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1 **Evidence that pain sensitivity is rhythmic in humans, mainly driven**
2 **by the endogenous circadian system and little by sleep**

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19 **Abstract**

20 Pain intensity has been reported to fluctuate during the day in some experimental and clinical conditions, but
21 the mechanisms underlying these fluctuations are unknown. Although the circadian timing system is known to
22 regulate a wide range of physiological functions, its implication in pain regulation is unknown. We show here,
23 using highly controlled laboratory constant routine conditions, that pain sensitivity is rhythmic over the 24-
24 hours and strongly controlled by the endogenous circadian timing system. We find that pain sensitivity follows
25 a sinusoidal circadian rhythmicity, with a maximum in the middle of the night and a minimum in the afternoon.
26 We also find a weak homeostatic control of pain sensitivity, with a linear increase over the 34 hours of prolonged
27 wakefulness, which parallels that of sleep pressure. Using mathematical modelling, we describe that the
28 circadian system accounts for 80% of the full magnitude of pain sensitivity over the 24 hours, and that sleep-
29 related processes account for only 20%. This result reveals that nocturnal analgesia is predominantly induced
30 by the circadian system and has been wrongly attributed only to sleep. Our findings highlight the need to
31 consider the time of day in pain assessment, and suggest that personalized circadian medicine may be a
32 promising approach to pain management.

33

34 **Significance statement**

35 We discovered that sensitivity to pain is rhythmic in healthy humans, that sensitivity is maximal at night and
36 minimal in the afternoon. Contrarily to the current thinking that sleep is the best painkiller, we find that the 24-
37 h rhythmicity of sensitivity to pain is mainly controlled by a biological circadian clock in our body, and very
38 little by our sleep. Our article reveals the neurobiological mechanisms involved in driving the rhythmicity of
39 pain perception in humans, with the main time-piece located in the brain (the suprachiasmatic nuclei in the
40 hypothalamus). Our findings challenge the current vision of pain physiology, and reveal the need to consider
41 time-of-day and internal biological time for pain evaluation and pain management.

42 **Introduction**

43 Pain intensity has been reported to fluctuate during the day in a number of clinical conditions (3). The cyclic
44 nature of some headaches (4, 5) and the diurnal variation of pain related to osteoarthritis are classical clinical
45 observations (6, 7). The mechanisms underlying these fluctuations, however, are unknown. In particular, it
46 remains unclear whether such daily variations are related to the internal circadian clock, or to behavioral or
47 environmental factors, such as the sleep/wake cycle or the rest-activity cycle.

48 Pain has two main interconnected components: a sensory-discriminative component (location, quality, duration,
49 intensity etc.) and an emotional component (unpleasantness, anxiety, motivation, etc.) (8). This
50 multidimensional nociceptive response involves the activation of numerous subcortical and cortical regions of
51 the brain (e.g. somatosensory cortices, insula, thalamus, prefrontal cortex), often referred to as the “pain matrix”
52 (9). These structures are known to be regulated by the sleep/wake cycle or the circadian clock (10–12), but it
53 remains unclear whether pain sensitivity is rhythmic and how it is regulated.

54 The circadian timekeeping system plays a key role in physiology by regulating the rhythmicity of numerous
55 functions, from gene expression to cortical activity and behavioral functions (10, 11, 13–16). It is, therefore,
56 also likely to be involved in pain perception. The surprising lack of knowledge about the rhythmicity of pain
57 sensitivity may result from the impact of timing on pain perception rarely having been taken into account (3),
58 and the use of inappropriate protocols for the exploration of pain rhythmicity from a neurobiological and
59 mechanistic point of view. The experimental studies performed to date to investigate pain sensitivity changes
60 during the day in healthy individuals have reported conflicting results (17–22). In both experimental and clinical
61 studies, the limited number of measurements and their timing (mostly during the daytime) made it impossible
62 to demonstrate unequivocally the existence of a 24-hour rhythmicity in pain sensation. It is also impossible to
63 determine the origin of any rhythmicity in pain from these studies, because neither of the two types of highly
64 controlled laboratory protocols (constant-routine and forced-desynchrony paradigms) capable of separating
65 endogenous and exogenous rhythms (23, 24) were used. Endogenous rhythms are controlled by the central
66 biological clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus, and exogenous rhythms
67 depend on behavioral or environmental changes, such as the sleep/wake cycle, the dark/light cycle, or the
68 rest/activity cycle. In real-life conditions, endogenous and exogenous influences are expressed simultaneously,
69 making it impossible to attribute rhythmicity to one or the other. In this study, we aimed to determine whether

70 sensitivity to heat pain displays rhythmicity over the 24-h day, and to assess the precise contribution of the
71 circadian clock and sleep-related processes, by systematically assessing pain sensation and gold-standard
72 markers of circadian rhythmicity in highly controlled constant-routine conditions.

73

74 **Results**

75 *Pain rhythmicity is regulated by homeostatic and circadian processes*

76 Twelve healthy men aged 22.7 ± 3.3 years (mean \pm SEM) participated in a 56-hour experimental protocol
77 (Figure 1) including a 34-hour highly controlled constant routine (CR) designed to unmask endogenous
78 rhythmicity (enforced wakefulness, constant posture, low physical and cognitive activity, constant dim light,
79 equicaloric snacks every hour)(23). We assessed the effect of time-of-day on pain sensitivity, by measuring heat
80 pain every two hours during the 34 h of constant routine. In accordance with the current view that two main
81 processes regulate sleep (25), and in agreement with studies showing that physiological functions (such as
82 executive functions (26)) and cortical brain responses (measured by EEG (16) and fMRI (10)) are influenced
83 by both sleep pressure and the circadian timing system, we then modeled the effect of time on pain with an
84 additive mathematical model including a linear component (sleep-related homeostatic drive - process S) and a
85 sinusoidal component (circadian drive - process C)(15).

86

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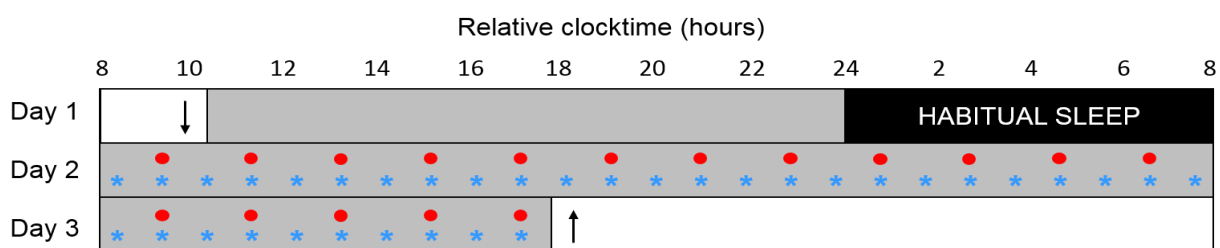


Figure 1. Overview of the experimental protocol. After a day of habituation (day 1) and an 8-h sleep episode, participants were subjected to a 34-hour constant routine (CR: days 2 and 3). Melatonin levels were assessed hourly (blue stars); pain sensitivity, temperature, heart rate and RMSSD were evaluated every two hours (red circles). Participants arrived at about 10:00 on day 1 (down arrow) and left the laboratory at about 18:00 on day 3 (up arrow). Gray rectangles represent wakefulness in dim light (~ 0.5 lux) and black rectangles represent scheduled sleep in darkness.

88

89 Pain sensitivity increases with sleep debt

90 We probed subjective pain (visual analog scale ratings) in response to two-second heat stimuli (42 °C, 44 °C
 91 and 46 °C) every two hours over the entire 34-hour constant routine (Figure 2A, 2B and 2C; all $R^2 > 0.72$). A

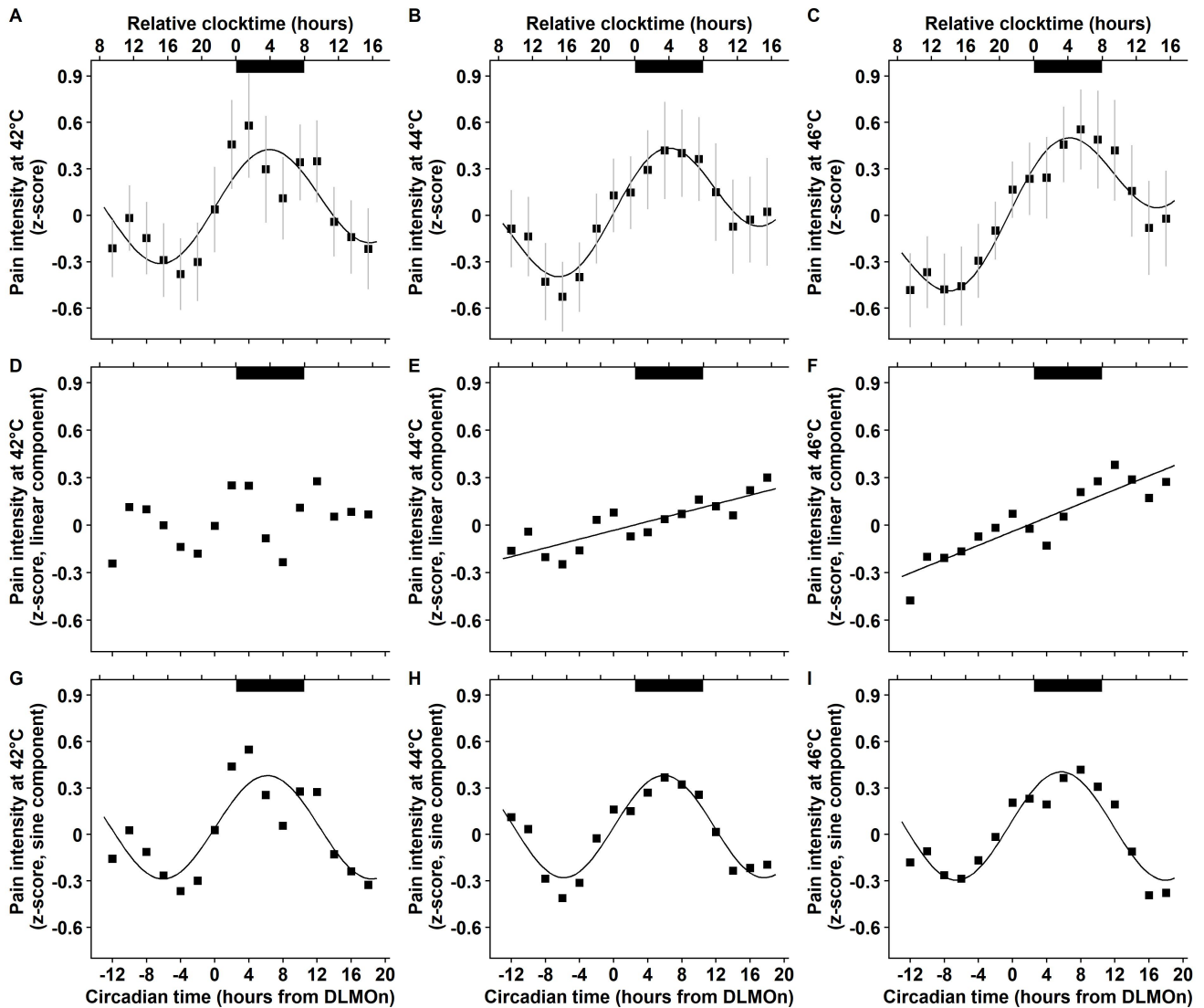


Figure 2. Mean pain intensities in response to 2-second heat stimuli at 42 °C, 44 °C and 46 °C are rhythmic across the 34-h constant routine protocol ($n = 12$). Dark bars correspond to the average timing of habitual sleep episodes (biological night). Circadian time 0 corresponds to dim light melatonin onset (DLMO_n, mean $\approx 21:30$). **A-C.** Combined models (sum of linear and sinusoidal components) applied to normalized data (mean \pm SEM) for stimuli at 42 °C (**A**, $R^2 = 0.72$), 44 °C (**B**, $R^2 = 0.92$) and 46 °C (**C**, $R^2 = 0.92$). **D-F.** Linear components for stimuli at 42 °C (**D**, $R^2 = 0.10$; $p = 0.23$), 44 °C (**E**, $R^2 = 0.73$; $p < 0.0001$) and 46 °C (**F**, $R^2 = 0.81$; $p < 0.00001$). Pain sensitivity increases with time spent awake for stimuli at 44 °C and 46 °C. **G-I.** Sinusoidal components for stimuli at 42 °C (**G**, $R^2 = 0.70$), 44 °C (**H**, $R^2 = 0.90$) and 46 °C (**I**, $R^2 = 0.86$). Pain sensitivity follows a circadian rhythm, with maximal pain at 3:30 (42 °C and 44 °C) or 3:00 (46 °C).

92 linear component was observed for the stimuli at 44 °C and 46 °C (Figure 2E and 2F; all $p < 0.0001$; all $R^2 >$
93 0.73), but not for the less painful stimuli at 42 °C (Figure 2D; $p = 0.23$; $R^2 = 0.10$). Our results thus confirm the
94 known relationship between sleep deprivation and greater pain sensitivity (27–29), but suggest that this
95 relationship may not apply to low levels of pain. As the participants were in a constant state of wakefulness
96 during the CR, the linear component of our model translates the effect of sleep debt and reflects homeostatic
97 sleep pressure. The slope of the linear regression line increased with stimulation temperature (Supplementary
98 Figure 1), so the largest changes in amplitude were observed for stimuli at 46 °C, which caused a change in pain
99 level of 1/10 on the visual analog scale (VAS). As pain responses were measured at three arbitrary temperatures,
100 we then used a modeling approach (classically used in pharmacology and photobiology) to extract an overall
101 pain sensitivity value (Figure 3). The mathematically modelled sigmoidal intensity response curve (based on
102 the combined results obtained at 42, 44 and 46 °C) yielded sensitivity values (ET_{50}) that confirmed the results
103 reported above; a linear increase in sensitivity to pain with time awake (lower ET_{50} values) (Figure 3C; $R^2 =$
104 0.81; $p < 0.01$).

105

106 *Pain sensitivity is driven by the circadian timing system, with maximal pain felt during the night*

107 Subjective measurements of pain in response to two-second thermal stimuli (42 °C, 44 °C and 46 °C) revealed
108 that pain sensitivity was influenced not only by sleep pressure, but also by the circadian timing system (Figure
109 2A, 2B and 2C; all $R^2 > 0.72$). Indeed, independently of the effect of sleep pressure, a sinusoidal component in
110 our model strongly accounted for changes in pain sensitivity across the 34 hours of constant routine, with a pain
111 sensitivity peak between 3:00 and 4:30 for both the responses to graded stimuli (Figure 2G, 2H and 2I) and heat
112 pain thresholds (Supplementary Figure 2C). These results were confirmed by the modeling of a sigmoidal
113 intensity response curve, which also showed a strong circadian rhythmicity of pain sensitivity (Figure 3D; $R^2 =$
114 0.93) and a pain peak in the middle of the night (at 4:30). Interestingly, the lack of circadian rhythmicity for
115 warm non-painful stimuli (Supplementary Figure 3C; $R^2 = 0.13$) suggests that the rhythmicity of pain sensitivity
116 is specific to pain and is not related to a general rhythmicity of thermal sensitivity. These results provide the
117 first evidence, to our knowledge, of a circadian rhythmicity of pain sensitivity in humans.

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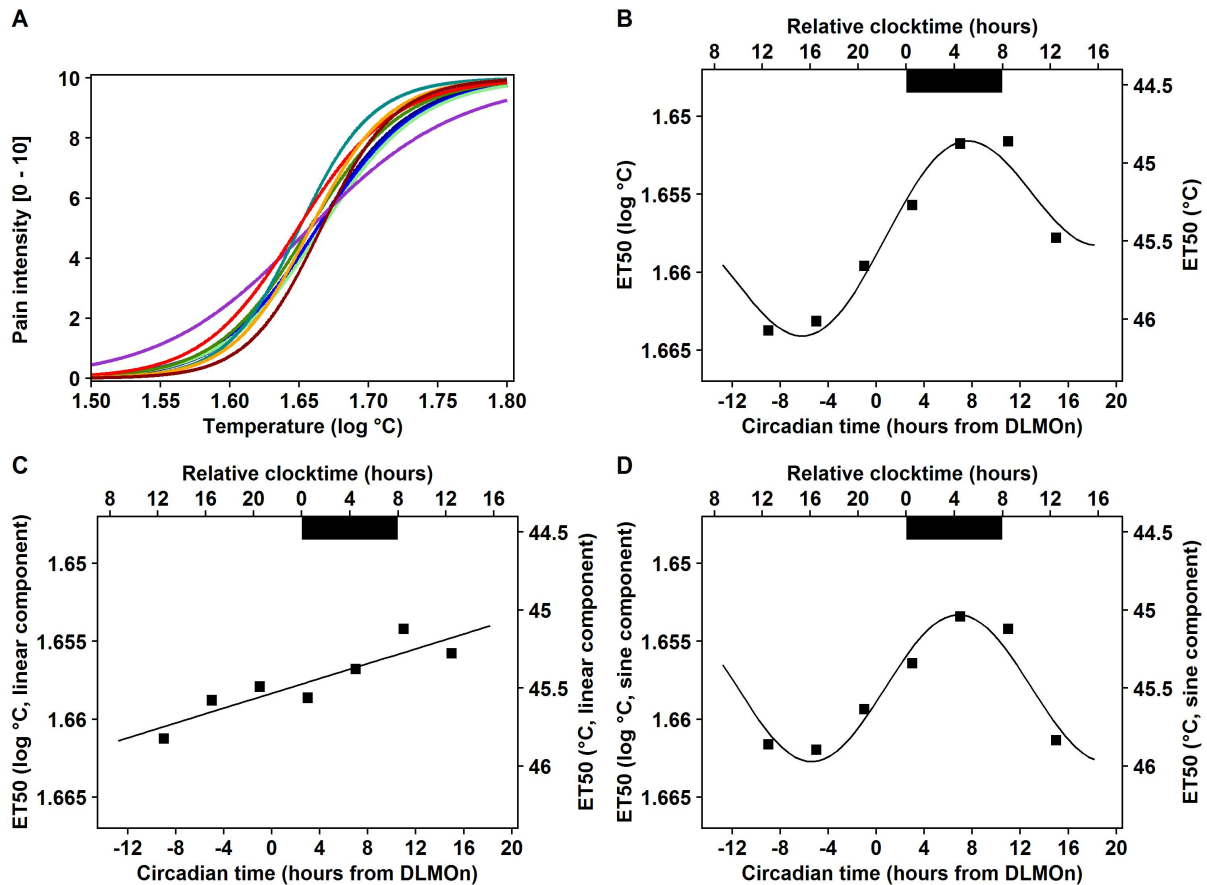


Figure 3. Mean pain sensitivity (ET_{50}) is rhythmic across the 34-h constant routine protocol ($n = 12$). **A.** Intensity response curves calculated on the 6 measures obtained at 42, 44, and 46 °C over two consecutive 2-hour segments (9 curves; all R^2 between 0.68 and 0.99). **B.** Combined model (sum of linear and sinusoidal components) applied to raw ET_{50} values ($R^2 = 0.96$). **C.** Linear component ($R^2 = 0.81$; $p < 0.01$). ET_{50} s decrease and pain sensitivity increases with time spent awake. **D.** Sinusoidal component ($R^2 = 0.93$). Pain sensitivity follows a circadian rhythm with maximal pain at 4:30. **B, C and D.** Dark bars correspond to average habitual sleep episodes (biological night). Circadian time 0 corresponds to DLMO_n (mean DLMO_n \approx 21:30).

119 Changes in pain sensitivity over the 24-hour day are mostly induced by the circadian system rather than a lack
 120 of sleep

121 We investigated the relative contributions of sleep and circadian drives to pain sensitivity, by calculating the
 122 mean changes in both these components and expressing them relatively to the total amplitude over 24 hours
 123 (Supplementary Figure 4). We found that the circadian system accounted for 80 % of the full magnitude of pain
 124 sensitivity changes over 24 hours, the remaining 20 % being accounted for by the homeostatic component.
 125 Surprisingly, the decrease in pain sensitivity attributable to sleep at night was very small.

126 Phase relationships between the circadian components of pain modulation and interoceptive responses

127 Having identified a circadian drive for pain, we investigated whether the rhythm of pain sensitivity displayed
128 phase relationships with interoceptive responses. Using cross-correlation analyzes, we identified a clear phase
129 opposition (~12-h lag) between the rhythms of pain sensitivity and core body temperature (Figure 4A), with the
130 acrophase of pain (at 3:30) occurring at about the same time as the nadir of core body temperature (at 3:00). We
131 also found that pain sensitivity peaked 1.5 hours after endogenous melatonin secretion (at 2:00) (Figure 4B).
132 Autonomic nervous system responses displayed strong circadian rhythmicity, with a nadir of vagal activity
133 (minimal heart rate) and a peak of parasympathetic activity (maximal RMSSD) at 2:00, preceding the pain
134 sensitivity peak by 1.5 hours (Figure 4C and 4D).

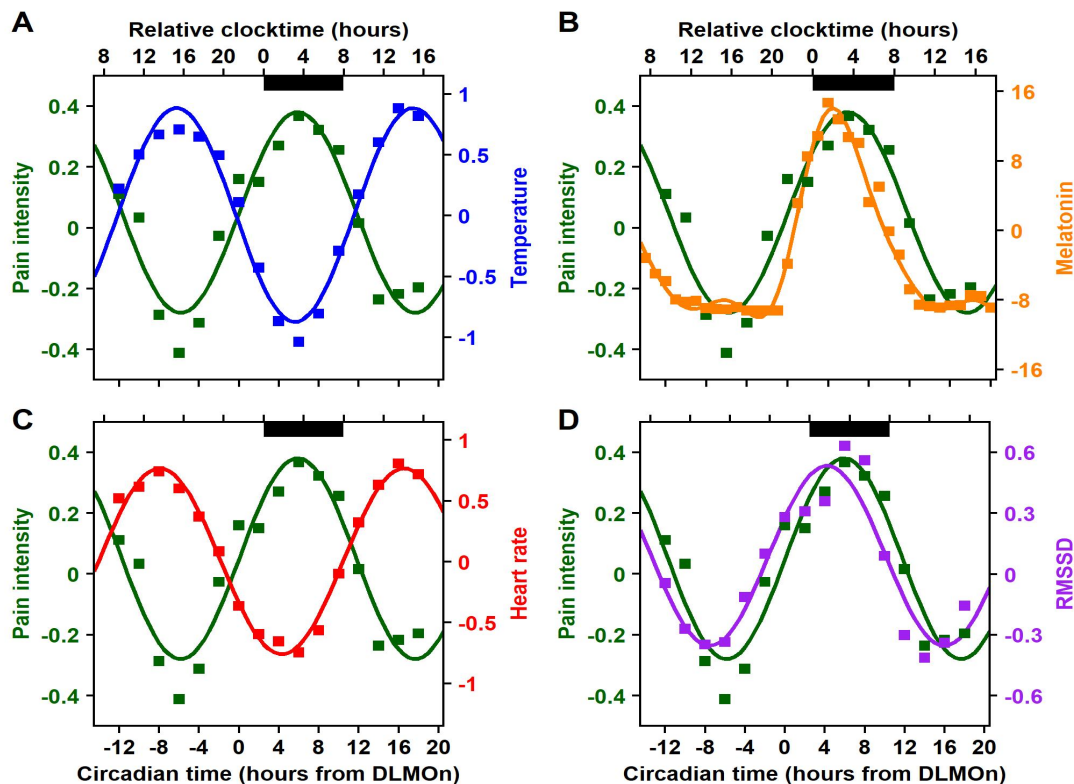


Figure 4. Phase relationships between circadian components of pain sensitivity and temperature (A), melatonin (B), heart rate (C) and parasympathetic activity (D) across the 34-hour constant routine protocol. Dark bars correspond to average habitual sleep episodes (biological night). Circadian time 0 corresponds to DLMO_n (mean DLMO_n \approx 21:30). All curves represent the sine component of the modeled parameter. **All panels.** Circadian rhythm of VAS pain intensity scores in response to a two-second stimulation at 44 °C, with a sensitivity peak at 3:30 (green curve; $R^2 = 0.90$). **A.** Circadian rhythm of baseline body temperature, with a minimal core body temperature at 3:00 (blue curve; $R^2 = 0.97$). **B.** Circadian rhythm of melatonin secretion (pg/mL), with a secretion peak at 2:00 (yellow curve; $R^2 = 0.98$). **C.** Circadian rhythm of heart rate, with a minimal heart rate at 2:00 (red curve; $R^2 = 0.99$). **D.** Circadian rhythm of RMSDD (parasympathetic activity), with an activity peak at 2:00 (purple curve; $R^2 = 0.89$).

135 **Discussion**

136 This is the first highly controlled laboratory study specifically designed to investigate pain rhythmicity and its
137 underlying mechanisms in healthy individuals. Our results unequivocally demonstrate that pain sensitivity is
138 endogenously driven by the circadian timing system, and that sleep and sleep deprivation have a much weaker
139 influence on pain sensitivity than previously thought.

140 A limited number of peer-reviewed studies have systematically investigated the rhythmicity of pain perception
141 in healthy individuals. Careful analysis reveals that published results are equivocal, some studies showing no
142 rhythmicity, others reporting maximal sensitivity either during the day or during the night (17–21) A recent
143 modelling work, using pooled datasets from four experimental studies, proposed a sinusoidal model of pain
144 sensitivity very similar to ours, with a peak sensitivity close to midnight (43). However, because the model was
145 built on data obtained from different populations and protocols, and collected during either sleep, wake, rest,
146 activity, light, or dark conditions, both the phase (timing) and the origin of this rhythmicity in pain sensitivity
147 cannot be attributed to any underlying timing mechanism, neither circadian, nor sleep-related. Overall, although
148 often claimed by the authors, none of the previous studies we have analyzed has demonstrated that pain
149 perception was circadian, i.e. of endogenous origin.

150 By contrast, our results, showing a strong sinusoidal oscillation of pain sensitivity in a constant routine protocol,
151 i.e. in the absence of rhythmic influences and times cues, provide unequivocal evidence that the rhythmicity of
152 pain sensitivity is driven from within, by the endogenous circadian timing system, and does not result from
153 influences evoked the light-dark cycle, the rest-activity cycle, or the sleep-wake cycle. Indeed, if pain sensitivity
154 were to be regulated exclusively by the sleep/wake cycle, as previously thought, we would have observed a
155 peak in pain sensitivity at the end of our 34-hour experimental constant-routine day and not in the middle of it
156 (after 20 hours) as we did. The very observation that the cyclicity of pain sensitivity is driven by the circadian
157 system, independently from the sleep/wake cycle or any other environmental cycle, demonstrates that both the
158 rhythmicity and its specific timing (its phase) are fundamental requirements in humans. Contrarily to the widely
159 held view that pain sensitivity is driven by the sleep-wake cycle (decreasing during sleep and increasing during
160 the day), our quantification that the circadian oscillation accounts for 80% of the full magnitude of pain
161 sensitivity over the 24-h, and that sleep deprivation accounts for only 20% of it, reveals that sleep and sleep

162 deprivation have in fact a very modest effect on pain. We also conclude that nocturnal analgesia is
163 predominantly due to the circadian system, that it has wrongly been attributed only to sleep in previous studies.
164 The pathways linking the circadian timekeeping system to pain perception cannot be inferred from this study,
165 but the suprachiasmatic nucleus is undoubtedly the starting point, and the subcortical and cortical regions of the
166 brain (e.g. somatosensory cortices, insula, thalamus, prefrontal cortex), often referred to as the “pain matrix”
167 (9) are likely to be involved, given that they have been shown to be regulated by the sleep/wake cycle or the
168 circadian clock (10–12). Our study suggests an interoceptive regulation of pain, where the circadian pacemaker
169 is likely to be central. Pain is traditionally regarded as an exteroceptive response depending on both the
170 somatosensory and emotional systems, however, it has been suggested that it may also be part of the
171 interoceptive system, relating to the condition of the body (8, 44). The interoceptive responses underlying the
172 maintenance of the internal environment of the body are organized in a hierarchical manner. They involve a
173 number of extensively connected physiological systems, so any change in one interoceptive function is usually
174 associated with changes in one or several other interoceptive functions. Our data are consistent with this view
175 as they show that, like other interoceptive functions, pain is driven by a time-specific circadian rhythm that is
176 directly related to the rhythmicity of other functions. The phase opposition we find between pain sensitivity and
177 core body temperature (CBT) suggests an interaction between thermoregulation and nociception (45), both of
178 which are components of the interoceptive system (8, 44). The phase relationships observed between the
179 rhythms of the sympathetic and parasympathetic systems (as assessed by cardiovascular measurements) and
180 pain are also consistent with this hypothesis and suggest the existence of strong interactions between the
181 nociceptive pathways and the autonomic nervous system (ANS) (46–48). The circadian timing system may, via
182 the SCN, serve as a key interface between pain and other interoceptive functions. The mechanisms underlying
183 these interactions are unclear, but, interestingly, our data suggest that they are probably not mediated by
184 melatonin. Melatonin, a nocturnal hormone released by the pineal gland, is generally reported to induce
185 antinociceptive effects (49–51). Such effects are not consistent with the temporal relationship between peak
186 pain sensitivity and peak endogenous melatonin secretion reported here, which instead suggests a
187 pronociceptive effect of melatonin. None of these mechanisms can be validated on the basis of our results as
188 we describe only temporal relationships between time series, but they could all be relatively easily tested
189 experimentally to determine their causality. Alternatively, the circadian rhythmicity of pain may be accounted

190 for by direct control of the nociceptive network (or the cognitive/emotional structures) by the SCN. In this
191 regulatory model of pain regulation, the circadian system may be responsible for controlling the precise timing
192 of nociception (8). As the thalamus is a key player in the nociceptive pathway and projections from the SCN to
193 the anterior paraventricular thalamus have been identified (52), pain sensitivity may be directly modulated by
194 this brain structure over the course of the 24-hour day. Multiple other pathways could be involved. Using the
195 same highly controlled experimental conditions we employed here, a study showed that ~15% of all identified
196 metabolites in plasma and saliva are under circadian control in humans (Dallmann 2012). These include
197 metabolites involved in pain pathways, and recently identified metabolites of neuroinflammation specifically
198 found elevated in patients with neuropathic pain compared to those without neuropathic pain (Pfyffer 2020).
199 Whether those metabolites are involved in all clinical conditions of pain or in experimentally induced pain is
200 unknown, but overlapping the human circadian metabolome and our results allows to propose that the circadian
201 system regulates pain sensitivity through multiple pathways, both in normal and pathological situations.
202 The influence of sleep and sleep deprivation on pain sensitivity is modest in terms of its impact on the full
203 magnitude of pain sensitivity over the 24-h, but it is not negligible. The linear increase in pain sensitivity that
204 we find during enforced wakefulness, after mathematically removing the circadian component, confirms that
205 pain sensitivity does increase with time spent awake and reveals that it is under the influence of an independent
206 (from the circadian system) homeostatic drive, possibly related to that involved in the buildup of sleep pressure
207 from waketime to bedtime (25). This finding is consistent with the studies we previously discussed (17-21, 43)
208 and with the classically described interaction between pain and sleep (3, 30–33), whereby pain sensitivity
209 appears to be driven by the sleep/wake cycle, with pain perception low in the morning after a night of good-
210 quality sleep, increasing during the day to reach a peak before bedtime, and then decreasing during sleep (29).
211 In the absence of sleep (after one night of total sleep deprivation), pain sensitivity has been shown to be higher
212 than it was at the same time on the previous day (27, 28), highlighting that there is an analgesic effect of sleep
213 and/or a hyperalgesic effect of sleep deprivation. This sleep drive is usually considered to explain why sleep
214 disorders, such as insomnia, are associated with an exacerbation of clinical pain (29, 34). The reciprocal
215 interactions between sleep homeostasis and pain may result from functional changes in the interconnected sleep
216 and pain systems. Consistent with this hypothesis, sleep loss is associated with an increase in the activation of
217 somatosensory brain areas induced by painful stimuli, potentially reflecting an amplification of neuronal

218 responses in the cortical nociceptive systems and/or a disinhibition of normal thalamocortical pain signaling
219 (33). In addition, sleep deprivation blunts activity in areas of the brain involved in endogenous pain modulation,
220 such as the striatum and insular cortex (33). The specific mechanisms underlying the interactions between pain
221 and sleep remain unknown, but may involve sleep-promoting factors, such as adenosine (35). Adenosine
222 accumulates with increasing homeostatic sleep pressure during wakefulness, reaching high levels at the end of
223 the day (36, 37), and then declining during sleep (38). In addition to its role in the sleep/wake cycle, adenosine
224 is also involved in the nociceptive system and may play an anti- or pronociceptive role, depending on the
225 receptors activated (39, 40). Thus, the hyperalgesic effect of constant wakefulness reported here may be at least
226 partly due to adenosine accumulation, leading to A1B receptor activation (37). Obviously, other mediators, such
227 as cytokines, which also play a role in both pain (41) and sleep regulation (42), may be involved in the sleep-
228 related modulation of pain sensitivity.

229 This study has a number of limitations. First, our protocol was conducted under non-ecological and highly
230 controlled laboratory conditions, which were nevertheless absolutely essential to dissect out the rhythmic and
231 endogenous elements of pain sensitivity. Pain sensation may be different in real-life conditions, but the
232 endogenous mechanisms controlling pain sensitivity are expected to be the same. The modest influence of sleep
233 deprivation on pain sensitivity that our model finds, may also be different in real-life conditions. Indeed, prior
234 to their experimental session in the laboratory, our participants underwent 3 weeks of sleep monitoring, during
235 which time they slept on average 8 hours per night, and ensured they were sleep satiated upon arrival. In real
236 life conditions, where sleep deprivation is common in our societies, the strength of sleep-related drive may be
237 higher than in our conditions. This does not invalidate our model, but asks for its careful interpretation in
238 different conditions (Prayag et al. 2021) and also for its evaluation in conditions of sleep deprivation. Second,
239 pain intensity was evaluated in healthy participants, with an experimental heat pain paradigm. It is conceivable
240 that sleep pressure and the circadian timing system have the same effect on any type of pain, but our results
241 cannot be directly extrapolated to clinical populations. Third, the population examined in this study consisted
242 exclusively of men. Circadian physiology is very similar in men and women, with only minor differences, such
243 as a slightly larger amplitude (53, 54), and a slightly shorter period (55) in women, but our results should not
244 be extrapolated to premenopausal women, in whom the menstrual cycle may modulate both the homeostatic
245 and circadian drives of pain sensitivity.

246 In conclusion, our results reveal the neurobiological mechanisms driving the rhythmicity of pain perception in
247 humans, with the main driving brain structure located in the suprachiasmatic nuclei of the hypothalamus. We show
248 that pain sensitivity is controlled by two superimposed processes: a strong circadian component and a modest
249 homeostatic sleep-related component. This finding may have clinical implications, as dysregulations of the
250 circadian system have been implicated in a number of diseases with major consequences for health (13). Such
251 alterations may also be involved in the pathophysiology of some chronic pain syndromes, as suggested for
252 cluster headaches, for example (56). The existence of a circadian rhythmicity in pain suggests that the efficacy
253 of pain management could be optimized using circadian medicine (1, 2). With this approach, analgesic
254 treatments could be administered according to the each patient's internal time (circadian time) rather than
255 according to a uniform timing schedule mostly based on pragmatic considerations (57, 58). Such circadian
256 approaches have already proved effective in cancer treatment (59), but have not been systematically evaluated
257 for the treatment of pain. Individually timed medication could improve chronic pain management and greatly
258 improve patients' quality of life, not only by improving treatment efficacy but also by reducing the adverse
259 effects of painkillers, including those pejorative to sleep and circadian physiology.

260

261 **Methods**

262 Participants

263 Twelve healthy men (20 - 29 years old, mean age = 22.7 ± 3.3 years; BMI = 21.8 ± 3.1 kg/m²) were included in
264 this study. Neurological, psychiatric and sleep disorders were excluded by clinical examination and
265 psychological questionnaires (Pittsburg Sleep Quality Index Questionnaire and Beck Depression Inventory)(60,
266 61). Participants had an intermediate chronotype (Horne and Ostberg Chronotype Questionnaire score between
267 31-69)(62) and had not done any shift work, or experienced transmeridian travel during the previous three
268 months. Participants had normal visual acuity (Landolt Ring Test and Monoyer scale), contrast vision
269 (Functional Acuity Contrast Test) and color vision (Farnworth D-15 and Ishihara Color Test). All experimental
270 procedures were carried out in accordance with the Declaration of Helsinki. The study was approved by the
271 local research ethics committee (CPP Lyon Sud-Est II) and participants provided written informed consent for
272 participation.

273

274 *Study design*

275 Participants were asked to maintain a regular sleep/wake schedule (bedtimes and waketimes within \pm 30 minutes
276 of self-targeted times) for an average of three weeks before admission to the laboratory, with verification by
277 wrist activity and light exposure recordings (ActTrust, Condor Instruments, São Paulo, Brazil). Subjects were
278 then admitted to the laboratory for a 56-hour experimental protocol (Figure 1), in which they were kept in an
279 environment free from external time cues (clocks, television, smartphones, internet, visitors, sunlight etc.).
280 Subjects maintained contact with staff members specifically trained to avoid communicating time-of-day
281 information or the nature of the experimental conditions to the subjects. Participants arrived at about 10:00 on
282 the first day. They were allowed to familiarize themselves with the laboratory environment, low light levels ($<$
283 0.5 lux), equipment, and measurements. Lunch and dinner were served at about 12:30 and 19:00. A series of
284 measurements were then performed until bedtime (participant's habitual bedtime), and an 8-hour sleep episode
285 was scheduled (constant darkness; recumbent position). This was followed by a 34-hour constant-routine
286 protocol beginning at the participant's usual waketime on day 2, and ending on day 3 (18:00 on average).
287 Habitual bedtimes were determined on the basis of sleep times averaged over the seven days preceding the
288 laboratory segment of the protocol. Average bedtime was 23:45 and average waketime was 8:00.

289

290 *Constant routine protocol*

291 A constant routine (CR) paradigm was used to reveal the endogenous circadian rhythmicity of various
292 parameters. The CR was conducted under constant environmental conditions, to eliminate, or distribute across
293 the circadian cycle, the physiological responses evoked by environmental or behavioral stimuli (i.e. sleeping,
294 eating, changes in posture, light intensity variations)(23, 63). In practical terms, participants were asked to
295 remain awake for 34 hours (starting at their habitual waketime), with minimal physical activity, while lying in
296 a semi-recumbent (45°) posture in bed. This posture was also maintained for the collection of urine samples
297 and bowel movements. Room temperature (mean = $23^\circ\text{C} \pm 0.6$ (SD)) and ambient very dim halogen light levels
298 were kept constant. Light intensity was homogeneous in the room ($<$ 0.5 lux at the participant's eye level in all
299 directions of gaze). Participants were given small equicaloric snacks and fluids at hourly intervals, to maintain
300 an equal nutritional caloric intake and stable hydration over the circadian cycle. Caloric requirements were
301 calculated on the basis of basal metabolic rate determined with the Wilmore nomogram and were adjusted

302 upward by a 7 % activity factor(64, 65). Fluid intake was calculated for each subject, to account for the sedentary
303 nature of the CR(65). A member of the study staff remained in the room with the participant at all times during
304 the CR, to monitor wakefulness and to ensure compliance with the study procedures.

305

306 Heat and pain evaluation

307 Thermal stimuli were applied to the forearm with a Peltier-type thermode (30 × 35 mm) connected to a
308 thermotest device (Somedic AB, Stockholm, Sweden). Heat detection and pain thresholds were determined
309 according to the method of limits (mean of three measurements).

310 Thermode temperature was gradually increased from a baseline temperature of 32 °C, at a rate of 1 °C/s, and
311 participants were asked to stop the increase in temperature when they started to feel a warm sensation (detection
312 threshold) or a pain sensation (pain threshold). At this point, the temperature returned to baseline at a rate of 1
313 °C/s. A minimum interval of 20 s was respected between each threshold measurement. If participants had not
314 pressed the button by the time the maximum temperature (50 °C) was reached, the stimulation was stopped and
315 the maximum temperature was recorded as the threshold value.

316 The pain induced by graded thermal stimuli was assessed with a 100-mm visual analog scale (VAS). All
317 participants received stimulation with three pseudorandomized heat stimuli (42 °C, 44 °C and 46 °C). For each
318 stimulus, participants were asked to rate the intensity of the pain on a VAS, extending from “no pain” to
319 “maximal imaginable pain”. For each stimulation, the thermode temperature gradually rose from baseline
320 temperature (32 °C) at a rate of 1 °C/s. Once the target temperature was reached, it was maintained for 2 s and
321 the temperature then returned to baseline. Stimuli were separated by an interval of at least 45 s. Pain sensitization
322 was prevented by applying the thermode to adjacent regions of the forearm, never using the same site for
323 consecutive stimuli.

324 For more precise assessments of pain sensitivity than could be achieved with the responses to arbitrary
325 temperatures, intensity response curves were calculated (Figure 3). This is a better approach to the assessment
326 of sensitivity, as it can be used to determine the half maximal effective temperature, or ET_{50} , corresponding to
327 the stimulation temperature required to induce 50 % of the maximal response (pain intensity of 5/10).

328

329

330 The data were modeled with a sigmoidal function:

$$331 \quad f(\text{temperature}) = \text{max} + \frac{\text{min} - \text{max}}{1 + \left(\frac{\text{temperature}}{\text{ET}_{50}}\right)^{\text{hillslope}}}$$

332 As the VAS is a bounded scale, minimum (min) and maximum (max) pain scores were set at 0 and 10,
333 respectively. Hillslope, the slope of the curve, and ET_{50} were left free. The statistical power of the modeling
334 approach was increased by calculating sigmoidal fits over 4-hour time epochs, corresponding to two evaluations
335 of pain sensitivity for each of the three stimuli (42 °C, 44 °C and 46 °C), providing six points on the regression
336 curve (Supplementary Figure 5). The ET_{50} values were extracted from each of the nine sigmoidal regressions
337 (see formula above; Figure 3A) and plotted over time (Figure 3B).

338

339 Body temperature

340 Core body temperature was measured every 2 h, with an ear thermometer (Braun Thermoscan Pro 6000, Welch
341 Allyn, New York, USA). Body temperature was measured within 2-3 seconds, with a precision of 0.2 °C.

342

343 Electrocardiogram

344 An electrocardiogram (ECG) was recorded with two adhesive skin electrodes (BlueSensor N, Ambu, Ballerup,
345 Denmark) positioned on the sternum and the lateral thorax (RA, LL, respectively, Fontaine bipolar precordial
346 leads). The signal was recorded at 256 Hz, with a Vitaport 4 digital recorder (Temec Instruments, Kerkrade,
347 The Netherlands), to assess autonomic nervous system activity. Heart rate (HR) and heart-rate variability (HRV)
348 were analyzed on the basis of the bipolar ECG signal. R-wave peak detection was performed over 10-second
349 windows during a 4.5-minute baseline resting episode. For interval analysis, data were resampled at a rate of 10
350 Hz. RMSSD was determined to estimate the vagally mediated changes reflected in HRV(66, 67). It was not
351 possible to obtain ECG data for the first participant, for technical reasons, so ECG analysis was performed for
352 11 participants.

353

354 Melatonin

355 Saliva was collected hourly, with cotton swabs placed directly in the mouth of the participant (Salivettes,
356 Sarstedt, Nümbrecht, Germany). Samples were stored at -20 °C until centrifugation and assay. Melatonin levels

357 were measured with an in-house radioimmunoassay ^{125}I (RIA). This assay was based on a competition
358 technique. The radioactive signal, reflecting the amount of ^{125}I -labeled melatonin, was therefore inversely
359 proportional to the concentration of melatonin in the sample. The sensitivity of the assay was 1.5 pg/mL. The
360 inter-assay coefficients of variation for high (18.5 pg/mL) and low (10 pg/mL) melatonin-concentration controls
361 were 19 % and 22 % respectively, and the mean intra-assay coefficient of variation was below 10 %. We
362 determined the circadian melatonin profile of each participant over a 24-hour day, by applying a three-harmonic
363 regression individually to the raw data collected during the CR (days 2 and 3)(68, 69). The model equation was:

$$364 \quad f(\text{time}) = \text{mesor} + \text{amplitude1} \times \cos\left(2\pi \times \frac{\text{time}}{\text{tau}} + \text{phase1}\right) + \text{amplitude2} \times \cos\left(2\pi \times \frac{\text{time}}{\frac{\text{tau}}{2}} + \text{phase2}\right) +$$
$$365 \quad \text{amplitude3} \times \cos\left(2\pi \times \frac{\text{time}}{\frac{\text{tau}}{3}} + \text{phase3}\right)$$

366
367 In the model, Tau (the circadian period) was constrained between 23.5 and 24.5 h; mesor, amplitudes (1 to 3)
368 and phases (1 to 3) were set free.

369 The dim light melatonin onset (DLMO), corresponding to the circadian phase, was calculated for each
370 participant. DLMO was defined as the time at which the ascending phase of the melatonin profile crossed the
371 25 % threshold of the peak-to-trough amplitude of the fitted curve. Due to technical problems with some saliva
372 samples, the full 24-hour melatonin profile could not be obtained for two participants. For one of these
373 participants, DLMO was calculated on the basis of melatonin levels during the habituation day (day 1), rather
374 than during the CR, for which we could not determine melatonin concentrations. For the second participant, in
375 the absence of melatonin concentration data (flat profile below the limit of quantification of the assay), DLMO
376 was estimated from the mean phase angle calculated between habitual bedtime and DLMO (calculated from
377 data published by Gronfier et al., 2004)(68).

378 379 Statistics

380 Outliers were identified on the basis of normalized data (z-scores) and were excluded from subsequent analyses
381 (outlier.test, R, Version 3.6.1 - 2019-07-05, R Foundation for Statistical Computing, Vienna, Austria). We
382 reduced inter-individual variability, by normalizing all data (except melatonin concentrations) by calculating
383 individual z-scores and smoothing them with a moving average (calculated on 3 points). The endogenous

384 circadian phase was taken into account for each participant, by aligning the data with the onset of melatonin
385 secretion (DLMO_n). As DLMO_n occurred at different times in different participants, individual melatonin onset
386 values were set to 0 (DLMO_n = circadian time 0), and all measurement times are expressed relative to melatonin
387 onset. We modeled the effects of time on the responses observed during the 34-hour constant routine, using an
388 additive model including a linear component (homeostatic, process S) and a sinusoidal component (circadian,
389 process C). The equation of the combined model was:

$$390 \quad f(\text{time}) = y_0 + a \times \text{time} + \text{mesor} + \text{amplitude} \times \cos\left(2\pi \times \frac{\text{time}}{\text{tau}} + \text{phase}\right)$$

391 Tau (circadian period) was constrained between 23.5 and 24.5 hours(70, 71), whereas all other parameters were
392 left free. Once the parameters of the combined model had been defined, process S and process C were modeled
393 separately. The homeostatic component (process S) was regressed against the linear component of the model:
394 $f(\text{time}) = y_0 + a \times \text{time}$. The circadian rhythmicity (process C) of the data was regressed against the sinusoidal
395 component of the model: $f(\text{time}) = \text{mesor} + \text{amplitude} \times \cos\left(2\pi \times \frac{\text{time}}{\text{tau}} + \text{phase}\right)$.

396 Statistics were calculated with R (Version 3.6.1 - 2019-07-05, R Foundation for Statistical Computing, Vienna,
397 Austria). Results were considered significant if $p < 0.05$. Unless otherwise stated, results are expressed as means
398 \pm SEM.

399 **Author contributions**

400 The experiment was conceived by CG, and designed by CG, DB and ID. Data were collected by ID and CG.
401 Melatonin assays were conducted by VR. Data were analyzed and interpreted by ID and CG. ID, CG and DB
402 wrote the manuscript, and VR revised the manuscript. All authors agree to be accountable for all aspects of the
403 work.

404

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409

410 **Conflicts of interest**

411 The authors declare that the research was conducted in the absence of any commercial or financial relationships
412 that could be construed as a potential conflict of interest.

413

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573 - Supplementary information -

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575 **Timing is everything: evidence that pain sensitivity is driven**
576 **by circadian and sleep processes in humans**

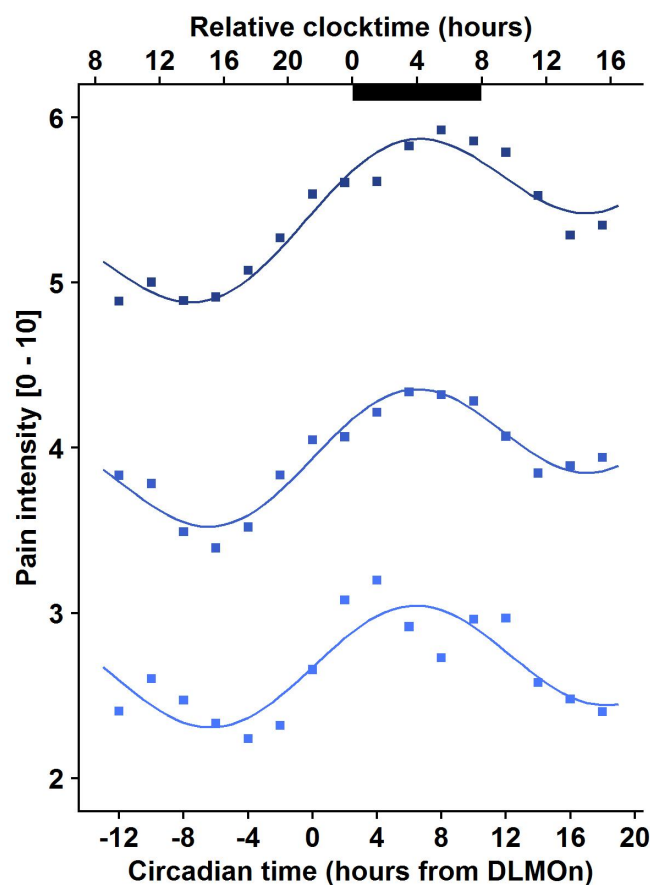
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583 **Supplementary Figure 1. Mean pain intensity in response to heat stimuli at 42 °C, 44 °C and 46 °C, across**
584 **the 34-hour constant routine protocol (n = 12).** Dark bars correspond to average habitual sleep episodes
585 (biological night). Circadian time 0 corresponds to DLMO (mean DLMO \approx 21:30). Combined models (sum
586 of linear and sinusoidal components) of pain sensitivity in response to heat stimuli at 42 °C (light blue curve),
587 44 °C (blue curve) and 46 °C (dark blue curve). The amplitude changes (between peak and trough) in pain
588 intensity for 42 °C, 44 °C and 46 °C are of 0.7, 0.8, and 1 VAS respectively.

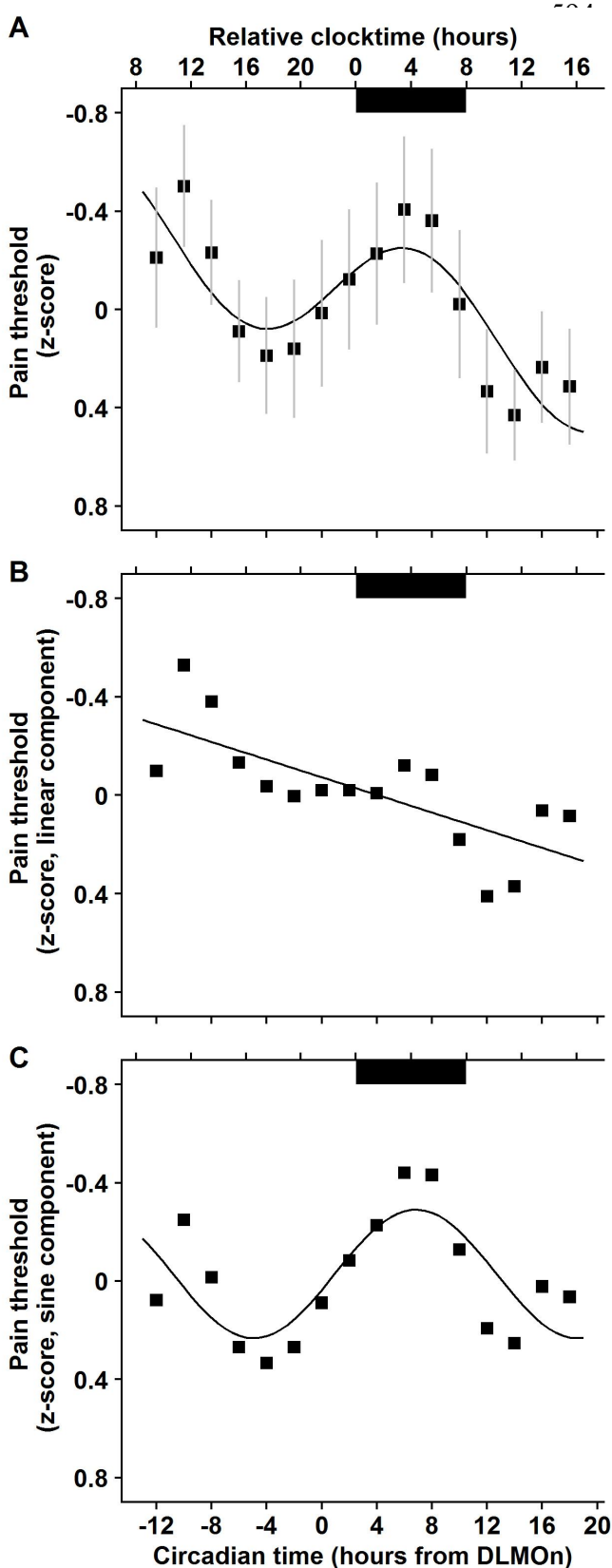
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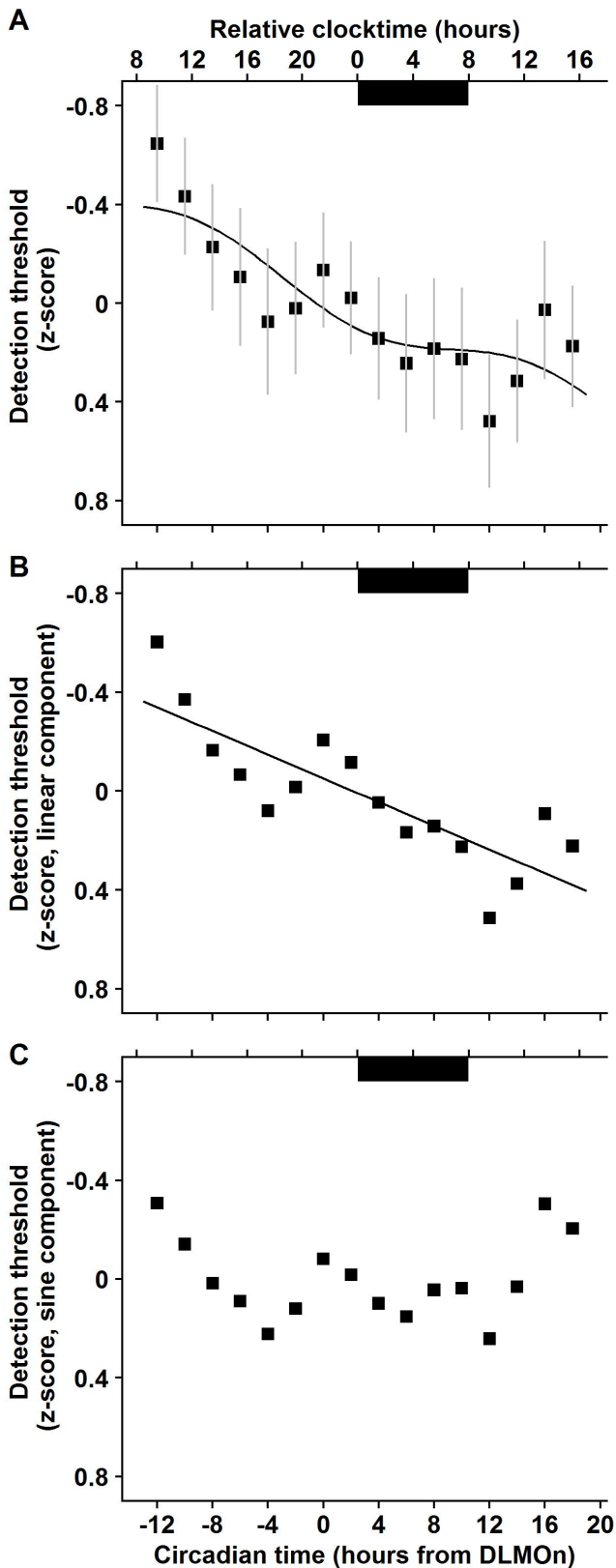
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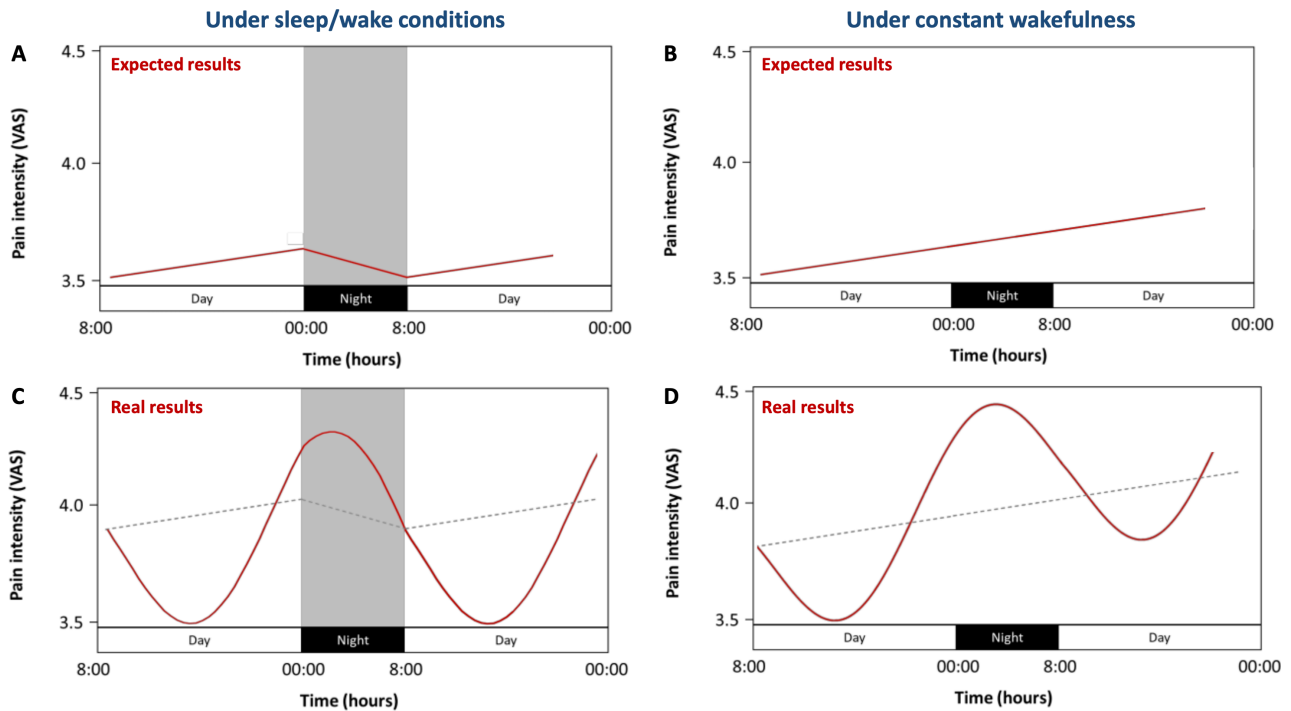
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Supplementary Figure 2. Mean heat pain thresholds, across the 34-h constant routine protocol (n = 12). Dark bars correspond to average habitual sleep episodes (biological night). Circadian time 0 corresponds to DLMO_n (mean DLMO_n ≈ 21:30). **A.** Combined model (sum of linear and sinusoidal components) applied to normalized data (mean ± SEM; R² = 0.69). **B.** Linear component (R² = 0.53; p < 0.01). Pain sensitivity decreases with time spent awake. **C.** Sinusoidal component (R² = 0.57). Pain sensitivity follows a circadian rhythm with maximal pain at 4:30.

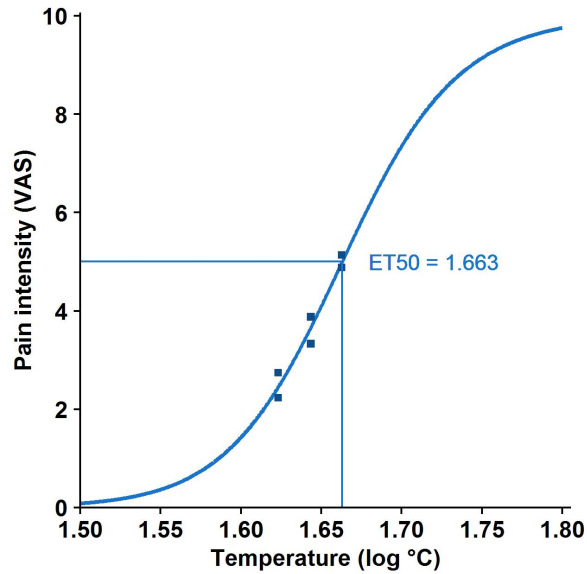


Supplementary Figure 3. Mean warm detection thresholds across the 34-h constant routine protocol (n = 12). Dark bars correspond to average habitual sleep episodes (biological night). Circadian time 0 corresponds to DLMO_n (mean DLMO_n ≈ 21:30). **A.** Combined model (sum of linear and sinusoidal components) applied to normalised data (mean ± SEM; $R^2 = 0.70$). **B.** Linear component ($R^2 = 0.68$; $p < 0.0001$). Heat sensitivity decreases with time spent awake. **C.** No sinusoidal component is found ($R^2 = 0.13$).



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Supplementary Figure 4. Variations of pain sensitivity across the 24h day. A-B. Expected pain sensitivity, according to the current homeostatic model based on data obtained in the literature (based on 3 articles only). Under regular sleep/wake conditions (A), pain sensitivity increases during wakefulness, in parallel to sleep pressure, and decreases during the night, due to an analgesic role of sleep. Under constant wakefulness conditions (B), pain sensitivity increases during wakefulness, and keeps increasing in the absence of sleep. In this model, pain sensitivity only depends on time since awakening (during the day), and time since bedtime (at night). C-D. Our results show that pain sensitivity is driven by two independent and additive components: a homeostatic drive and a circadian drive. With sleep at night (C) both mechanisms co-exist. Pain oscillates sinusoidally (circadian drive) and pain increases linearly during wakefulness and decreases during sleep (homeostatic drive – grey dotted line). Without sleep at night (D), our results under constant wakefulness show the superimposed additive homeostatic (grey dotted line) and circadian regulation of pain, with both a linear increase with time spent awake, and a sinusoidal oscillation.



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675 **Supplementary Figure 5. Pain Intensity Response Curve (IRC) at circadian time -9 (~ 12:30).** Sigmoidal
676 regression, calculated on a 4-hour time epoch (10:30 – 14:30), corresponding to 2 evaluations of pain sensitivity
677 to each of the 3 heat stimulations (42 °C, 44 °C and 46 °C), providing 6 points for the regression. The ET_{50}
678 value, corresponding to a stimulus inducing a pain of 5/10, is extracted from the sigmoidal regression (here ET_{50}
679 = 1.66 log[temperature]).

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683 **Time-of-day mechanisms of heat detection and pain thresholds**

684 Heat pain thresholds and warm detection thresholds were measured every 2 hours throughout the whole 34-hour
685 constant routine (Supplementary Figures 2 and 3; all $R^2 > 0.69$). The significant linear trend observed for heat
686 pain thresholds (Supplementary Figure 2; $p < 0.01$; $R^2 = 0.53$), suggests a decrease in pain sensitivity with sleep
687 pressure. This result might reflect a deterioration of cognitive-motor performances (slower reaction times)
688 associated with sleep pressure (1,2). The fact that this effect was not specific to the heat pain threshold, since a
689 similar relationship was seen for the warm detection threshold (Supplementary Figure 3; $R^2 = 0.68$; $p < 0.0001$),
690 is consistent with this hypothesis. The strong circadian rhythm of pain sensitivity (with a peak at 4:30), assessed
691 through heat pain threshold measures, confirms the results found with graded heat stimuli (and presented in the
692 main article). The lack of circadian rhythmicity for warm non-painful stimuli (Supplementary Figure 3C; $R^2 =$
693 0.13) suggests that the rhythmicity of pain sensitivity is specific to pain and is not related to a general
694 rhythmicity of thermal sensitivity.

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697 **References**

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