.8 ± 0.3	10.2 ± 0.6	2.67 ± 0.08	1.34 ± 0.03	147 ± 27	189 ± 14
	10 <sup>6</sup> ng/l	11.3 ± 0.6	9.9 ± 0.5	2.53 ± 0.09	1.35 ± 0.18	101 ± 13	159 ± 24
<b>ob/ob</b>	0	30.4 ± 0.8	7.2 ± 0.7	7.88 ± 0.38	1.27 ± 0.12	708 ± 50	451 ± 24
	10 ng/l	27.7 ± 1.5	10.2 ± 3.1	7.43 ± 0.65	1.12 ± 0.09	672 ± 62	674 ± 105
	10 <sup>2</sup> ng/l	31.7 ± 1.6	8.1 ± 0.9	7.81 ± 0.29	1.06 ± 0.02	674 ± 75	587 ± 85
	10 <sup>3</sup> ng/l	30.0 ± 0.6	9.5 ± 1.2	7.73 ± 0.44	1.16 ± 0.08	533 ± 58†	372 ± 42
	10 <sup>4</sup> ng/l	31.3 ± 0.9	8.9 ± 0.7	8.16 ± 0.32	1.19 ± 0.08	683 ± 43	748 ± 230
	10 <sup>5</sup> ng/l	33.3 ± 0.5	9.8 ± 1.0	8.46 ± 0.29	1.07 ± 0.03	844 ± 104	759 ± 133
	10 <sup>6</sup> ng/l	29.1 ± 0.9	9.4 ± 0.5	8.83 ± 0.28†	1.29 ± 0.05	936 ± 56†	702 ± 76
<b>Two-way ANOVA analysis</b>		<b>G,T</b>		<b>G,T</b>		<b>G,T,GxT</b>	<b>G</b>

502 Note: Plasma parameters were determined in the fed state. Body weight gain was calculated for each mouse over  
503 the 4 months of treatment. Results are mean ± SEM for 5 to 7 mice. Statistical analysis was performed with a two-  
504 way ANOVA: G, effect of genotype, T, effect of treatment, GxT, interaction between genotype and treatment.  
505 Individual means were then compared with the *post hoc* Bonferroni test. †Significantly different from untreated  
506 mice of the same genotype ( $P < 0.05$ ).

507

508 **Table 2.**

509 Top 10 well-annotated genes that were up or down-regulated in liver of wild-type and ob/ob mice treated with  
 510 the cocktail containing  $10^6$  ng/l of each drug.

<b>Up-regulated in both lean and ob/ob mice<sup>a</sup></b>	<b>Name (Accession number)</b>	<b>Gene ontology<sup>b</sup></b>
Dbp (lean 3.70 ; ob 2.08) <sup>c</sup>	D site albumin promoter binding protein (NM_016974)	Circadian rhythm. Positive regulation of transcription from RNA polymerase II promoter
Alas1 (lean 2.56 ; ob 1.52)	Aminolevulinic acid synthase 1 (NM_020559)	Biosynthetic process. Heme biosynthetic process
Ypel1 (lean 1.89 ; ob 1.54)	Yippee-like 1 (Drosophila) (NM_023249)	Negative regulation of protein kinase B signaling cascade. Regulation of myelination
Gnat1 (lean 1.79 ; ob 1.79)	Guanine nucleotide binding protein, alpha transducing 1 (NM_008140)	Adenylate cyclase-modulating G-protein coupled receptor signaling pathway. Cell proliferation
Pfkfb3 (lean 1.79 ; ob 1.59)	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (NM_133232)	Fructose 2,6-biphosphate metabolic process. Phosphorylation
Arrdc3 (lean 1.79 ; ob 1.52)	Arrestin domain containing 3 (NM_001042591)	Fat pad development. Negative regulation of heat generation
<b>Down-regulated in both lean and ob/ob mice</b>	<b>Name (Accession number)</b>	<b>Gene ontology</b>
Cry1 (lean 0.38 ; ob 0.65)	Cryptochrome 1 (photolyase-like) (NM_007771)	Circadian rhythm. DNA repair
Hmgcs1 (lean 0.45 ; ob 0.61)	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (AK078743)	Cholesterol biosynthetic process. Isoprenoid biosynthetic process
Trim27 (lean 0.45 ; ob 0.63)	Tripartite motif-containing 27 (AK049314)	Interferon gamma secretion. Negative regulation of adaptive immune response
Cstad (lean 0.50 ; ob 0.63)	CSA-conditional, T cell activation-dependent protein (NM_030137)	Mitochondrial membrane organization
Avpr1a (lean 0.54 ; ob 0.67)	Arginine vasopressin receptor 1A (NM_016847)	Calcium-mediated signaling. Elevation of cytosolic calcium ion concentration
Lonrf3 (lean 0.56 ; ob 0.58)	LON peptidase N-terminal domain and ring finger 3 (NM_028894)	Proteolysis
Cdca2 (lean 0.56 ; ob 0.61)	Cell division cycle associated 2 (NM_175384)	Cell cycle. Cell division
Arntl (lean 0.57 ; ob 0.36)	Aryl hydrocarbon receptor nuclear translocator-like (NM_007489)	Circadian regulation of gene expression. Circadian rhythm
Chka (lean 0.60 ; ob 0.56)	Choline kinase alpha (NM_013490)	CDP-choline pathway. Glycerophospholipid biosynthetic process
Cxadr (lean 0.62 ; ob 0.56)	Coxsackie virus and adenovirus receptor (NM_009988)	Actin cytoskeleton reorganization. AV node cell to bundle of His cell communication
<b>Up-regulated only in lean mice</b>	<b>Name</b>	<b>Gene ontology</b>
Cfd (4.35)	Complement factor D (adipsin) (NM_013459)	Complement activation, alternative pathway. Innate immune response
H19 (3.85)	H19 fetal liver mRNA (AK003142)	Negative regulation of cell proliferation. Regulation of gene expression
Tppp3 (2.94)	Tubulin polymerization-promoting protein family member 3 (NM_026481)	Microtubule bundle formation
Inhbb (2.63)	Inhibin beta-B (NM_008381)	Activin receptor signaling pathway. Cellular response to cholesterol
Kif23 (2.50)	Kinesin family member 23 (NM_024245)	Spindle midzone assembly involved in mitosis
E2f7 (2.44)	E2F transcription factor 7 (NM_178609)	Cell cycle. Chorionic trophoblast cell differentiation
Pdlim3 (2.44)	PDZ and LIM domain 3 (NM_016798)	Actin filament organization. Heart development
Igfbp6 (2.38)	Insulin-like growth factor binding protein 6 (X81584)	Regulation of cell growth
Esm1 (2.33)	Endothelial cell-specific molecule 1 (NM_023612)	Biological process. Regulation of cell growth
Il1rap (2.33)	Interleukin 1 receptor accessory protein (NM_008364)	Cytokine-mediated signaling pathway. Innate immune response
<b>Down-regulated only in lean mice</b>	<b>Name</b>	<b>Gene ontology</b>
Fos (0.19) <sup>2</sup>	FBJ osteosarcoma oncogene (NM_010234)	Cellular response to cellular calcium ion. Cellular response to extracellular stimulus.
Nr4a1 (0.21)	Nuclear receptor subfamily 4, group A, member 1 (NM_010444)	Apoptotic process. Cell migration involved in sprouting angiogenesis
Egr1 (0.31)	Early growth response 1 (NM_007913)	Bone morphogenetic protein signaling pathway. Cellular

		response to gamma radiation
Dusp1 (0.36)	Dual specificity phosphatase 1 (NM_013642)	Cell cycle. Dephosphorylation
Igh-VJ558 (0.40)	Immunoglobulin heavy chain (J558 family) (XM_001474025)	
Igfbp1 (0.40)	Insulin-like growth factor binding protein 1 (NM_008341)	Regulation of cell growth
Fam65b (0.42)	Family with sequence similarity 65, member B (NM_029679)	Biological process
Zfp36 (0.44)	Zinc finger protein 36 (NM_011756)	3'-UTR-mediated mRNA stabilization. Intracellular protein kinase cascade
Fam110c (0.45)	Family with sequence similarity 110, member C (NM_027828)	Positive regulation of cell migration. Positive regulation of protein kinase B signaling cascade
Slc34a2 (0.46)	Solute carrier family 34 (sodium phosphate), member 2 (NM_011402)	Cellular phosphate ion homeostasis. In utero embryonic development
<b>Up-regulated only in ob/ob mice</b>	<b>Name</b>	<b>Gene ontology</b>
Clec2d (2.86)	C-type lectin domain family 2, member d (NM_053109)	Cellular defense response. Negative regulation of osteoclast differentiation
Usp2 (2.78)	Ubiquitin specific peptidase (NM_198092)	Cell cycle. Muscle organ development
Mt2 (2.63)	Metallothionein 2 (NM_008630)	Cellular response to drug. Cellular zinc ion homeostasis
Upp2 (2.56)	Uridine phosphorylase 2 (NM_029692)	Metabolic process. Nucleoside metabolic process
Mt1 (2.44)	Metallothionein 1 (NM_013602)	Cellular metal ion homeostasis. Cellular response to chromate
Krt20 (2.33)	Keratin 20 (NM_023256)	Apoptotic process. Cellular response to stress
Rgs16 (2.27)	Regulator of G-protein signaling 16 (NM_011267)	G-Protein coupled receptor signaling pathway. Negative regulation of signal transduction
Car3 (2.08)	Carbonic anhydrase 3 (AK075630)	One-carbon metabolic process. Response to oxidative stress
Grm8 (1.92)	Glutamate receptor, metabotropic 8 (NM_008174)	Adenylate cyclase inhibiting G-protein coupled glutamate receptor signaling pathway. G-protein coupled receptor signaling pathway
Cdc6 (1.85)	Cell division cycle 6 homolog ( <i>S. cerevisiae</i> ) (NM_011799)	Cell cycle. Cell division
<b>Down-regulated only in ob/ob mice</b>	<b>Name</b>	<b>Gene ontology</b>
Moxd1 (0.18)	Monoxygenase, DBH-like 1 (NM_021509)	Catecholamine metabolic process. Oxidation-reduction process
Hsd3b5 (0.18)	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 5 (NM_008295)	Oxidation-reduction process. Steroid biosynthetic process
Man2a2 (0.40)	Mannosidase 2, alpha 2 (NM_172903)	Carbohydrate metabolic process. Mannose metabolic process
Usp26 (0.45)	Ubiquitin specific peptidase 26 (NM_031388)	Protein deubiquitination. Proteolysis
Gadd45g (0.45)	Growth arrest and DNA-damage-inducible 45 gamma (NM_011817)	Activation of MAPKK activity. Apoptotic process
Atp2b2 (0.45)	ATPase, Ca <sup>2+</sup> transporting, plasma membrane 2 (NM_009723)	ATP catabolic process. Calcium ion export
Ppp1r9a (0.46)	Protein phosphatase 1, regulatory (inhibitor) (NM_181595)	Actin filament organization. Calcium-mediated signaling
Kalrn (0.47)	Kalirin, RhoGEF kinase (AK051053)	Axonogenesis. Intracellular signal transduction
Murc (0.48)	Muscle-related coiled-coil protein (NM_026509)	Cardiac myofibril assembly. Cell differentiation
Elovl3 (0.48)	Elongation of very long chain fatty acids-like 3 (NM_007703)	Fatty acid biosynthetic process. Fatty acid elongation, monounsaturated fatty acid

511 Note:<sup>a</sup>Less than 10 genes were found to be modulated by the drug mixture in this category. <sup>b</sup>The first 2 terms were selected by  
512 using the Amigo Gene Ontology website, with “*Mus musculus*” and “*Biological process*” as filters. <sup>c</sup>The number in parentheses  
513 is the fold change given by the microarray analysis.

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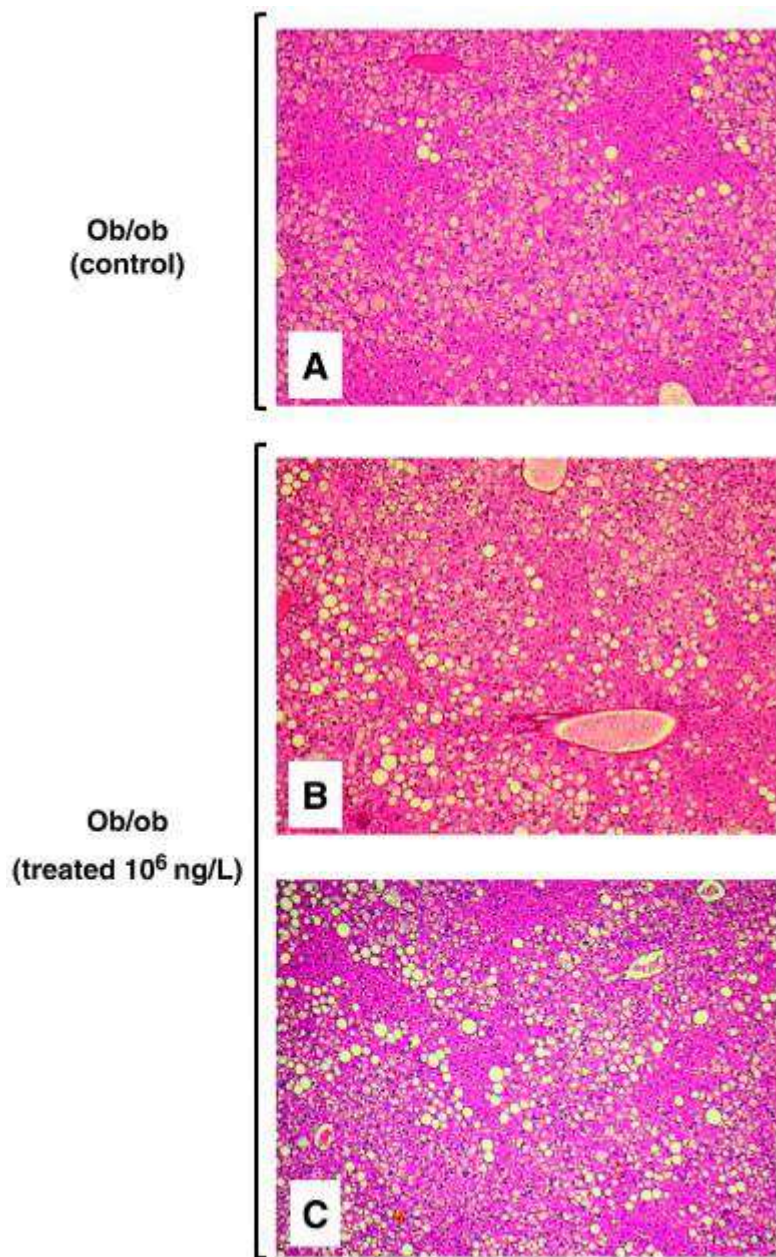
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520 **Legends to the figures**

521



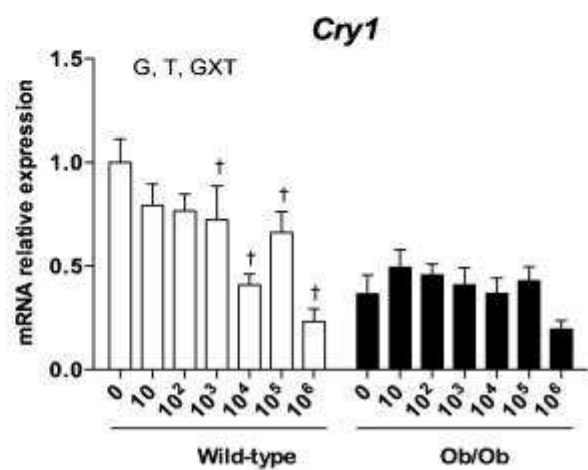
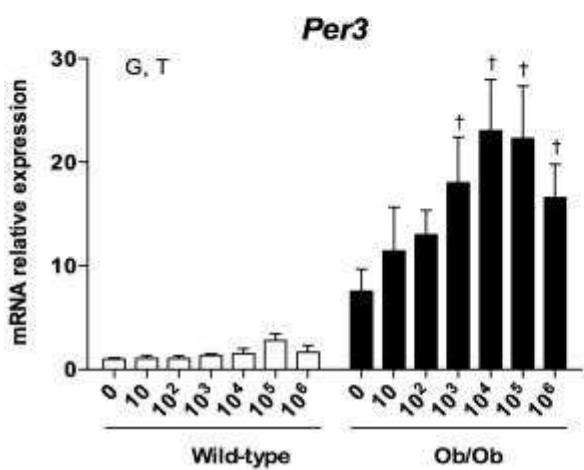
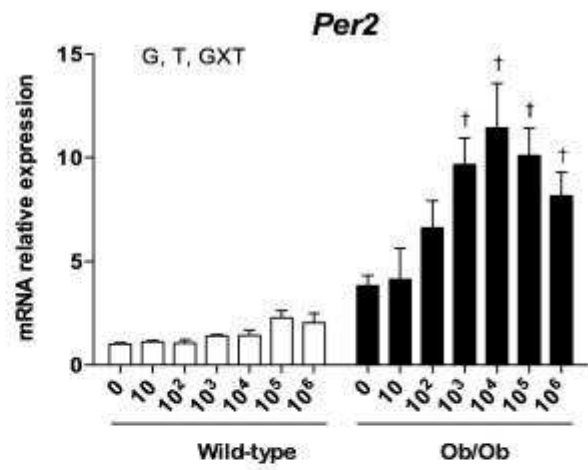
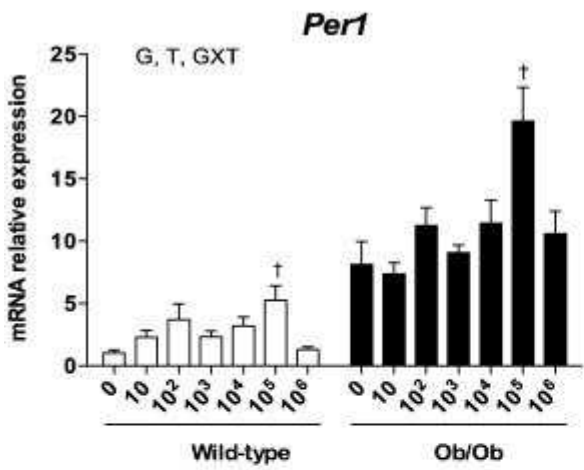
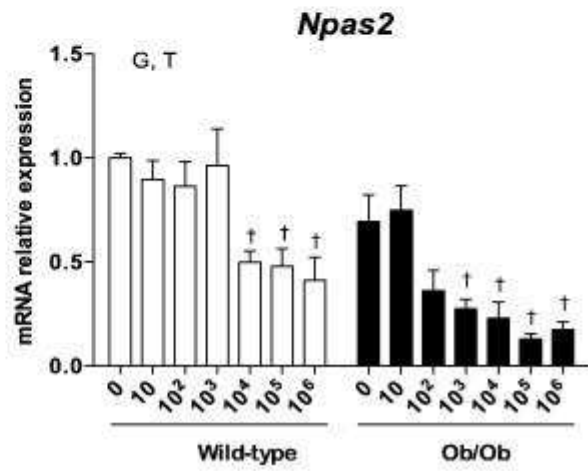
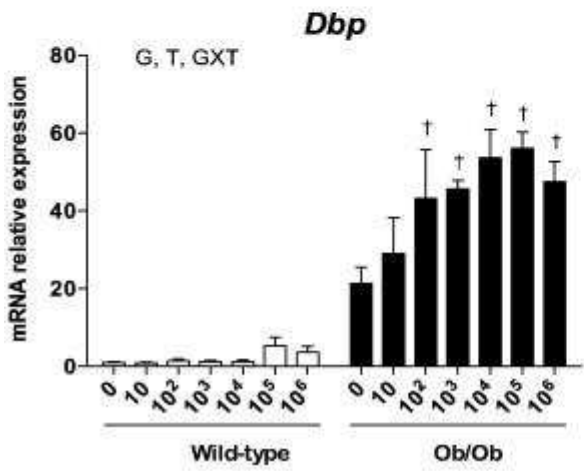
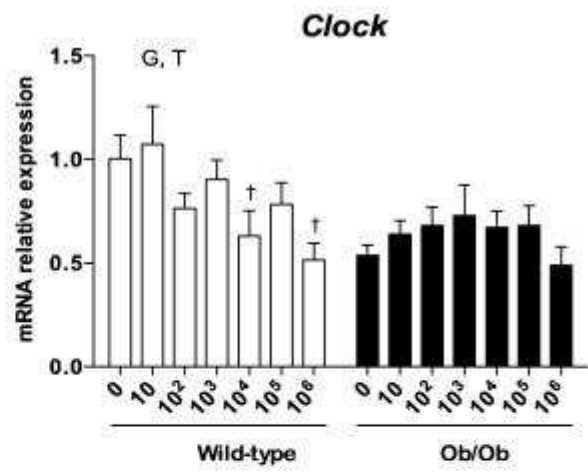
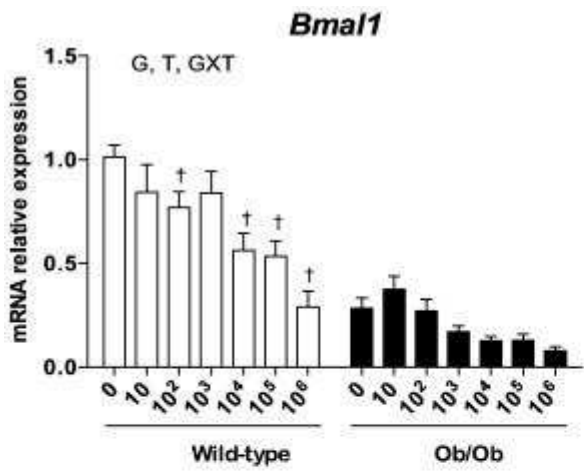
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523

524 **Figure 1.** Hepatic steatosis in obese ob/ob mice treated or not with the cocktail containing  $10^6$  ng/l

525 of each drug. Liver sections were stained with H&E and pictures were taken at 200x magnification.

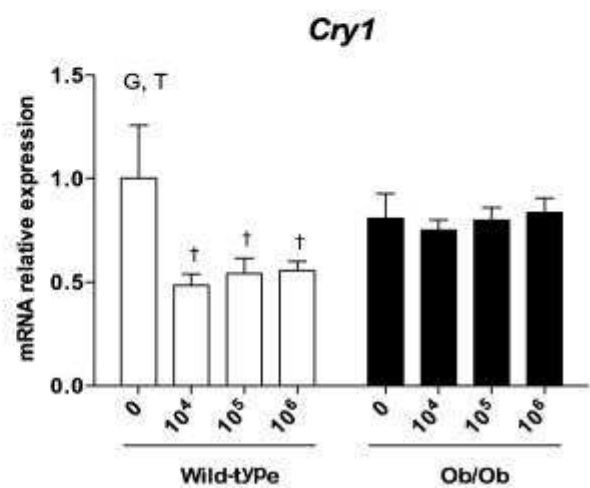
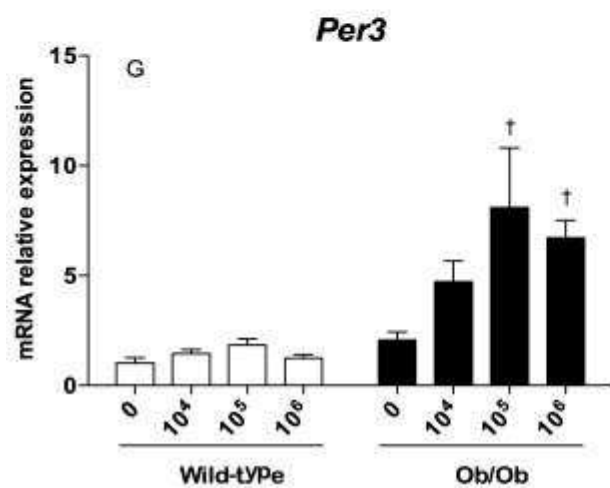
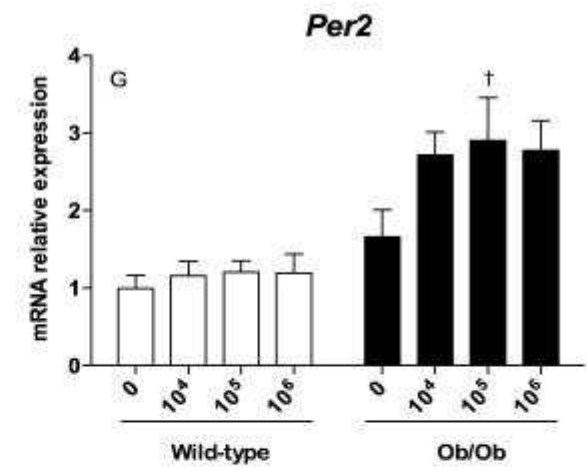
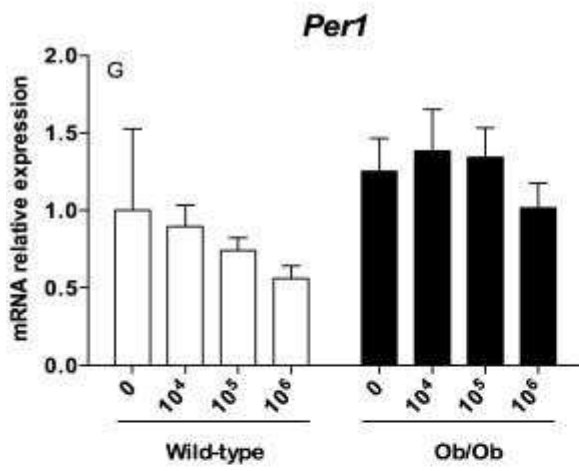
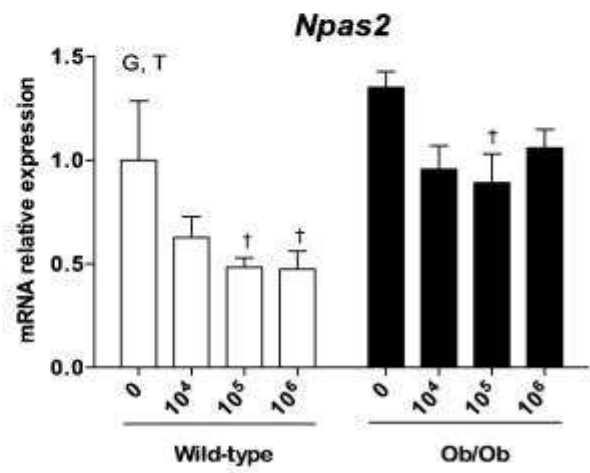
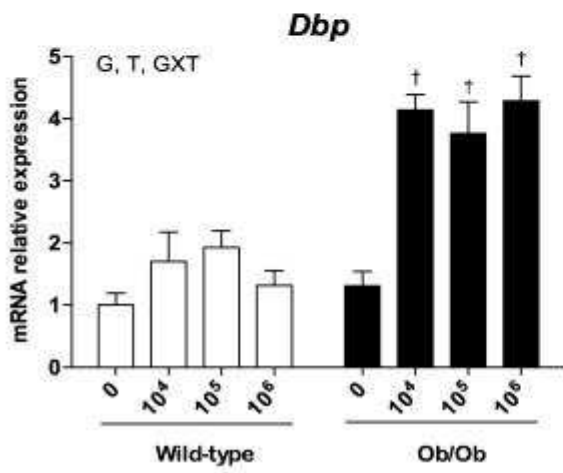
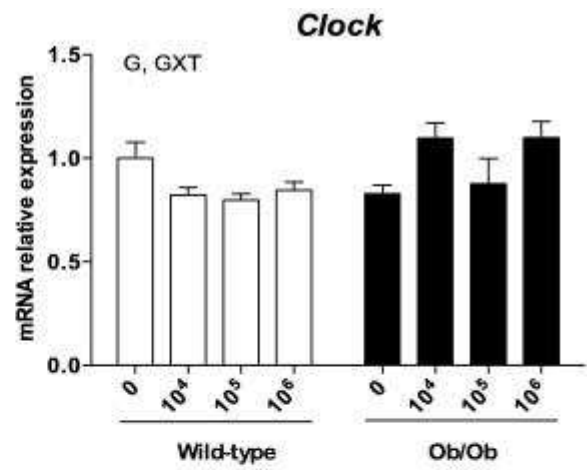
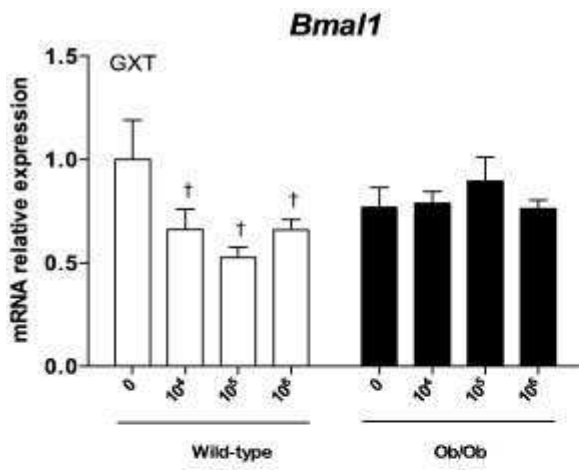
526 Mice presented in panels A, B and C are those numbered 1, 14 and 18 in Supplementary Table 1.



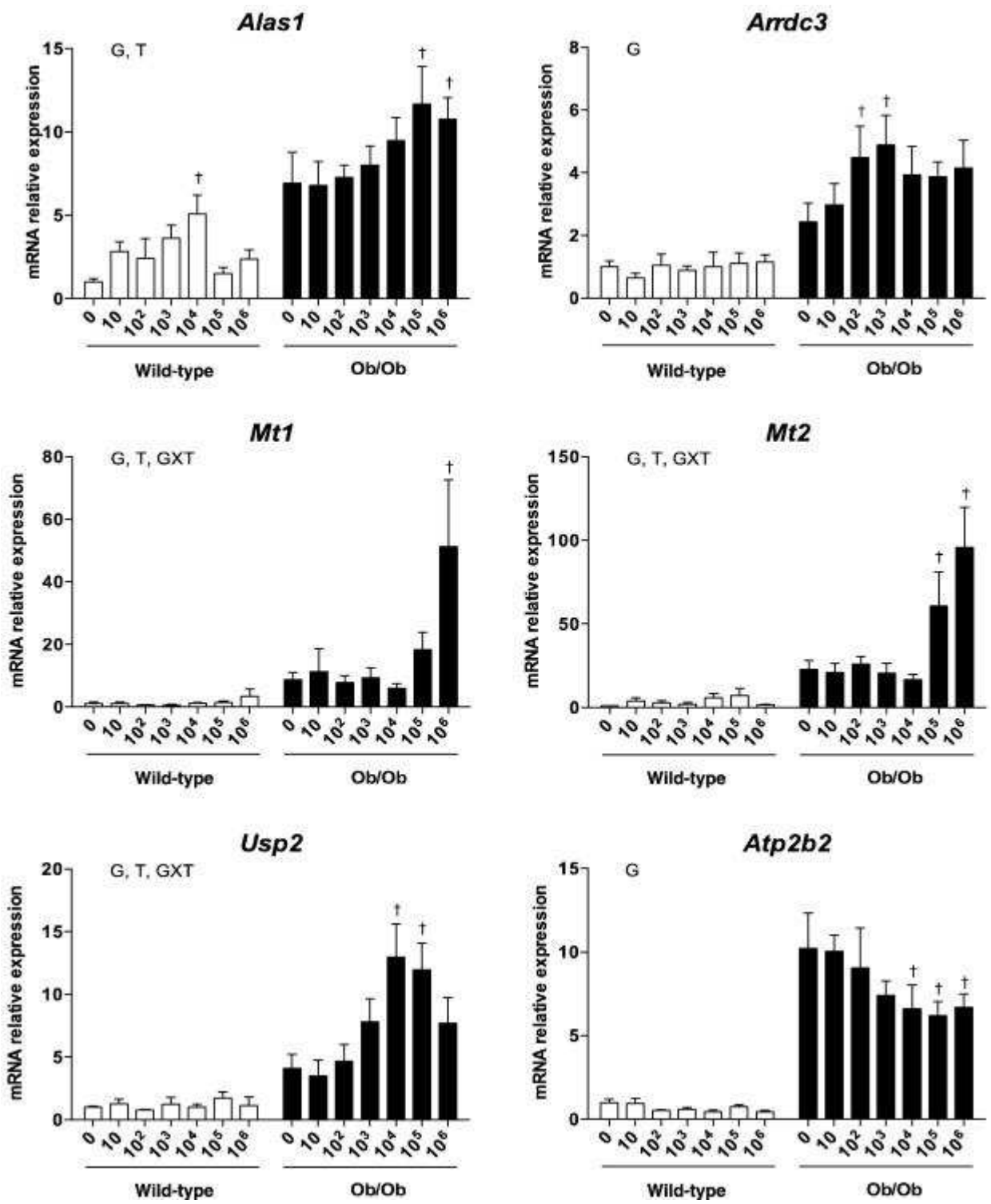
528

529 **Figure 2.** Hepatic mRNA expression of 8 circadian genes in male lean and obese mice treated or not  
530 with the cocktail containing the 11 drugs at concentrations ranging from 10 to 10<sup>6</sup> ng/l. Results are  
531 mean ± SEM for 5 to 7 mice. Statistical analysis was performed with a two-way ANOVA: G, effect  
532 of genotype, T, effect of treatment, GxT, interaction between genotype and treatment. Individual  
533 means were then compared with the *post hoc* Bonferroni test. †Significantly different from  
534 untreated mice of the same genotype ( $P<0.05$ ).





536 **Figure 3.** Hepatic mRNA expression of 8 circadian genes in female lean and obese mice treated or  
537 not with the cocktail containing the 11 drugs at concentrations ranging from  $10^4$  to  $10^6$  ng/l. Results  
538 are mean  $\pm$  SEM for 4 to 8 mice. Statistical analysis was performed with a two-way ANOVA: G,  
539 effect of genotype, T, effect of treatment, GxT, interaction between genotype and treatment.  
540 Individual means were then compared with the *post hoc* Bonferroni test. †Significantly different  
541 from untreated mice of the same genotype ( $P<0.05$ ).  
542  
543



544

545

546 **Figure 4.** Hepatic mRNA expression of 6 non-circadian genes in male lean and obese mice treated  
 547 or not with the cocktail containing the 11 drugs at concentrations ranging from 10 to 10<sup>6</sup> ng/l.  
 548 Results are mean ± SEM for 5 to 7 mice. Statistical analysis was performed with a two-way  
 549 ANOVA: G, effect of genotype, T, effect of treatment, GxT, interaction between genotype and  
 550 treatment. Individual means were then compared with the *post hoc* Bonferroni test. †Significantly  
 551 different from untreated mice of the same genotype ( $P < 0.05$ ).