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Short-term propofol anaesthesia down-regulates clock genes expression in the master clock

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Introduction

10 Circadian rhythmicity is of crucial relevance for anaesthesia, not only for chronopharmacological aspects, but also for the clinical management of patients (Dispersyn et al. 2009).

15 Mammalian physiology is endogenously rhythmic on a daily basis, due to a network of circadian clocks that generate an internal cyclic timing. At the top of the circadian system is a master clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus. This structure provides timing
20 signals to many secondary clocks and oscillators in the brain and peripheral organs, thus controlling indirectly many circadian rhythms in behaviour (e.g., the sleep-wake cycle), physiology (e.g., hormonal secretion, like pineal melatonin or
25 adrenal glucocorticoids) (Kalsbeek et al. 2011) and sensitivity to drugs (e.g., anaesthetics) (Dispersyn et al. 2010). Furthermore, the SCN clock can be reset by environmental cues, allowing the phase-adjustment of internal rhythmicity
30 to the external cues.

Although the main environmental synchronizer (also called “Zeitgeber” or “time-giver”) is ambient light, the SCN clock can also be reset by other, non-photic cues. The most common
35 non-photic factors are behavioural manipulations, although nutritional cues may also impact SCN function (Challet 2010). *Period (Per)1* and *Per2* are genes participating in the main feedback loop of the molecular clockwork and involved in
40 resetting of the SCN (Ko and Takahashi 2006).

More precisely, expression of both genes in nocturnal rodents’ SCN is up-regulated by light exposure during nighttime (i.e., period of minimal daily expression) and down-regulated by behavioural activation during daytime (i.e., period of maximal daily expression) (Albrecht et al. 1997; Challet and Pévet 2003).

The chronobiotic impact of general anaesthetics (i.e., their effect on the circadian clock) is not fully characterized yet. Propofol is a widely used intravenous anaesthetic. On the one hand, general anaesthesia induced by propofol triggers phase-advances of SCN-controlled circadian rhythms only at certain times of the day (late resting period and early active period (Dispersyn et al. 2009; Challet et al. 2007). Moreover, general anaesthesia induced by propofol has been associated with a subsequent reduction of *Per2* mRNA levels in the whole brain (Yoshida et al. 2009). On the other hand, inhalational anaesthesia with sevoflurane leads to a down-regulation of *Per2* in the SCN, but its shifting effects of the sleep-wake cycle have not been clearly identified (Ohe et al. 2011). One critical point that remains to be characterized is the acute effects of propofol anaesthesia *per se* on the SCN molecular clockwork. For that purpose, anaesthesia has to be of short duration (to limit secondary effects, such as hypothermia), without concomitant surgery (to avoid confounding effects on the read-out parameters) and in the absence of light (to avoid stimulating effects of light on clock gene expression). In this context, we studied *Per1*

and *Per2* expression in the SCN of rats exposed to constant darkness after a single-dose injection of propofol (or vehicle) when the SCN clock is shifted by propofol (i.e., late afternoon in rats).

Material and methods

Animals and housing conditions

Adult, 2-months-old, male Long-Evans rats (*Rattus norvegicus*) were obtained from Janvier® (Le Genest-St-Isle, France). The experiment was performed in accordance with the Principles of Laboratory Animal Care (NIH published 86–23, revised 1985) and the French laws (License n°67–88 to E.C.).

Animals were housed in Plexiglas cages ($n = 5$ per cage) at 23°C under a 12 h light/12 h dark cycle (LD) for 2 weeks. Food and water were available *ad libitum*. On the day of the experiment, all rats were transferred to constant darkness by not allowing lights on in the morning. Because previous studies (Dispersyn et al. 2009; Challet et al. 2007) showed that propofol administration at the end of the resting period leads to phase-advanced sleep-wake and body temperature rhythms, rats received a single-dose injection of propofol 1% (120 mg/kg, $n = 15$) or vehicle (Intralipid® 10%, $n = 15$) in late day (i.e., projected circadian time (CT) 10, that is, 10 h after the expected time of lights on). Thereafter, rats were decapitated after CO₂ killing in darkness 1 h, 2 h or 3 h after the treatment (i.e., projected CT11, CT12 or CT13; $n = 5$ per time and treatment). Brains were quickly removed, frozen with –30°C isopentane and stored at –80°C.

Radioactive *in situ* hybridization

Twenty- μ m brain coronal sections at the SCN level were made on a cryostat, mounted in series on SuperFrost®Plus slides (VWR, USA) that were stored at –20°C. We analysed expression of *Per1* and *Per2* mRNA in the SCN. The *rPer1* and *rPer2* clones are a gift from Prof. H. Okamura (Graduate School of Pharmaceutical Sciences, Kyoto University, Japan). The sense (to check specificity of the signal) and antisense cRNA riboprobes were transcribed in the presence of ³⁵S-UTP (0.4625 Mbq/ μ l, PerkinElmer, France) according to the manufacturer's protocol (MAXIscrip, Ambion, Austin, TX, USA).

Briefly, sections were postfixed in 4% phosphate-buffered paraformaldehyde for 15 min, rinsed in 1× phosphate buffer saline (PBS) for 2 min, and then acetylated for 10 min in 0.1M triethanolamine and acetic anhydride. After a 1× PBS rinse for 2 min, sections were dehydrated in graded ethanol series and finally dried at room temperature (RT). Hybridization was carried out at 54°C overnight with riboprobe in a solution containing 100% deionized formamide, 4 g of sulfate dextran, 50× Denhardt's solution, 20× sodium citrate saline (SSC), 40 mg salmon sperm DNA, 10 mg transfer RNA, 1M dithiothreitol. After hybridization, the sections were washed for 5 min in 2× SSC before being treated with ribonuclease A (Sigma-Aldrich; 10 mg/ml) and a buffer solution for 30 min at 37°C. The slides were then rinsed and stringency washes were carried out (1× SSC for 5 min at RT, and 0.2× SSC for 30 min at 62°C). Finally, the sections were dehydrated in a graded ethanol series and air-dried at RT. Slides were exposed with ¹⁴C standards to an autoradiographic film (BioMax® MR, Kodak, Lyon, France) for 3 days. Analysis of the autoradiograms was performed using NIH-ImageJ software. Background was subtracted. For each probe and each animal, quantification was calculated and averaged on three sections of middle SCN for a given brain by subtracting the intensity of staining of background area (diameter: 200 μ m) measured in the anterior hypothalamic area above the SCN from an area (diameter: 200 μ m) measured in the left and right SCN.

Statistical analysis

Data are presented as mean \pm SEM. Data were analysed with two-way analyses of variance (ANOVA; IBM® SPSS® Statistics version 25, Chicago, USA). Bonferonni post hoc tests were used if main effects or interactions were found to be significant.

Results

- **Expression of *Per1*** ($n = 27$, not 30 due to lost sections)

We found a time (CT11, CT12, CT13; $F(2,27) = 15.103$; $p < 0.001$) and a treatment effect (Propofol, Intralipid®; $F(1,27) = 4.407$; $p = 0.048$).

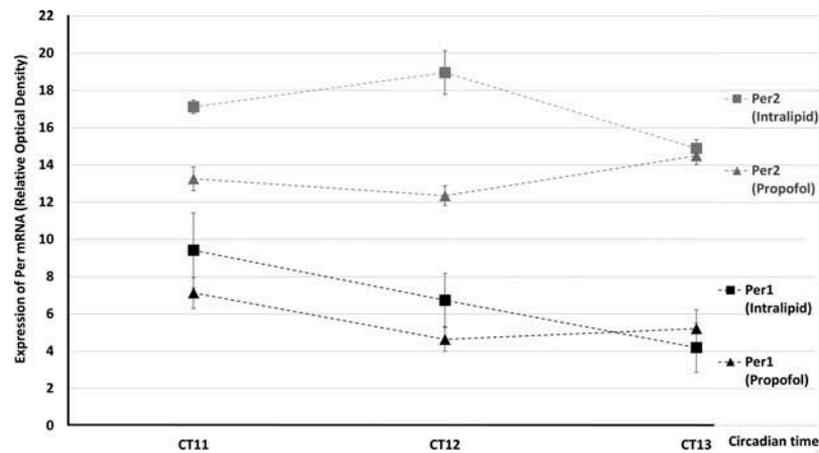


Figure 1.

The interaction between time/treatment was also statistically significant ($F(2,27) = 4.310$; $p = 0.027$). The post-hoc test showed only differences between CT11 versus CT12 ($p = 0.005$) and CT11 versus CT13 ($p < 0.001$) (Figure 1). Together, these results indicate that propofol anaesthesia induced a decrease in *Per1* expression in the SCN at CT11 and CT12 when compared with vehicle group.

- **Expression *Per2*** ($n = 26$, not 30 due to lost sections)

We observed a treatment effect (Propofol, Intralipid®; $F(1,26) = 13.382$; $p = 0.002$), but no time effect (CT11, CT12, CT13; $F(2,26) = 0.338$; $p = 0.717$) was detected. No difference between different time points was found with the post hoc test. Finally, the interaction between time/treatment was statistically significant ($F(2,26) = 3.535$; $p = 0.048$) (Figure 1). Thus, the expression of *Per2* in the SCN is decreased at CT11 and CT12 after propofol anaesthesia.

Discussion

At behavioural level, propofol anaesthesia in rats produces phase-advances in the late (subjective) daytime, an effect reminiscent of the chronobiotic consequences of classical non-photic cues (*i.e.*, transient behavioural arousal associated with motor activity) studied in nocturnal mammals (Mrosovsky 1996; Challet and Pévet 2003) exogenous melatonin (Pitrosky et al. 1999) and dark pulses in constant light (Dwyer and Rosenwasser 2000; Mendoza et al. 2004).

At molecular level, we show here for the first time that short-term anaesthesia induced by propofol leads to a transient down-regulation of *Per1* and *Per2* expression in the SCN. These acute effects already known for behavioral arousal and dark pulses (Maywood et al. 1999; Mendoza et al. 2004) play a causal role in the resetting properties of non-photic cues (at least for *Per1*: see Hamada et al. (Hamada et al. 2004). Of interest, even if treatment with exogenous melatonin can shift the SCN clock as propofol anaesthesia does, the initial molecular targets are not *Per* genes, but *Rev-erba* and *Rorβ* (Agez et al. 2007). Together, these data suggest that propofol anaesthesia shares activation of common transduction SCN pathways with classical non-photic cues and dark pulses, but not with pharmacological doses of melatonin.

Per genes and anaesthesia

Other studies have already found that general anaesthesia influences *Per2* expression in the brain of rodents. They show a decrease in *Per2* expression after inhalational anaesthesia (sevoflurane) (Sakamoto et al. 2005; Kobayashi et al. 2007) or after propofol anaesthesia (Yoshida et al. 2009). In these studies, gene expression was assessed in the whole brain and the authors have maintained anaesthesia for 2 h or more. Their animals were exposed to hypothermia and hypoxia, both being able to interact with circadian rhythmicity (Herzog and Huckfeldt 2003; Touitou et al. 2010). Ketamine, an intravenous anaesthetic acting as N-methyl-d-aspartate (NMDA) receptors

220 antagonist (Solt and Forman 2007) alters *Per2*
 expression in an *in vitro* model (Bellet et al.
 2011). Recently, a decrease in *Per2* expression in
 SCN of rodents after 4–8 hours exposition to sevo-
 225 flurane (Ohe et al. 2011; Kadota et al. 2012; Anzai
 et al. 2013) or isoflurane anaesthesia (Xia et al.
 2015) in subjective daytime is reported. Besides,
 Wang et al. show a suppression in *Per1* expression
 in the middle prefrontal cortex (mPFC) of rats
 (Wang et al. 2016). To our knowledge, the conse-
 230 quence of general anaesthesia on *Per1* expression
 in the SCN has not been studied yet.

Cerebral targets of propofol

To understand the molecular changes induced in the
 master clock by propofol, we need to investigate the
 235 neurochemical mechanisms of general anaesthesia,
 especially the targets of propofol. Molecular studies
 have demonstrated that the γ -aminobutyric acid type
 A (GABA_A) receptors are the main target of volatile
 anaesthetics (isoflurane, sevoflurane) and of propofol
 240 (Solt and Forman 2007; Rudolph and Antkowiak
 2004). GABA_A receptors are the major inhibitory
 neurotransmitter-gated ion channels in the human
 brain (Solt and Forman 2007) and GABA is the
 principal neurotransmitter of the circadian system
 245 (Moore and Speh 1993; Albers et al., 2017). More
 than 90% of SCN neurons contain GABA as a neu-
 rotransmitter and express both GABA_A and GABA_B
 receptors (Albers et al., 2017; Han et al. 2012).
 Propofol decreases neuronal excitability by enhan-
 250 cing the activity of GABA_A receptors. The hypnosis
 and immobility induced by propofol are mediated by
 GABA_A receptors containing β_3 subunits, while
 sedation is linked to receptors containing β_2 subunits
 (Solt and Forman 2007). Propofol also acts through
 255 other receptors like glycine receptors or calcium
 channels (Rudolph and Antkowiak 2004).

GABAergic system in the SCN as a putative target of propofol anaesthesia

The data mentioned above suggest that the propofol
 260 action can be mediated by GABA_A receptors in the
 SCN. *In vitro*, GABA can induce phase-shifts in
 clock cells kept in culture and the daily treatment
 by GABA synchronizes cultured clock cells (Liu and
 Reppert 2000). In SCN explants of luciferase

transgenic rats, the suppression of *Per2* expression 265
 observed after sevoflurane application is blocked by
 the co-application of both GABA_A and GABA_B
 receptor antagonists, suggesting an GABAergic
 mechanism (Matsuo et al. 2016). *In vivo*, the
 GABA_A receptor activation by muscimol (a selective 270
 GABA_A receptors agonist) during the mid-subjective
 day not only induces phase advances of locomotor
 activity in hamsters (Smith et al. 1989), but also
 suppresses *Per1* mRNA and *Per2* mRNA expression
 in the SCN of both diurnal and nocturnal rodents 275
 (Ehlen et al. 2006; Novak et al. 2006). Yokota and al.,
 report also phase advances in hamster locomotor
 activity and reduced expression of *Per1* and *Per2* in
 the SCN 1 and 2 h after injection of brotizolam 280
 (which is a GABA_A receptor agonist belongs to ben-
 zodiazepines family) during mid-subjective daytime
 in hamsters (Yokota et al. 2000). The effects are quite
 comparable to those observed after propofol injec-
 tion. However, the injection of muscimol (Smith
 et al. 1989) or brotizolam (Yokota et al. 2000) during 285
 the mid-subjective night leads to phase-delays of
 locomotor activity, while propofol anaesthesia has
 no longer any shifting effects at this time of day
 (Challet et al. 2007). In mutant mice with loss of
 function of voltage-gated Na⁺ channel NaV1.1 290
 (which is the primary voltage gate Na⁺ channel in
 several classes of GABA interneurons), Han et al.
 demonstrated important disruption in the circadian
 master clock with longer circadian period, delayed
 activity onset and decreased circadian amplitude. 295
 This impairment is related to reduction of
 GABAergic transmission (Han et al. 2012). In a
 recent abstract, the suppression of *Per1* expression
 in the mPFC of rats following a propofol infusion is
 described. However, this result is not observed when 300
 propofol is co-administered with GABAzine (a
 GABA_A receptor antagonist) (Wang et al. 2016).

Other propofol targets?

The molecular interaction of propofol with GABA_A
 receptors is largely studied, but its action on glycine 305
 receptors is not fully clear. Recently, Nguyen et al.
 demonstrated that the glycine receptors partially
 contribute to propofol-induced hypnosis (Nguyen
 et al. 2009). An electrophysiological study on SCN
 cells revealed that glycine application induces phase 310
 advances during the subjective day and phase-delays

during the early subjective night (Mordel et al. 2011). The timing of these phase-shifts, however, is very different from those induced by *in vivo* injections of propofol (Challet et al. 2007), suggesting that the chronobiotic effects of propofol are not mainly mediated via glycinergic signalling.

Another hypothesis for propofol action is a modulation of the gap junctions in the SCN. These junctions made of connexins are responsible for the intercellular coupling between SCN cells (Aton and Herzog 2005). An ultrastructural study reported the immunocytochemical evidence that these neuronal gap junctions are composed of connexin36 (Rash et al. 2007). Previously, Mantz and colleagues showed that propofol and other general anaesthetics differentially reduce permeability of gap junctions, at least in cultured astrocytes (Mantz et al. 1993). In rat SCN neurons cultured *in vitro*, the gap junction communication is involved in interneuronal communication and the coupling state between neurons via GABA_A receptor. The GABA_A receptor agonist muscimol acts as a gap junction blocker (Shinohara et al. 2000). In the same way, connexin36-knockout mice show dampened circadian activity rhythms and a delayed onset of activity during transition to constant darkness, as compared to wild-type mice (Long et al. 2005). Of note, these knockout mice show a greater sensitivity to anaesthetics, including propofol (Jacobson et al. 2011). Together, these data suggest that the phase-shifts induced by propofol anaesthesia may be related to an uncoupling effect between SCN neurons mediated by GABA_A receptors.

Some data suggest that circadian effects of anaesthetics result from the inhibition of the binding of the complex CLOCK:BMAL1 (Circadian Locomotor Output Cycles Kaput: Brain and Muscle Arnt-like 1 protein) via glycogen synthase kinase 3 β (GSK3 β) (Poulsen et al. 2018). In physiological conditions, CLOCK:BMAL1 complex promotes *Per* genes transcription (Ko and Takahashi 2006). Ketamine, propofol anaesthesia or long-term sevoflurane exposure leads to (GSK3 β) inactivation (Poulsen et al. 2018). This effect has also been observed in the presence of dizocilpine maleate (an NMDA receptor antagonist) (Hetman et al. 2000) suggesting an anti-NMDA mechanism of ketamine and sevoflurane, but another not yet elucidated mechanism for propofol.

Further studies are needed to better understand the neurochemical and chronobiological mechanisms

underlying propofol anaesthesia and propose preventive treatments of the circadian disturbances, fatigue and sleep disorders reported by patients after general anaesthesia (Dispersyn et al. 2009).

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Conflicts of interest

The authors declare no conflicts of interest.

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