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Normalization of disrupted clock gene expression in males with tetraplegia.

A crossover randomized placebo-controlled trial of melatonin

supplementation

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Running title:

Clock genes in males with tetraplegia

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Abstract

Study design Crossover double blind, randomized placebo-controlled trial.

Objective Circadian oscillators are located both in the brain and in peripheral organs. Melatonin, the main brain-derived hormone governing circadian variations, is highly associated with daylight patterns. However, in subjects with tetraplegia the melatonin levels are blunted. Here we studied peripheral oscillators in peripheral blood mononuclear cells (PBMCs) in males with tetraplegia by examining how exogenous melatonin may influence the expression of clock gene mRNAs.

Setting Sunnaas Rehabilitation Hospital, Nesoddtangen, Norway.

Methods Six males with tetraplegia received 2 mg of melatonin or placebo 4 days before the study period. We also included six able-bodied men sleeping or kept awake during the night. Plasma samples were collected four times during a 24-h period. The mRNA expression levels of the clock genes *PER1*, *PER2*, *BMAL1* and *REV-ERBα* were quantified in PBMCs using quantitative RT-PCR.

Results The mRNA expression levels of *PER-1 and -2* and *REV-ERBα* were increased at 04:00 h compared to the able-bodied controls ($p < 0.05$). Melatonin supplementation changed mRNA peak-time towards the time of supplementation.

Conclusions Several peripheral clock genes displayed distorted expression levels in tetraplegia. Supplementation with melatonin changed the mRNA expression levels of these genes towards those observed among able-bodied.

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Introduction

In all species many biochemical, physiological, and behavioural processes oscillate with a 24-h period. These rhythms are driven by endogenous circadian clocks, which function through interacting with positive and negative transcriptional/translational feedback loops. The main murine genes of the negative-feedback loop are the *Per*s and *Cry*s, whereas *Clock* and *Bmal1*, coding for two basic helix-loop-helix transcriptional activators, are important genes of the positive loop [1]. These positive and negative feedback loops are interconnected by a second loop where the transcription of *Rev-Erbα* and *Rora*, two nuclear orphan receptor genes, is regulated by *Clock:Bmal1* heterodimers. *Rev-Erbα* and *Rora* compete for the same element on the *Bmal1* promoter, but have opposing actions. This circadian timing system is governed by a master circadian pacemaker located in the suprachiasmatic nucleus of the anterior hypothalamus as well as peripheral oscillators located in most organs and tissues [2]. Also in humans the expression of *PER1*, *PER2*, and *BMAL1* mRNAs show circadian rhythmicity in peripheral tissues (e.g. the skin and the oral mucosa), and in the peripheral blood mononuclear cells [PBMCs] [3-6].

Following a complete cervical spinal cord injury (SCI) in humans, nervous input through somatic and autonomic afferent fibres from the body below the SCI level is disrupted and the efferent sympathetic innervation of the pineal gland via the superior cervical ganglion is lacking control from higher autonomic centres. It has been hypothesized that these disrupted nervous connections abolish rhythmic melatonin production. In line with this we and others have reported blunted circadian rhythm and low blood levels of melatonin in persons with cervical SCI [7-9] and indeed, in healthy adults, melatonin levels range from approximately 10 pg/mL at the end of the light period up to 200 pg/mL near the midpoint of the dark period, whereas in tetraplegic subjects the corresponding values are closer to 2 and 15 pg/mL.

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Melatonin has been used as a marker of the central circadian pacemaker in humans [10-11], however, it is unclear how the peripheral oscillators are influenced by the absence of melatonin rhythmicity in humans with complete cervical SCI and blunted melatonin levels. To study the effect of melatonin on the circadian variations of markers of haemostasis (many of which show 24-h rhythms [7 and references therein]), we performed a cross-over double-blind, randomized placebo-controlled trial of melatonin supplementation in tetraplegia [7].We could not attribute any major role of melatonin in regulating the circadian variation of a wide range of hemostatic factors [7]. However, in a previous investigation we did find melatonin to reduce peak thrombin generation [12]. Although melatonin supplementation did not change the levels of many other hemostatic factors, it could modify circadian variations of peripheral clock genes. We therefore planned for and used specially prepared blood samples (PAX gene RNA Blood collection tubes (PreAnalytiX) obtained in our randomized trial to examine the effect of melatonin supplementation on the expression of four cardinal circadian clock genes (*Per1*, *Per2*, *Bmal1* and *Rev-Erbα*), in PBMCs sampled 4 times throughout a 24-h cycle in six tetraplegic subjects. Blood was specifically collected four times during a 24-h period, namely at 07:00, 22:00, 04:00 and 07:00 h to capture possible changes in clock gene expression levels around the time of melatonin supplementation. We also included six able-bodied subjects sleeping or kept awake during the night as controls.

Methods

Subjects and design of study

The study was approved by the Regional Committee for Medical Health Research Ethics in Norway and is registered with Clinicaltrials.gov identifier: NCT 01741389 and with the Norwegian Medicines Agency EUDRACT no. 2010-021212-24. Details of the study design and randomization have been described previously [7]. Briefly, we designed a cross-over double-blind, placebo-controlled trial of six tetraplegic men in addition to a control group of six able-bodied men, i.e. four study-groups: tetraplegic men given placebo, the same tetraplegic men given melatonin, and able-bodied men sleeping or kept awake during the night. During the time of the trial sunset and sunrise occurred around 07:00 and 19:00 h, respectively. The trial was performed in the south of Norway. The tetraplegic men were invited through the hospital's own in-patient coordinator. The able-bodied participants were all hospital staff and were invited through intranet or by direct request. The tetraplegic men were randomized to first receive 2 mg of melatonin (Circadin; Neurim Pharmaceuticals, Zug, Switzerland) or placebo (Kragerø Tablettproduksjon AS, Kragerø, Norway) daily at 22:00 h for 4 days before they were subjected to a 24-h period of blood sampling (see figure 1a). The dose of 2 mg of melatonin is recommended for the treatment of insomnia, and in a pilot study we found that this dose markedly increased the blood concentration of melatonin (data not published). Blood was collected four times during a 24-h period, namely at 07:00, 22:00, 04:00 and 07:00 h. The "wash-out" period lasted 4 days in the tetraplegic group before the cross-over, which is assumed to be sufficient since the half-life of melatonin is about 35-50 min, thus ensuring minimal, if any, carry-over effect. The able-bodied men were subjected to a similar two 24-h periods of blood sampling, with two weeks in-between sampling. They slept or were kept awake during the night with group-common low-intensity activities as playing computer games, table tennis or watching movies (see figure 1b). All the participants

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120 received standardized meals at regular time-points. No other restrictions except zero alcohol

121 intake and maximum two cups of coffee were required from the participants.

122

123 **Blood sampling**

124 Venous blood samples were collected in 5 ml Vacutainer vacuum tubes containing 0.5 ml

125 buffered sodium citrate (0.129 M) (Becton-Dickinson, Plymouth, UK) and 2.5 ml PAX gene

126 RNA Blood collection tubes (PreAnalytiX, Hombrechtikon, Switzerland). Citrated blood was

127 kept at room temperature and immediately centrifuged at 2000 g for 15 min. Platelet-poor

128 plasma aliquots and PAX gene RNA tubes were stored at -70 °C until assayed. All analyses

129 were performed examiner-blind, and the samples were run in-batch using a balanced set-up

130 with equal number of cases and controls in each run.

131

132 **Assays**

133 Melatonin concentrations were assayed with an ELISA-kit (Buhlmann Lab. AG, Basel,

134 Switzerland) as described earlier [7]. For clock gene expression analysis, DNase-treated total

135 RNA was isolated from PBMCs and used for cDNA synthesis (iScript™ cDNA synthesis kit,

136 Biorad, Milan, Italy). cDNA was PCR-amplified in a CFX Connect Real-Time PCR Detection

137 System [Biorad, Milan, Italy] using SsoFast EvaGreen Supermix (Biorad). The following

138 primers were used:

139

140 *Per1* F: GTGCGGAGGACACTCCTG, R: TTGGCTGAGGGAGTGAGGT;

141 *Per2* F: TCGTTTGAACTGCGGTGAC, R: GTATCCATTCATGCTGGGCT;

142 *Bmal1* F: AGCCACGGTGGTGCTGGCTA, R: AACCAATGAAGGCCCAGGATTCCAC;

143 *Rev-Erbα*: F: CGCAACCTCTAGTTTGAGTCAAGGTCC, R:

144 ACGCCACCTGTGTTGTTGTTGGA;

145 *18S rRNA* F: CGAGCCGCCTGGATACC, R: CATGGCCTCAGTTCCGAAAA;

146 *GAPDH* F: GATGACATCAAGAAGGTGGTGAAGC, R:

147 TTCGTTGTCATACCAGGAAATGAGC;

148 *CDK4* F: ATCCCAATGTTGTCCGGCTG, R: TGATCTCCCGGTCAGTTCGG.

149

150 We used NormFinder (Aarhus University Hospital, Denmark) to evaluate and screen the

151 following three housekeeping genes: *GAPDH*, *CDK4* and *18S rRNA*. Based on the rankings,

152 we have chosen to normalize to the geometric mean of CDK4 and 18S, and the expression of

153 genes of interest using the $2^{-\Delta\Delta Ct}$ method (arbitrary units (AU)) [13]. We furthermore, scaled

154 the AU values to the mean overall expression of each respective gene for every patient and

155 time point. This allowed us to plot expression of several genes on the same graph in order to

156 visualize the daily cycle of genes relative to their own expression level.

157

158 **Statistics**

159 The statistical analyses were performed with SPSS version 25.0 (Chicago, IL, USA) and the

160 MedCalc Software (Mariakierke, Belgium). Values are given as mean absolute values with

161 standard error of the mean (SEM) or as median (range) as appropriate. Differences in the

162 plasma concentrations of the various parameters between the study groups were evaluated

163 with two-ways ANOVA and Dunnett's post hoc test, profile differences were evaluated with

164 mixed models (time (continuous) versus group (categorical)). We considered *p*-values less

165 than 0.05 to indicate statistical significance.

166

167 **Statements of ethics**

168 We certify that all applicable institutional and governmental regulations concerning the

169 ethical use of human volunteers were followed during the course of this research.

170

171 # Results

172 **Characteristics of the study participants**

173 The mean (range) age of the males with tetraplegia was 46 (27-60) years. Their injury level

174 ranged from the cervical vertebra 5 to 8, all diagnosed with a complete injury according to the

175 American Spinal Cord Injury Association International Standards For Neurological

176 Classification of SCI [14], and the mean (range) time since injury was 18 (3-43) years. Their

177 mean (range) body mass index (BMI) value was 25.4 (23.8-26.6) kg/m^2. The corresponding

178 values among the controls were not significantly different from the tetraplegic men; age 43

179 (34-54) years and BMI 26.6 (20.1-35.3) kg/m^2. All participants completed the study protocol

180 except for one male with tetraplegia who withdrew from one of two 24-h blood samplings.

181

182 **Plasma melatonin profiles in the two study groups**

183 Fig. 2 shows the 24-h plasma melatonin levels in the four study groups. The plasma melatonin

184 levels among the able-bodied increased in the evening (22:00 h), irrespective of whether they

185 slept or not. A similar pattern was observed upon melatonin supplementation to the tetraplegic

186 group, where the night-time melatonin plasma levels were elevated about 50-fold. As

187 expected, the plasma melatonin levels remained low and unaltered in the tetraplegic group

188 given placebo.

189

190 **Disrupted PBMC clock-gene rhythmicity in tetraplegia**

9

191   To visualize the rhythmicity of genes and present the expression of core clock genes on

192   a single graph for each group, mean scaling was performed and presented in figure 3. Only

193   the sleeping able-bodied group had a visual diurnal rhythmicity, i.e. the two 07:00 h

194   measurement-points being similar for each of the four clock gene expression levels. In

195   contrast, when these able-bodied were awake they had slightly downward flattened profiles

196   for the four clock gene expression levels. The maximum mRNA expression level for the

197   sleeping able-bodied apparently occurred at 07:00 h for *Per1*, *Per2* and *Bmal1*, whereas the

198   mRNA for *Rev-Erbα* had a maximum at 22:00 h. In the tetraplegic group the maximum

199   mRNA expression level of all four clock-genes apparently occurred at 22:00 h in the

200   melatonin-supplemented and at 04:00 h in the placebo-supplemented group. We examined the

201   overall rhythm using mixed model (time versus group) analysis in the four study groups and

202   found that the tetraplegia group receiving placebo had a different profile compared to the

203   able-bodied group (awake) for BMAL1 and PER-1 expression ($p = 0.01$ and $p = 0.002$,

204   respectively).   Males with tetraplegia receiving placebo had a different mRNA expression

205   profile for all clock gene investigated than the same males receiving melatonin (*Rev-Erbα*: p

206     $= 0.001$, *Bmal1*: $p = 0.03$ *Per1*: $p = 0.02$, *Per2*: $p = 0.004$). There were no other significant

207   differences in any of the other mRNA levels or profiles of the four clock genes among other

208   study-group comparisons.

209

210   We next examined the mRNA expression levels of the clock genes separately among

211   the four study groups (Fig. 4). We observed increased *Per1*, *Per2* and and *Rev-Erbα* mRNA

212   expression levels at 04:00 h in the tetraplegic group receiving placebo compared to sleeping

213   able-bodied ($p = 0.04$, $p = 0.03$ and $p = 0.02$, respectively). However, the variation (SEM) of

214   mRNA expression levels in both tetraplegic groups (placebo or melatonin) was large. The

215   mRNA expression levels of BMAL1 remained unchanged ($p > 0.05$) among the four study

216   groups.

Discussion

218   To our knowledge, this is the first study of mRNA expression levels of the clock genes *Per1*,

219   *Per2, Bmal1* and *Rev-Erbα* in males with tetraplegia, a condition leading to disrupted efferent

220   input to the pineal gland from the superior cervical ganglion and thus blunted plasma

221   melatonin levels. Our results suggest disrupted peripheral clock regulation in males with

222   cervical SCI. In line with this we found that the tetraplegic groups receiving placebo had

223   increased *Per1*, *Per2* and *Rev-Erbα* expression levels at 04:00 h compared to awake able-

224   bodied controls. Furthermore, the melatonin supplementation changed the expression profile

225   in the tetraplegic group by changing the maximum value from 04:00 h to 22:00 h, i.e. towards

226   the time point of supplementation of melatonin. Thus the males with SCI receiving melatonin

227   behaved more like the able-bodied males staying awake overall, with lower expression of all

228   clock genes measured at 07:00 h in contrast to the able-bodied sleep group. This may be a

229   result of clearance of melatonin in the SCI group related to the 50 times higher plasma levels

230   of melatonin.

231   It is well known that in addition to the disrupted efferent input to the suprachiasmatic

232   nucleus, tetraplegic subjects have a low-grade chronic inflammation [15]. Inflammation has

233   been shown to disrupt the expression of clock genes [16]. Our results showing reduced clock-

234   gene mRNA levels in some of the tetraplegic subjects are in accordance with other studies of

235   PCBMs during ongoing inflammation and disease processes [17]. For example, patients with

236   chronic lymphatic leukemia have significantly down-regulated expression of both melatonin

237   plasma levels and mRNA of clock genes in peripheral blood (*Bmal1*, *Per1* and *Per2*) [18].

238   Sleep deprivation also leads to decreased clock gene expression levels [19, 20], which we also

239   found among some of our able-bodied study subjects.

11

240   On the other hand increased clock-gene mRNA expression levels in pathological

241   conditions have been reported, *e.g.* a study found increased mRNA *Bmal1* expression levels

242   in prostate cancer cells [21]. Furthermore, these authors also reported that melatonin

243   supplementation reversed and normalized the expression levels [21], *i.e.* similar to our

244   findings showing a shift in the peak expression closer to melatonin supplementation. An

245   increase in clock gene mRNA expression could be explained by melatonin receptor

246   hypersensitivity in tetraplegia. For example, hypersensitive receptors in various organs after a

247   SCI are recognised as parts of the mechanism behind vascular autonomic dysreflexia and

248   changes of bladder function [22-23].

249   Our findings with large inter-individual variation in the mRNA expression levels may

250   mirror the heterogeneity of the SCI among the study participants and the complex feedback

251   system of peripheral oscillators in humans [24]. Regulators other than melatonin, e.g. food

252   and social activities, may also affect peripheral clock genes differently in subjects with SCI

253   compared with able-bodied. Disruption of rhythms has been shown to lead to a variety of

254   conditions including sleeping-disorders, depression and cancer [25], conditions found to be

255   more frequent in SCI [25-29]. A dysregulated peripheral clock in tetraplegia may be an

256   contributing factor of the increased risk of such disorders and indeed there is evidence of that

257   the use of Circadin (melatonin) over prolonged period of time has a positive effect on sleep

258   related disturbances in elderly people with low melatonin levels (30)

259   Despite the low number of participants, our study was robustly designed and the study

260   subjects were carefully monitored under standardized conditions during the 24-h study period.

261   The washout time for plasma or brain drug levels is not necessarily the same as for the

262   downstream effects on receptor pathways and gene translation and/or transcription. Our study

263   design was not designed to observe these downstream effects. In-hospital-induced stress can

264   modify clock gene expressions [31], but this effect should have been minimized by the double

265 blinded and randomized cross-over design. Moreover, the tetraplegic group was rather

266 uniform as only males were included and they all had a complete and stable, long-standing

267 injury (> 3 years). Importantly, the tetraplegic and able-bodied subjects were matched

268 regarding gender, age and BMI.

269 **Conclusions**

270 To our knowledge this is the first study to describe disrupted 24-h clock gene expressions

271 PBMCs in males with tetraplegia. Our main result was that tetraplegic males receiving

272 placebo have increased *Per1*, *Per2* and *Rev-Erbα* mRNA expression levels in the early

273 morning compared with able-bodied. Specifically, melatonin supplementation for four days

274 changed mRNA expression profile in PBMCs in tetraplegia by shifting the peak expression

275 towards the time point of melatonin supplementation. More studies in larger SCI patient

276 cohorts are needed to map the regulatory function of melatonin on peripheral clock genes in

277 various organs.

278

285 **Conflict of interest statement**

286 The authors declare no conflict of interest.

287

288 **Authors' contributions**

289 EK was responsible for designing the study, collecting data and interpreting them and writing

290 the paper.

291 EF was responsible for analyzing data and interpreting them and writing the paper.

292 MS was responsible for interpreting data and writing the paper.

293 AD was responsible for designing the study, interpreting data and writing the paper.

294 PMS was responsible for designing the study, interpreting data and writing the paper.

295 MCM was responsible for analyzing data and writing the paper.

296 GS was responsible for interpreting data and writing the paper.

297 BØ was responsible for analyzing data and interpreting them and writing the paper.

298 CB was responsible for analyzing data and interpreting them and writing the paper.

299 POI was responsible for designing the study, interpreting data and writing the paper.

300

301 **Text summary supplementary data file**

302 mRNA expression levels of *Per1*, *Per2*, *Rev-Erbα*, *Bmal1, CDk4* and *s18* measured at 07:00

303 (time = 1), 22:00 (time = 2), 04:00 (time =3) and 07:00 (time = 4) h. Melatonin (group = 3) or

14

304   placebo (group = 4) where given to the tetraplegia group, the able-bodied slept (group = 1) or

305   were awake (group = 2).

306

307

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Figure legends

Fig. 1 a. Experimental protocol, males with tetraplegia. Blood samples, collected 4 times over a continuous 24 h period beginning on experimental day 4 and 8, were assayed for their melatonin concentration and for the expression of clock genes in PBMCs. Crossover from placebo or melatonin were scheduled to experimental day 5. At the start of the study, participants lived on their habitual sleep/wake schedule. Wake episodes were spent in normal indoor light intensities, and sleep episodes took place in darkness.

Fig. 1 b. Experimental protocol, able-bodied males. Blood samples, collected 4 times over a continuous 24 h period beginning on experimental day 1 and 16 (with a 14 days crossover time) were assayed for their melatonin concentration and for the expression of clock genes in PBMCs. At the start of the study, participants lived on their habitual sleep/wake schedule. Wake episodes were spent in normal indoor light intensities, and sleep episodes took place in darkness.

Fig. 2 Plasma melatonin concentrations (pg/ml) in the four study groups during the 24-h observation period. Values are means (SEM). Melatonin or placebo where given orally to the tetraplegia group every night at 22:00 h for four continuous days before blood sampling. Melatonin was measured at 07:00, 22:00, 04:00 and 07:00 h during the 24-h observation period. The able-bodied males slept (from 23:00 to 07:00 h) or were kept awake during the 24-h observation period. SCI-spinal cord injured.

Fig. 3. Overall mRNA expression levels of the clock genes in PBMCs during the 24-h observation period. Values are means ($\pm$SEM). The expression of a gene in each sample was scaled to a mean expression of the same gene in all samples (AU / AU_{mean}) and presented as

421   scaled AU units. a: Spinal cord injured (melatonin); b: Spinal cord injured (placebo); c: Able-

422   bodied males (awake) and d: Able-bodied males (sleeping). The mRNA expression levels

423   were measured at 07:00, 22:00, 04:00 and 07:00 h during the 24-h observation period.

424   Melatonin or placebo where given orally to the tetraplegia group every night at 22:00 h for

425   four continuous days before blood sampling. The able-bodied males slept (from 23:00 to

426   07:00 h) or were kept awake during the 24-h observation period.

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429   **Fig. 4.** Comparison of genes among groups, mRNA expression levels of the clock genes in

430   PBMCs during the 24-h observation period. Values are means (±SEM). Gene expression was

431   calculated using the $2^{-\Delta\Delta Ct}$ methods and presented as arbitrary units (AU). a: *Per1*; b: *Per2*; c:

432   *Rev-Erbα*, and d: *Bmal1*. The mRNA expression levels were measured at 07:00, 22:00, 04:00

433   and 07:00 h during the 24-h observation period. Melatonin or placebo where given orally to

434   the tetraplegia group every night at 22:00 h for four continuous days before blood sampling.

435   The able-bodied males slept (from 23:00 to 07:00 h) or were kept awake during the 24-h

436   observation period.

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Figure 1 a. Males with tetraplegia

Placebo or Circadin 2mg
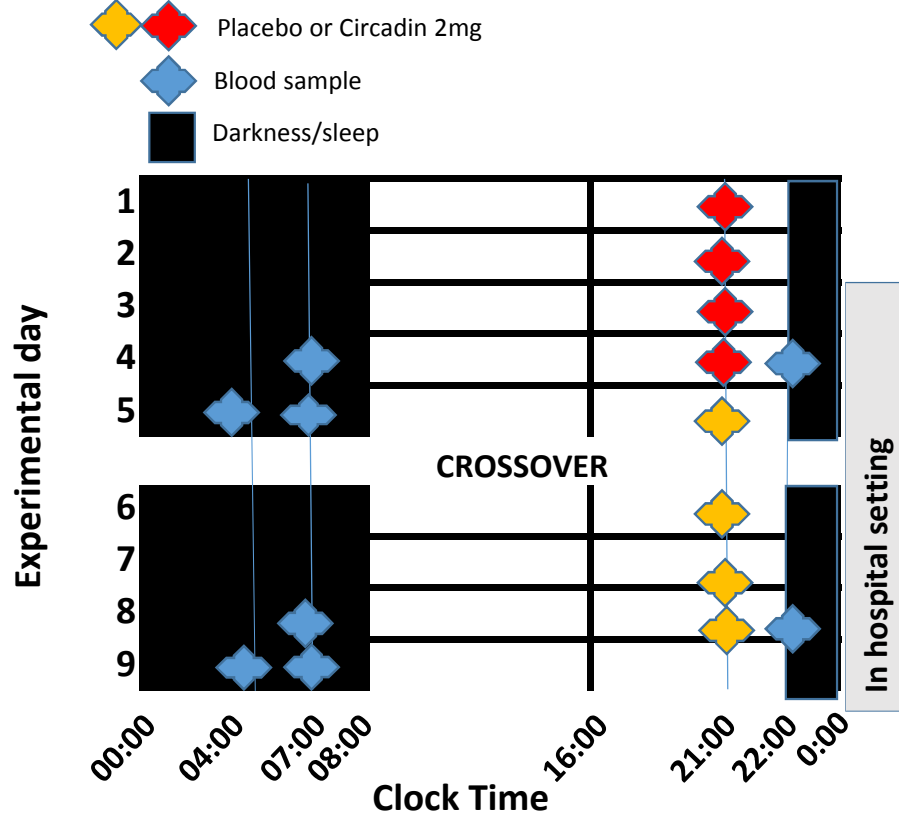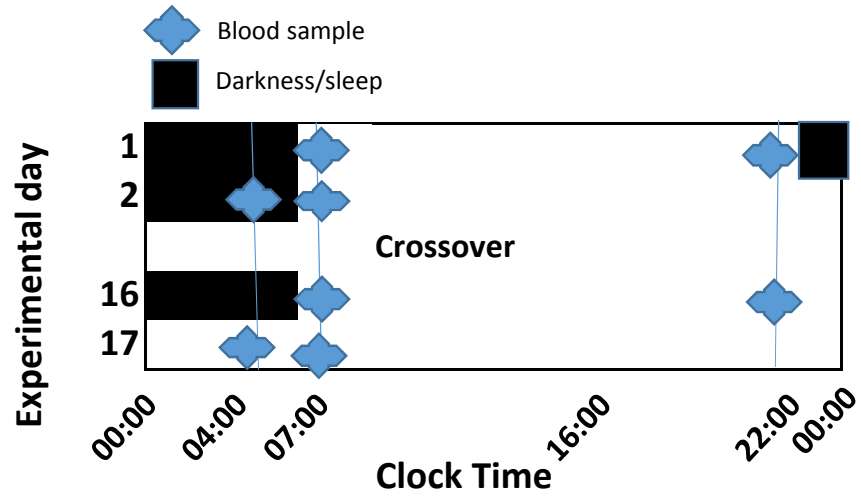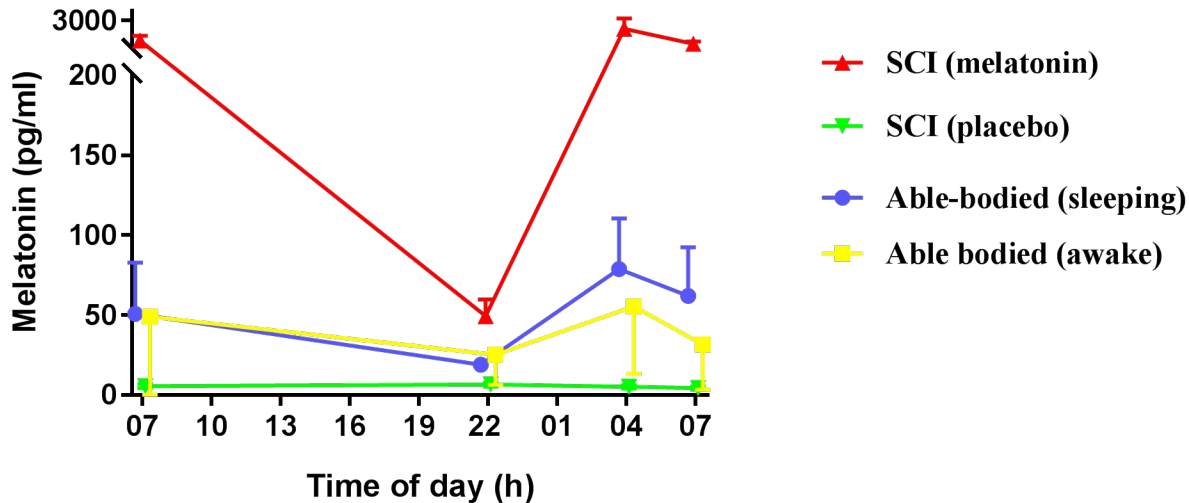
Blood sample

Darkness/sleep

CROSSOVER

In hospital setting

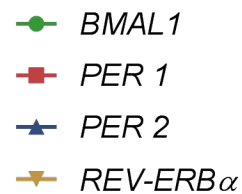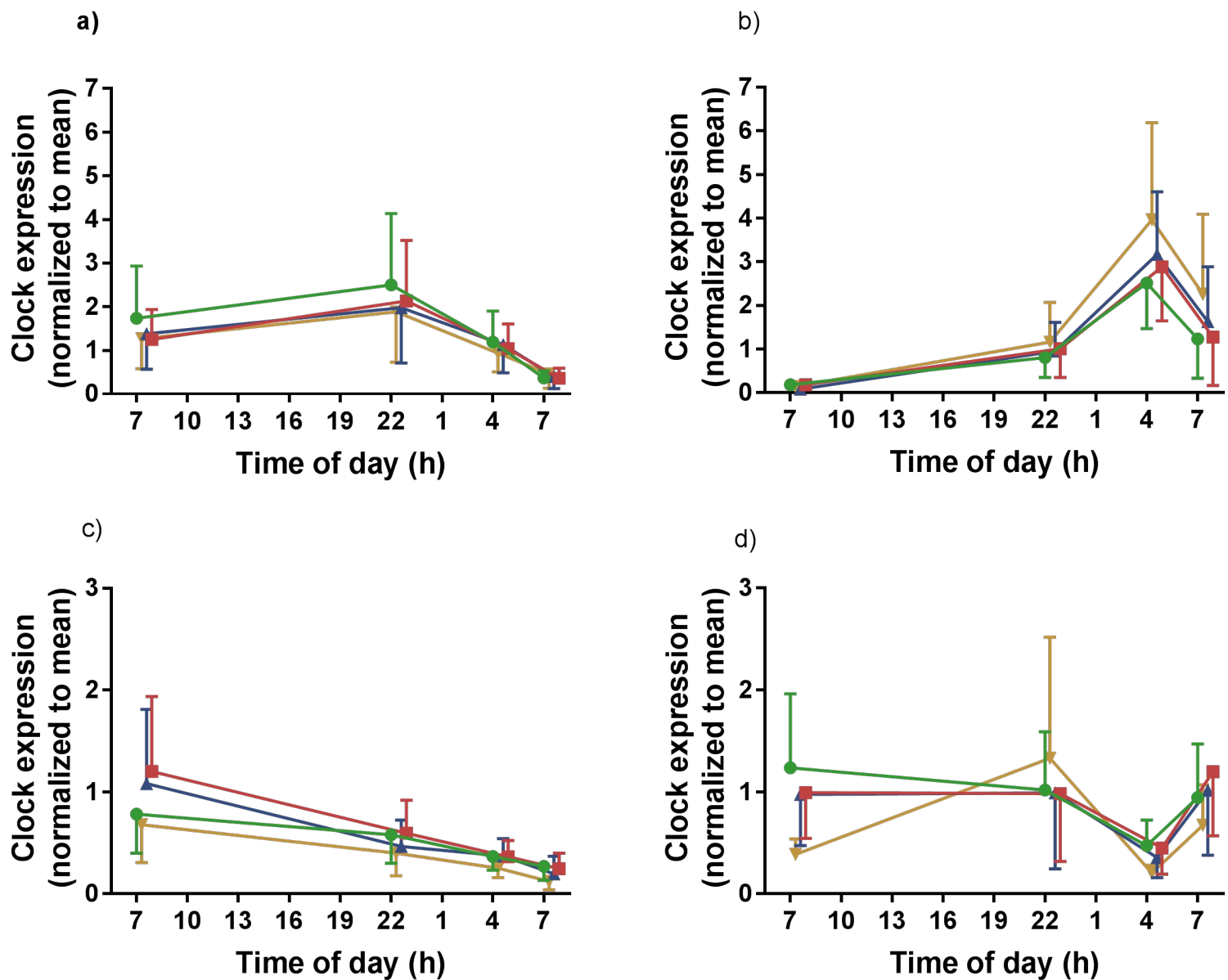Experimental day

Clock Time

Figure 1 b. Able-bodied males

Figure 2

Figure 3.
Overall rhythm in the groups.

Figure 4
Comparison of genes among the groups.

a)

b)

c)

d)

Able-bodied (sleeping)
Able-bodied (awake)
Spinal Cord-Injured (placebo)
Spinal Cord-Injured (melatonin)