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Cirkadiánní hodiny v hipokampu

Circadian Clock in the Hippocampus

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Abstract

To adapt to the daily changing environment, living organisms on Earth have developed an inner clock. In mammals, the clocks are organized in a circadian system which is governed by the central oscillator in suprachiasmatic nuclei (SCN) and consists of peripheral oscillators located in tissues and organs throughout the body. The synchrony of the entire system allows proper alignment of physiological processes to the optimal time of day. It was shown that cognitive performance and memory formation are also subjected to daily variations and many brain regions involved in these processes, especially hippocampus (HPC), were recently found to harbor circadian clocks. Nevertheless, the precise function of these clocks and the mechanism of their synchronization to the external conditions remain to be elucidated.

The focus of my PhD thesis was a detailed analysis of the circadian clocks in rodent HPC. We aimed to characterize the oscillators in distinct HPC subregions, identify signals responsible for their synchronization and compare the HPC clock properties to those of the clock in the choroid plexus (CP) which is the adjacent non-neuronal structure. Moreover, we examined the role of clocks in HPC and other brain regions in the process of memory formation, focusing on time memory acquisition.

First, we characterized the clocks in individual HPC subregions *in vivo* and, using a newly established technique, also *in vitro.* Then, using both approaches we tested the role of glucocorticoid hormones (GCs) as a possible synchronizing signal for the HPC clocks. We showed that GCs, which exhibit daily rhythms in secretion and provide the animal with information about arousal state, are necessary for the HPC clock function *in vivo* and are able to directly synchronize the HPC clock in the *in vitro* conditions. Additionally, we explored the role of GSK3β signaling, an important pathway involved in memory and cognition-related processes and demonstrated that the GCs and GSK3β signaling both affect the HPC clock, however with opposite effects.

Next, we compared properties of the HPC clock to the recently discovered circadian oscillator in the CP. We demonstrated that even though both the neuronal and non-neuronal tissues harbor circadian clock, the two oscillators differ in robustness and phases of their rhythms *in vivo*. Importantly, our results show, that HPC and CP clocks share the GCs as a synchronizing signal, however, surprisingly we provide evidence showing that the mechanism of GC-mediated synchronization varies between the two closely associated tissues.

Finally, our goal was to elucidate the involvement of HPC clock and other brain oscillators in the process of memory formation, namely in time memory, and analyze the potential changes of the brain clocks following its acquisition. Surprisingly, our results show that the clocks in HPC subregions are not affected during the process of time memory formation, however, our findings highlight the potential involvement of dorsal striatum oscillator in this process.

Abstrakt

Cirkadiánní systém se vyvinul jako mechanismus adaptace živých organismů na pravidelně se střídající podmínky na Zemi. Jedná se o systém vnitřních hodin, který je u savců řízen centrálním oscilátorem v suprachiasmatických jádrech (SCN) hypotalamu, jenž řídí periferní oscilátory, nacházející se v tkáních a orgánech těla. Synchronizace tohoto systému s vnějším prostředím umožňuje správné načasování fyziologických procesů včetně kognice a paměti v průběhu dne. V nedávné době byla popsána přítomnost periferních cirkadiánních hodin v oblastech mozku, jako je hipokampus (HPK), které se na těchto procesech podílí. Přesná úloha těchto oscilátorů a mechanismus jejich synchronizace s vnějšími podmínkami nicméně dosud nejsou známy.

Cílem mé doktorské práce byla detailní analýza cirkadiánních hodin v HPK hlodavců. Naším záměrem bylo charakterizovat oscilátory v jednotlivých částech HPK, identifikovat signály zodpovědné za jejich synchronizaci a porovnat vlastnosti hodin v HPK s neneuronálním oscilátorem v choroidním plexu (CP), který se nachází v těsné blízkosti HPK. Naším dalším cílem pak bylo prozkoumat úlohu hodin v HPK a dalších oblastech mozku v procesu tvorby paměti, a to se zaměřením na paměť časovou.

Nejprve jsme charakterizovali hodiny v jednotlivých částech HPK, a to jak *in vivo*, tak za použití nově zavedené *in vitro* techniky. Následně jsme za využití obou přístupů testovali schopnost glukokortikoidních hormonů (GK) hodiny v HPK synchronizovat. Naše výsledky ukazují, že GK, vykazující denní rytmus v sekreci a poskytující zvířeti informaci o momentálním stavu bdělosti, jsou nezbytné pro fungování HPK hodin *in vivo* a jsou schopné tyto hodiny přímo synchronizovat v *in vitro* podmínkách. Zkoumali jsme také vliv GSK3β signalizace, důležité signální dráhy účastnící se procesů spojených s pamětí a kognicí, a ukázali jsme, že jak GK, tak GSK3β signalizace hodiny v HPK ovlivňují, ovšem s opačnými účinky.

Dále jsme provedli srovnání vlastností charakterizovaných HPK hodin s nově objeveným oscilátorem v CP. Naše výsledky prokazují, že tyto struktury neuronálního a neneuronálního původu obsahující cirkadiánní hodiny se liší svou robustností a fází cirkadiánních rytmů *in vivo*. Ukázali jsme také, že jak HPC, tak CP hodiny jsou synchronizovány GK. Naše experimenty ovšem překvapivě signalizují, že mechanismus účinku GK se v těchto dvou přilehlých tkáních liší.

Závěrem jsme demonstrovali, že vznik časové paměťové stopy oproti očekávání nevede ke změnám v chodu cirkadiánních hodin v jednotlivých částech HPK. Naše výsledky nicméně naznačují možný podíl cirkadiánních hodin v dorsálním striatu na tomto procesu.

Introduction

Circadian system
In mammals the synchronization of physiological processes with the light dark cycle is carried by the circadian system, a hierarchical system governed by the central pacemaker, located in the suprachiasmatic nuclei (SCN) in the hypothalamus (Hendrickson *et al.*, 1972; Moore and Lenn, 1972). Other clocks in tissues and organs throughout the body are synchronized by the SCN and act as the so-called peripheral oscillators.

The central oscillator
The SCN receives information about the light/dark cycle in the environment from the retina via the retinohypothalamic tract (Abrahamson and Moore, 2001). Its outputs then synchronize clocks in the rest of the body accordingly and enable the proper timing of physiological processes carried out by the peripheral tissues (Saini *et al.*, 2011). The SCN itself is composed of tightly packed neurons coupled by synapses and produces a robust rhythmic signal (Hastings *et al.*, 2018).

Molecular mechanism of the circadian clock
At the molecular levels, the function of circadian clocks is driven by a set of transcription-translational feedback loops, composed of the so-called clock genes and their protein products (Takahashi *et al.*, 2008). As a result, these genes and proteins exhibit daily expression rhythms. The clock genes can then drive the expression of the so-called clock-controlled genes (CCGs), influencing other processes in the tissues (Bass and Takahashi, 2010; Asher and Schibler, 2011).

Peripheral oscillators in the brain
Outside of the SCN, other organs and tissues in the body have been found to act as peripheral oscillators. The cells of these tissues contain functional molecular clock machinery, which influences many cellular, tissue and organ level processes and synchronizes their occurrence to the daily light/dark cycle. These clock however do not receive information about outside conditions directly from the retina, but instead rely on other synchronizing factors, such as neural, hormonal, and metabolic signals, orchestrated by the SCN (Ishida *et al.*, 2005; Segall *et al.*, 2006, 2008; Verwey *et al.*, 2007; Buhr *et al.*, 2010; Hood *et al.*, 2010). Many brain regions have been found to harbor circadian clocks, influencing the function of these brain parts (reviewed in Paul *et al.*, 2020).

Hippocampus
Hippocampus (HPC) is a brain region responsible for memory formation, spatial orientation, and other cognitive processes. It is a part of the limbic system and also a target of dopaminergic pathways. It is composed of distinct subregions: *Cornu Ammonis* 1-4 (CA1-4) and dentate gyrus (DG). HPC functions have been shown to vary across the circadian cycle (Holloway and Wansley, 1973; Chaudhury and Colwell, 2002) and it has also been observed that the disruption of circadian rhythms has a detrimental effect on these processes (Ruby *et al.*, 2008; Phan *et al.*, 2011). Based on these findings, the possibility of an HPC oscillator has been addressed by many studies. Rhythms of clock gene and protein expression have been found in the HPC, however sometimes with contradictory results and the question of the self-sustainability of the HPC oscillator still remains controversial. Importantly, genetic disruption of the molecular clock mechanism (either global or forebrain-specific) by either *Per1* or *Bmal1* deletion produced deficits in HPC-dependent memory and related processes, highlighting the role of the circadian clock in regulation of the HPC function (Wardlaw *et al.*, 2014; Rawashdeh *et al.*, 2016; Snider *et al.*, 2016). However, the synchronization of hippocampal clock to the external light-dark cycle and thus the proper timing of memory acquisition and related processes has been sparsely studied and this thesis is devoted to contributing to this knowledge.

Other extra-SCN brain oscillators
Peripheral oscillators have been described in other brain structures as well. These are often parts of the limbic system and dopaminergic brain regions, e.g. cingulate cortex, dorsal striatum, nucleus accumbens and amygdala. In general, the findings mostly describe rhythmical expression of clock genes and proteins in these tissues and not much is known about the functions of these circadian oscillators (reviewed in Paul *et al.*, 2020).

Another robust brain oscillator has been only recently discovered in the choroid plexus, an epithelial tissue located in the brain ventricles, responsible for CSF production and composition (Myung, Schmal, *et al.*, 2018; Quintela *et al.*, 2018). The non-neuronal oscillator in the CP is capable of influencing the SCN clock in vitro probably via paracrine signals (Myung, Schmal, *et al.*, 2018), making it a potentially important player in the network of distinct extra-SCN brain clocks. This could be particularly important in the case of HPC, which is in close contact with the brain ventricle system.

Synchronization of peripheral oscillators

In rats and mice, the *in vivo* phases of the clock in HPC and other brain areas are phase delayed to the SCN clock (Wang *et al.*, 2009; Jilg *et al.*, 2010), which likely reflects the nocturnal behavioral patterns of these animals. Entrainment of circadian clocks with environmental cycles is believed to be mediated via the light-entrainable SCN clock, however, the signals that the brain oscillators use for setting the phase of their clocks are unknown.

Glucocorticoid hormones

The synthesis of glucocorticoid hormones (GCs) is controlled by the SCN and follows daily oscillations. The maximum of the plasma GC rhythm occurs at the beginning of the active phase of the organism (Cheifetz, 1971; Qian *et al.*, 2012). Because of their daily rhythm in plasma levels and high abundance of GRs in peripheral tissues, glucocorticoids present a convenient hormonal signal carrying out synchronization of peripheral oscillators. This was confirmed in various cellular models, tissues and organs (reviewed in Spencer et al., 2018), including several extra-SCN brain oscillators (Amir *et al.*, 2004; Lamont *et al.*, 2005; Segall *et al.*, 2006; Woodruff *et al.*, 2016). Several molecular clock components were shown to contain GREs in their promoters, including clock genes *Per1*, *Per2*, *Nr1d1* and *E4bp4* (Torra *et al.*, 2000; Yamamoto *et al.*, 2005; So *et al.*, 2009; Surjit *et al.*, 2011; Cheon *et al.*, 2013). Moreover, the clock molecular mechanism is also able to modulate the GC signaling. Several GR phosphorylation sites, modulating the receptors' function, has been shown to be regulated in a circadian manner and the phosphorylation of GRs can be carried out directly by the clock protein CLOCK (Nader *et al.*, 2009; Robles *et al.*, 2016). Additionally, clock proteins CRY1 and CRY2 also regulate GC signaling by rhythmically repressing the GR activation by GCs (Lamia *et al.*, 2011) adding even more possible layers to the interplay between circadian clocks and glucocorticoid signaling.

GSK3β signalling

Glycogen synthase kinase 3 beta (GSK3β) is a serine/threonine kinase participating in a wide range of signaling pathways. In neuronal tissue, the regulation of GSK3β activity has been shown to play a role in synaptic plasticity and the enzyme has also been studied in the context of neuropsychiatric disorders, namely in connection with Alzheimer's disease pathology (reviewed in King *et al.*, 2014). GSK3β has also been found to take part in regulation of the molecular mechanism of circadian clocks. In its active state, GSK3β phosphorylates several clock proteins. The phosphorylation of PER2 by GSK3β increases nuclear translocation of the protein, which is necessary for its role in the molecular feedback loop (Iitaka *et al.*, 2005). Another phosphorylation target of the kinase is the clock protein NR1D1. In this case the phosphorylation increases stability of the protein, which then leads to a decrease in levels of BMAL1 (Yin *et al.*, 2006). Additionally, GSK3β also phosphorylates CLOCK (Spengler *et al.*, 2009) and BMAL1 (Sahar *et al.*, 2010), targeting BMAL1 for degradation. Specific inhibition of GSK3β using small molecule inhibitors, such as CHIR99021 consistently causes a shortening of circadian rhythm period in the U2OS cell line (Hirota *et al.*, 2008) and also in SCN and HPC organotypic explants (Besing *et al.*, 2015, 2017). The GSK3β phosphorylation state, reflecting its endogenous activity state, was found to follow a circadian rhythm in the SCN and liver (Iwahana and Akiyama, 2004; Iitaka *et al.*, 2005; Besing *et al.*, 2015) and interestingly also in HPC (Kinoshita *et al.*, 2012; Kaasik *et al.*, 2013; Besing *et al.*, 2017). The physiological consequences of GSK3β activity oscillations in these tissues remain unclear, but together with the extensive interaction with the circadian clock molecular machinery, they make GSK3β an interesting candidate for an intrinsic signaling molecule possibly connecting the synaptic plasticity processes with the circadian clock.

The role of brain oscillators in time memory

Physiological processes undergoing daily variation include memory formation, learning and related processes (Holloway and Wansley, 1973; Monk *et al.*, 1997; Chaudhury and Colwell, 2002; Wright *et al.*, 2002; Gritton *et al.*, 2012). The structural substrates of this modulation are not known but are likely to involve the oscillators located in memory-related brain regions, including HPC. One particularly interesting type of memory tightly related to the circadian system is the so-called time memory, enabling animals to anticipate the timing of significant day-to-day events, such as food availability or predation, and thus providing them with a competitive advantage. Importantly, the central oscillator in the SCN is not essential for time memory formation. Animals with SCN lesions were shown to produce time-specific responses to several conditioned learning paradigms (Ko *et al.*, 2003; Cain and Ralph, 2009; Cain *et al.*, 2012). Furthermore, the 24 h period of time memory is preserved in hamsters carrying a mutation, making their central oscillator generate rhythmical behavior with a 20 h period (Cain *et al.*, 2014). The question therefore remains whether the other brain oscillators outside of the SCN could play a role in the formation of time memory.

Aims of the thesis

To characterize circadian oscillators in distinct regions of rodent hippocampus and adjacent choroid plexus.

Our objective was to identify and characterize the circadian clocks in distinct regions of the rodent hippocampus in vivo and in vitro and to identify potential synchronizing signals, entraining the HPC clock. Additionally, our goal was to compare the HPC clock properties and synchronization mechanism with adjacent non-neuronal oscillator in the 3rd ventricle choroid plexus.

Our hypothesis was that the HPC clock would exhibit robust rhythmicity in vivo and in vitro and that the HPC oscillator would be synchronized by glucocorticoid hormones, reflecting the arousal state of the animal, as well as by GSK3β signaling, involved in memory-related processes in the HPC. We expected the neuronal and non-neuronal peripheral clock to share the same phase, relative to the external environment and presumed that glucocorticoid hormones would act as a synchronizing signal in both closely associated oscillators.

To determine the role of rodent hippocampal circadian clock, and clocks in other brain regions, in time memory formation.

In this study we aimed at finding the role of circadian clock in hippocampus and other brain regions related to memory, in the specific process of time memory.

Our hypothesis was that the process of acquiring of a time memory would be accompanied by phase-shifting of circadian clock phases in brain regions involved in this process, including hippocampus.

Methods

Animals
Adult male Wistar rats (Institute of Physiology, the Czech Academy of Sciences), adult *mPer2^{Luc}* mice of both genders (strain B6.129S6-Per2tm1Jt/J, JAX, USA; a colony maintained at the Institute of Physiology, the Czech Academy of Sciences and a separate colony maintained at the Biological Sciences Facility at the University of Toronto) and a group of wild type C57B16 mice (Institute of Physiology, the Czech Academy of Sciences) were housed individually under a 12-h light/12-h dark cycle (LD12:12); light on at 6:00 corresponded to Zeitgeber time (ZT) 0, light off at 18:00 corresponded to ZT12.

Adrenalectomy experiment
Wistar rats was subjected to ADX or sham surgery (the same procedure but without removal of adrenal glands) as described previously (Soták et al., 2016). The ADX animals were either untreated (ADX; n = 35) or treated with dexamethasone (1 mg/kg b.w.) at ZT12 (before lights off, 18:00) for 8 days (ADX+DEX; n = 27). The sham operated rats (SHAM; n = 35) were untreated and used as controls. All rats were sacrificed 8 days after surgery under deep isoflurane anesthesia in 4 h intervals during 24 h. The brains were frozen on dry ice and kept at -80°C.

Acute GC effect on gene expression
Wistar rats were injected with DEX (1 mg/kg) or vehicle (VEH; phosphate-buffered saline) at ZT3 (3 hours after lights on) for the HPC experiment and at ZT16 (4 h after lights off) for the CP experiment. Animals were sacrificed under deep thiopental anesthesia at 0, 30, 60, 120, 240 and 480 min following the injection (5 animals per time point and group). The brains were frozen on dry ice and kept at -80°C.

Conditioned place avoidance (CPA)
mPer2^{Luc} mice were pre-exposed to two identical chambers, which differed in the pattern on their walls. For conditioning each mouse was placed into one of the chambers for 10 minutes at either ZT3 or ZT11. The next day, at the same time, the animal was placed into the other chamber. Animals were thus exposed to each chamber four times over 8 consecutive days. For each animal, one of the chambers was the shock chamber, where they received three 0.3mA foot shocks during each 10-minute conditioning trial. After the end of the conditioning procedure, animals were placed in the alley connecting the two chambers at ZT3 or ZT11 and given access to both chambers. Time spent in each chamber was recorded. Naïve mice and mice exposed to the CPA apparatus without conditioning were used as controls. All animals were anesthetized with isoflurane and sacrificed at ZT04 or ZT12, one hour following the testing.

Laser capture microdissection
Frozen brains were sectioned on cryostat into 20 or 30-µm coronal sections, stained with cresyl violet (Sigma Aldrich, St. Louis, USA) and dissected using a laser microdissector (LMD6000, Leica) (Houdek and Sumová, 2014). In the hippocampus study, rat CA1, CA3 and DG were separated bilaterally from 30-µm sections. In the choroid plexus study, CP tissue from the 3rd ventricle was dissected from 30-µm coronal sections of rat brains. In the time memory study, sections containing SCN, Ci, DS, NAS, NAC, DG, CA3, CA1, BLA and CeA were dissected - 20-µm sections were used for smaller nuclei, whereas 30-µm sections were used in larger structures.

RNA isolation and qRT-PCR
Total RNA was isolated using the RNeasy Micro kit (Qiagen, Valencia, USA), reverse transcribed into cDNA (SuperScript VILO cDNA Synthesis Kit, Invitrogen, Carlsbad, USA) and analyzed by qRT-PCR using 5x HOT FIREPol Probe qPCR mix Plus (Solis Biodyne, Tartu, Estonia) and TaqMan Gene Expression Assays (Thermo Fisher Scientific, Waltham, MA, USA). Relative cDNA concentrations were quantified using the Pfaffl ΔΔCt method. In the HPC and the CP study, TaqMan Assays for rat genes *Gilz* (Rn00580222_m1), *Per1* (Rn01325256_m1), *Per2* (Rn01427704_m1), *Nr1d1* (Rn01460662_m1) and *Bmal1* (Rn00577590_m1) were used. In the time memory study, TaqMan Assays (Life Technologies) for mouse gene *Per2* (Mm00478113_m1), *Nr1d1* (Mm00520708_m1), *Bmal1* (Mm00500226_m1), *N3c1* (????) and *Sgk1* (?????) were used. The mRNA concentrations were normalized relative to the rat *Beta-2-Microglobulin* (Rn00560865_m1) housekeeping gene measured in a duplex reaction.

In situ hybridization
Brain sections were processed for *in situ* hybridization as described previously (Sumová et al., 2003). The probes were labelled using 35S-UTP. Autoradiographs of the sections were analyzed with Image Pro (Olympus, New York, USA). Each optical density (OD) measurement was corrected for nonspecific background. Data were normalized to the highest value of each of the daily profiles. The cDNA fragments of rat rPer1 (980 bp; 581-1561; GenBank AB_002108), rPer2 (1512 bp; 369-1881; GenBank NM_031678), rBmal1 (841 bp; 257-1098; GenBank

AB012600) and rNr1d1 (1109 bp, BC_062047) were used as templates for the in vitro transcription of cRNA probes.

Immunohistochemistry

Frozen rat brains were sectioned into 15-µm thick coronal sections and processed for standard immunohistochemistry using diaminobenzidine (DAB; CAT# D5905, Sigma Aldrich, St. Louis, MO, USA) and fluorescence-labelled secondary antibodies. The final concentrations of secondary antibodies used were 1:600 (anti-rabbit antibody conjugated with Alexa Fluor® 594, Cat# A-11037, RRID:AB_2534095, Thermo Fisher Scientific; anti-mouse antibody conjugated with FITC, Cat# F-2761, RRID:AB_2536524, Thermo Fisher Scientific; anti-chicken antibody conjugated with Cy3®, Cat# ab97145, RRID:AB_10679516, Abcam, Cambridge, UK). The primary antibodies used in this study were: rabbit GR polyclonal antibody (M-20, CAT#:sc-1004, RRID:AB_2155786, Santa Cruz Biotechnology, Dallas, TX, US); rabbit PER2 (CAT#:AB2202, RRID:AB_1587380, Millipore, Burlington, MA, US); rabbit phospho-GSK-3β (Ser9) (D85E12, CAT#:5558, Cell Signaling, Danvers, MA, USA); chicken GFAP polyclonal antibody (CAT#:ab4674, RRID:AB_304558, Abcam) and mouse HuC/HuD monoclonal antibody (15A7.1, CAT#:MABN153, Millipore). The immunofluorescence was detected using Leica SP8 WLL MP laser scanning confocal microscope, the images with the DAB staining were taken using Olympus BX53 microscope.

Organotypic explants

Organotypic explants of selected brain regions were prepared from *mPer2^{Luc}* mice (adult or 6-day old pups) sacrificed by rapid cervical dislocation. All explants were made from 300-µm coronal sections cut using vibratome (Leica, Wetzlar, Germany). HPC explants were prepared in cold HBSS (adult) or EBSS (6-day old pups). Explants were placed on Millicell Culture Inserts (Merck) inside petri dishes containing 1 ml of air-buffered media (adult) (DMEM with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% GlutaMAX (all Thermo Fisher; Waltham, MA, USA), 2% B27 supplement (Thermo Fisher) and 0.5mM D-Luciferin (Biosynth, Staad, Switzerland)) or CO2-buffered recording media (pups) (MEM supplemented with 25% heat inactivated horse serum, 25% EBSS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM GlutaMAX (all Thermo Fisher Scientific) and 0.5mM D-Luciferin (Biosynth)). All explants were placed in motorized Luminoview LV200 luminescence microscope (Olympus, Tokyo, Japan) with exposure time of 3 - 6 min (pups) or up to 1 h (adult explants). HPC explants from pups were cultured in fresh recording media for 3 days and then exposed to a treatment with 1 µl of DEX (Sigma-Aldrich) (100µM), CHIR-99021 (Sigma-Aldrich, CAT#:SML1046) (CHIR, 20mM), or with 1 µl of the corresponding vehicles (0.001% ethanol in ddH2O for DEX, and DMSO for CHIR-99021), per 1 ml of media. A separate set of explants was co-treated with 1 µl of both CHIR-99021 (or corresponding vehicle – DMSO) and DEX.

CP explants were prepared from 3rd ventricle CP and processed the same way as adult HPC explants. The bioluminescence traces were recorded in Lumicycle (Actimetrics; Wilmette, IL, USA) and analyzed using LumiCycle Analysis software (Actimetrics). Explants were treated with 1 µl of 100 µM DEX per 1 ml of media or 1 µl of the corresponding vehicle: 0.001% ethanol in ddH2O. A separate set of explants treated with DEX was pretreated with 1 µl of mifepristone (MIF): 1mM; Chelerythrine (Chel): 10mM; H89: 10mM; U0126: 25mM; or VEH (ethanol for MIF, water for Chel and H89, DMSO for U0126). For repeated treatments, the explants were washed with 37°C PBS (2x5 minutes), synchronized using serum shock (2 h in 50% horse serum, 37°C), washed again and placed in fresh media.

Viability test

Adult *mPer2^{Luc}* mouse organotypic explants were stained with Hoechst (5 µg/ml, Sigma Aldrich, 20 min at 4°C) and propidium iodide (PI) (ReadyProbes, Thermo Fisher, two drops of PI added into media. 30 min incubation at 37°C). Fluorescent signal and PER2-driven bioluminescence were detected using LV200 bioluminescence microscope (Olympus) at 2, 26 and 50 h following explant preparation.

PER2LUC degradation assay

CP explants were synchronized using serum shock and recorded in LumiCycle until the first peak in bioluminescence. Cycloheximide (Sigma Aldrich) (CHX, 40µg/ml) was added alone (CHX) or with 1 µl of 100 µM DEX (CHX+DEX). Bioluminescence was recorded for 8 h and one-phase exponential decay curves were fitted. The explants were then washed, placed into fresh media, and immediately recorded using LumiCycle again. The rate of PER2 accumulation was expressed as time between the wash and the first peak in bioluminescence of individual explants.

Data analysis

Daily profiles of normalized clock gene expression were analyzed for rhythmic expression by fitting two alternative regression models: horizontal straight line (null hypothesis) or cosine curve model defined by the equation Y=mesor+(amplitude*cos(2*π*(X-acrophase)/wavelength)) with a constant wavelength of 24 h (alternative hypothesis). P values, R2 (goodness of fit), amplitudes and acrophases were determined. Fold changes in gene expression detected after acute DEX injection in vivo based on VEH controls were analyzed using two-way ANOVA.

The relative periods and amplitudes of PER2-driven bioluminescence rhythms of HPC organotypic explants were compared by unpaired t-test; the periods before and after treatment were evaluated by paired t-test.

The microscopic images obtained by the Luminoview LV200 were analyzed using ImageJ (NIH, Bethesda, MD, USA). The data were fitted with a damped sine wave to calculate the period, amplitude, mesor and R2. For assessment of the effects of various treatments of explants on the parameters of bioluminescence rhythms (amplitude, mesor, R2), ratios of their values before and after each treatment were calculated (value 1 means no change, values above and below 1 mean increase and decrease, respectively) and compared by unpaired t-test (two groups) or one-way ANOVA (three groups) with the post hoc analysis of Sidak's multiple comparison method. Period changes (values before and after the treatment for each explant) were statistically compared by paired t-test.

The phase shifts were quantified by fitting a sine curve to at least three full circadian cycles of a 24-h running average baseline-subtracted rhythm and then extrapolating beyond the time of treatment. The calculated phase shift was designated as a phase advance (+) or a delay (-). The phase response curves (PRCs) were constructed by plotting the calculated phase shift as a function of treatment time normalized to the endogenous period in vitro and expressed relative to the trough (time 0) or peak (time 12) of the rhythm. The effects of inhibitor or VEH pretreatment before DEX on the phase were plotted as PRCs. The PRCs were binned into 3h intervals and compared using two-way ANOVA (effect of group) with the post hoc analysis of Sidak's multiple comparison method. The effect of inhibitor versus VEH pretreatment to DEX application was tested using F-test comparing linear regression curves fitted to the phase responses. For $P < 0.05$ a single curve, and for $P > 0.05$ two separate curves were plotted. The half-life and rate constant (K) of one-phase exponential decay curves of PER2 degradation in organotypic explants and the accumulation times were compared using unpaired t-test.

For the time memory study, both behavioral and molecular data were analyzed using planned comparisons and unpaired t-tests with Welch's corrections. Dwell time spent in shock vs. safe chambers was used to analyze the behavioral data and relative clock gene expression and ratio in animals exposed to the testing apparatus vs. conditioned groups and the naïve controls sacrificed at two different times was used to analyze the molecular data.

All statistics were performed using GraphPad Prism 7 software (GraphPad, CA, USA).

Results

The circadian clock in hippocampus and its synchronization

Characterization of clocks in individual HPC regions

To elucidate the role of chosen synchronizing signals on clock in the HPC, we first aimed to localize the cells expressing GR, PER2 clock protein and the inactivated GSK3β enzyme in the HPC and to compare their levels in individual HPC regions: CA1, CA3 and DG, using immunohistochemistry. The three HPC regions show a homogenous expression of GR, however CA1 and DG vary in levels of a core clock protein PER2 and in the activation state of the GSK3β kinase. These differences between the circadian clocks in the individual HPC regions lead us to compare their sensitivity to pathways involving GR activation and GSK3β inhibition in the possible mechanism of their synchronization.

Glucocorticoids are necessary for HPC clock gene rhythms in vivo and the response to GC signaling varies between regions

To test the role of glucocorticoids in synchronization of HPC clock we first examined their role *in vivo*. We performed intraperitoneal (i.p.) injections of either dexamethasone (DEX, 1 mg/kg), a synthetic GR agonist, or vehicle (VEH, PBS), and detected the acute effect of a GC pulse on mRNA levels of clock genes using qRT-PCR. In both HPC regions, the acute DEX injection resulted in an increase in transcription of the GC-immediate response gene *Gilz*. The induction of expression was seen at 120 min following the injection (CA1, $P = 0.0164$ and DG, $P = 0.0045$) and persisted in the elevated state until 240 min following the injection in CA1 ($P < 0.0001$). Interestingly, none of the analyzed clock genes (*Per1, Per2, Nr1d1*) possessing the GRE in their promotors showed any response to the DEX injection over the 4 h period.

In the next experiment we first used adrenalectomized Wistar rats (ADX) to assess the effect of removing the endogenous glucocorticoids on the HPC clock. The expression of clock genes *Per1, Per2, Nr1d1* and *Bmal1* in individual HPC region was evaluated using in situ hybridization. In the control group of sham operated animals (SHAM) all four clock genes showed a circadian rhythm in their expression. With the exception of *Nr1d1* the rhythms were quite shallow in their amplitude, but their expression was mutually synchronized in the CA1, CA3 and DG HPC regions. Following ADX, the circadian rhythms in clock gene expression were almost completely abolished in all three regions. Finally, in the third group of animals, we tested the effect of daily DEX injections in ADX animals on the clock gene expression profiles (ADX+DEX). The injections (1mg/kg, i.p.) were carried out for 8 consecutive days preceding the sample collection at the beginning of the animals' active phase (ZT12, before lights-off) to mimic endogenous GC rhythm. The injections of DEX were able to reinitiate robust oscillations of all four clock genes expression in DG. On the other hand, in CA1 and CA3 the effect was only partial, with only some of the clock genes being expressed rhythmically.

Hippocampal clock in vitro - caveat

To study the HPC clock separately, while excluding all signals coming from the rest of the body, and to assess its ability to sustain oscillation on its own we needed to establish an *in vitro* model. Based on approaches used in published studies (Loh *et al.*, 2015; Ma *et al.*, 2016; Besing *et al.*, 2017) we prepared organotypic explants from adult *mPer2*^{Luc} transgenic mice containing HPC and monitored PER2-driven bioluminescence signals using LumiCycle photomultiplier. The explants exhibited robust rhythms in bioluminescence with a sharp decline of total bioluminescence levels shortly after explanting. While working on our study, we used a newly acquired luminescence microscope (LV200, Olympus) and found out that we were wrongly attributing the bioluminescence to the HPC tissue. The microscopic resolution showed that the HPC tissue was in fact only responsible for the initial bout of bioluminescence and the circadian rhythms generated by the organotypic explants that were measured in LumiCycle were actually originating from closely associated tissue of the choroid plexus (CP) in the 3rd ventricle or the epithelium lining the lateral ventricle. We examined the viability of the tissue using propidium iodide (PI) stain. PI staining showed a rise in the number of dying cells in the HPC shortly following the explanting, with CP remaining viable. Based on these findings we withdrew a manuscript, which was already accepted into the British Journal of Pharmacology (Šuchmanová *et al.*, 2019).

To proceed with in vitro examination of the HPC clock we then established a new protocol for preparation and cultivation of HPC explants from neonatal (6-day old) *mPer2*^{Luc} mice, widely used for preparation of explants from other tissues, probably because of better viability of the young tissues (du Pré *et al.*, 2017; Patton *et al.*, 2020). These HPC organotypic explants were viable in long-term culture and it was possible to use the bioluminescence microscope for analysis of bioluminescence signal.

Effect of glucocorticoids and GSK3 inhibition on hippocampal clock in vitro
We used the organotypic explants containing HPC from 6-day old *mPer2*^{Luc} mouse pups and monitored PER2-driven bioluminescence signal coming from CA1, CA3 and DG using a luminescence microscope (LV200). The baseline bioluminescence rhythms were recorded for three days and the explants were then treated with either DEX (100nM), CHIR99021 (CHIR) (20μM), a selective inhibitor of GSK3β, or corresponding vehicles (VEHs) (either 0.001% ethanol in ddH2O or DMSO, respectively) to observe the effects of GCs and GSK3β inhibition on HPC clock.

The application of VEHs had only minor effects on the bioluminescence rhythms in all three HPC regions. Decreased amplitude and mesor are the result of the dampening of these rhythms and VEH_{CHIR} treatment slightly shortened the rhythm periods. The treatment with DEX significantly increased the amplitude of the rhythms in CA1 and CA3, but not in DG. The mesor of the PER2-driven bioluminescence on the other hand was not affected by DEX application in any HPC region. The treatment with CHIR had an inverse pattern of effects on these parameters. Its application significantly increased the amplitude of the rhythms in CA3 and had no effect on this parameter in CA1 and DG. However, CHIR administration consistently and significantly raised the mesor values in all three studied HPC regions. DEX and CHIR also had opposite effects on the periods of PER2-driven bioluminescence rhythms in HPC explants. DEX application prolonged periods of the rhythms, whereas CHIR significantly shortened them. The quality of CA1 rhythms was not affected by DEX, but DG rhythms were significantly improved. The treatment with CHIR on the other hand did not affect the rhythms in DG, but significantly worsened the bioluminescence rhythms in CA1. The CA3 region rhythms were slightly improved following DEX application and worsened after the administration of CHIR. These results show that GC signaling and GSK3β inhibition have opposite effects on circadian rhythms in the CA1, CA3 and DG HPC regions. Interestingly the individual regions show differences in their sensitivity to these input signals, and notably, the DG shows the most robust effects in both cases.

Inhibition of GSK3β overrides DEX effect on the period length in individual HPC regions
The application of VEH+DEX did not have a significant effect on the amplitudes or mesors of PER2-driven bioluminescence rhythms in any of the HPC regions, with the ratios of before and after treatment values remaining close to 1. The co-treatment with CHIR+DEX did not change the amplitudes significantly, compared to VEH+DEX. The mesors however, were significantly increased in all three HPC regions following CHIR+DEX co-treatment, similar to the effect of the application of CHIR alone. In case of period length, VEH+DEX administration resulted in lengthening of periods in CA1 and DG, but the effect was not significant in CA3. The co-treatment with CHIR+DEX again mimicked the effect of CHIR administration, with significant shortening of periods of rhythms in CA3 and DG and a suggested shortening trend in CA1. These results show that the co-treatment with CHIR and DEX resulted in a CHIR-like response of the clocks in the studied HPC regions.

The non-neuronal oscillator in choroid plexus, its properties and synchronization
Endogenous rhythm in glucocorticoid hormones is necessary for robust clock gene rhythms in choroid plexus in vivo
First, we used immunohistochemistry to confirm the presence of GR protein in the cytoplasm of rat CP cells. Then we examined the role of endogenous glucocorticoids in the CP clock by subjecting rats to adrenalectomy (ADX), removing the source of endogenous GCs, or sham surgery (SHAM). The expression profiles of clock genes *Per1, Per2, Nr1d1* and *Bmal1* in CP were analyzed using in situ hybridization.

In the SHAM control group of animals all studied clock genes exhibited robust daily rhythms in expression. The phases of individual clock genes matched the TTFL, confirming the presence of an intrinsic molecular oscillator in the tissue. ADX had a detrimental effect on the CP circadian clock, causing decreased amplitude of *Per2, Nr1d1* and *Bmal1* expression rhythms and abolishing the *Per1* rhythm altogether. The daily administration of DEX to ADX animals increased the amplitudes of *Per2, Nr1d1* and *Bmal1* rhythms in CP. *Per1* expression remained arrhythmical in ADX+DEX group according to cosinor analysis, however *Per1* levels were significantly increased at the time corresponding to the DEX injection. Interestingly the phases of the strengthened clock gene rhythms following ADX+DEX still adhered to the TTFL model but were shifted compared to the control animals (SHAM group).

DEX resets the CP clock in vitro
The CP explants exhibited circadian rhythms in PER2-driven bioluminescence. The application of DEX caused a significant increase in the amplitude of CP PER2-driven bioluminescence rhythms when compared to the effects of VEH. Neither DEX nor VEH had affected the period of bioluminescence rhythms and their effects were not

significantly different. To compare the effects of DEX and VEH on the phase of the CP clock we used a phase response curve (PRC). The PRCs for both DEX and VEH were constructed by plotting individual phase shifts according to the treatment time relative to the phase of PER2-driven bioluminescence rhythm (peak of PER2 was assigned time 12). The VEH treatments had only minor effects on the phase of the bioluminescence rhythms. DEX treatments however, produced phase shifts corresponding to the PRC type 0 (Johnson, 1992). The maximal phase shifts caused by DEX occur around the peak of PER2-driven bioluminescence (time 12), with phase shift size of up to 12 h. Statistical analysis confirmed significant differences between the two treatments. A separate set of explants was pretreated with MIF before DEX application showed that MIF was able to completely block the DEX effects on the bioluminescence rhythms. These results clearly show that GCs are able to reset the CP clock in vitro and that this effect is mediated by a GR-dependent pathway.

DEX resets the CP clock by inducing Per1 expression and via CRE-dependent pathways

After showing the ability of DEX to reset the CP circadian clock *in vitro* we further explored the involved mechanisms. To examine the effects of DEX on gene expression in CP we injected intact rats with DEX (i.p., 1 mg/kg at ZT16, 4 h after lights off) or corresponding vehicle (VEH; PBS) and analyzed the expression of *Gilz*, a GC-responsive gene, and clock genes *Per1, Per2, Nr1d1, Bmal1* and *E4bp4* using laser dissection of CP and qRT-PCR. Samples were collected before (time 0), and then 1, 2, and 4 hours following the DEX or VEH injection.

Compared to VEH injection, DEX caused an increase in *Gilz* expression, which became significantly higher 1 hour after the injection and increased even more markedly 2 and 4 hours following the DEX administration. This confirmed the validity of our protocol, showing that CP cells were reached by the GC pulse and responded rapidly. Out of the studied clock genes, only Per1 expression became increased 2 hours following the injection ($P = 0.0305$) and remained elevated after 4 hours ($P = 0.0221$). The rest of the clock genes did not show any response to DEX during the 4-hour time window. These results suggest the involvement of acute stimulation of Per1 expression in the mechanism responsible for DEX-mediated resetting of the CP clock.

Apart from the triggering of gene expression, signaling pathways involving PKC, PKA and MEK/ERK were previously reported to contribute to GC effects (Groeneweg *et al.*, 2012). Therefore, we tested the involvement of these kinases by using their specific inhibitors to block the DEX-induced phase shifts of CP clock in vitro. A separate set of organotypic CP explants was pretreated with either PKC inhibitor chelerythrine (Chel; 10μM), PKA inhibitor H89 (10μM), MEK inhibitor U0126 (25μM) or corresponding VEHs before DEX was added to the cultivation media. The treatments were timed to the phase-delaying part of the PRC and the resulting shifts of PER2-driven bioluminescence rhythms were measured. Pretreatment with PKC inhibitor Chel had no effect on the DEX-induced phase shifts ($P = 0.9581$), however inhibition of PKA by H89 slightly decreased the magnitude of phase shifts ($P = 0.0211$). Notably, the inhibition of MEK, downstream of PKA, lead to a more pronounced decrease in the DEX-induced phase shifts of the CP clock ($P = 0.0001$). None of the inhibitors caused phase shifting of the rhythms when applied on their own. These findings reveal a partial involvement of PKA-ERK1/2 pathway in the resetting of CP clock by DEX in vitro.

DEX affects the stability of PER2 protein in CP in vitro

To address the possible involvement of posttranslational mechanisms affecting PER2 protein turnover in the DEX-mediated resetting of the CP clock we tested the effect of DEX on stability of PER2 in the CP organotypic explants from *mPer2^{Luc}* mice. To demonstrate the effect of DEX on the dynamics of PER2 degradation we added translation inhibitor cycloheximide (CHX; 40μg/ml) into the cultivation media of explants. We then analyzed the dynamics of PER2 degradation (measured as the rate of decrease in levels of PER2-driven bioluminescence generated by the explant) following the termination of de novo translation either in the presence or absence of DEX.

Using this approach, we showed that DEX significantly decreases the PER2 protein half-life ($P = 0.0048$) and increases the values of K ($P = 0.0026$). Interestingly, after a wash removing the proteosynthesis inhibitor, DEX also significantly increased the rate of new PER2 accumulation ($P < 0.0001$). These findings demonstrate that even though the expression of Per2 is not acutely induced by DEX, it can still affect the molecular clock mechanism by modulating the turnover dynamics of the protein. This is corroborated by the pattern of phase shifting properties of DEX application on the rise versus on the decline of PER2-bioluminescence rhythm. Treatment with DEX on the rise of the rhythm increased the rate of PER2 accumulation and prolonged its duration, thus phase delaying the clock. On the other hand, the application of DEX after the peak of bioluminescence rhythm accelerated the decrease of PER2 levels, which in turn caused the rhythm to advance in its phase.

The time memory study

To study the involvement of extra-SCN brain oscillators in the process of time memory formation we analyzed clock gene expression following a conditioning protocol. We implemented a model detecting and predicting the phase of the whole molecular mechanism of the circadian clock based on relative changes in clock genes and their ratios using only two time points. The procedural controls were chosen to evaluate the effect of the conditioning procedure alone.

Conditioned place avoidance and time memory

One set of mice, used as procedural controls, was subjected to the conditioning procedure daily either at ZT3 or ZT11, but without any foot shock being delivered. Following eight days, these animals were tested for preference of chambers at the two Zeitgeber times and no significant change in their preference was found. Another set of mice was subjected to the CPA conditioning protocol, receiving a foot shock in one of two context chambers at either ZT3 or ZT11 for eight days. After the end of conditioning the mice were tested for preference of chambers either at the time matching their conditioning or at the other, non-matching time.

Animals that were conditioned using a foot shock at ZT3 were significantly less likely to visit the same chamber when tested at the same time of day ($p=0.0003$), however when tested at ZT11 no significant avoidance was measured ($p=0.7948$). Correspondingly, animals trained at ZT11 only significantly avoided the foot hock chamber if they were tested at ZT11 ($p=0.0458$) and not at ZT3 ($p=0.1446$).

Conditioning effects on the expression of Nr1d1 and Per2 in brain oscillators

Once we established that the animals were able to remember the timing of conditioned responses from the CPA paradigm, we examined the expression of clock genes *Nr1d1* and *Per2* and their ratios (N/P) in samples from the SCN and other brain oscillators implicated in memory formation: hippocampal regions CA1, CA3 and DG, dorsal striatum (DS), two parts of nucleus accumbens: shell (NAS) and core (NAC), cingulate cortex (Ci), basolateral amygdala (BLA) and the central nucleus of amygdala (CeA). Gene expression was compared between control animals (without conditioning, tested either at ZT3 or ZT11) and mice that underwent conditioning either at ZT3 or ZT11. Conditioned animals were either tested at the time corresponding to the timing of their conditioning training or at the non-matching *Zeitgeber* time.

In the SCN, there was a significant difference between the levels of *Nr1d1* at the two times in control animals ($p=0.0066$). This difference remained significant in animals conditioned at ZT03 but not in the ZT11 conditioned group. The expression of *Per2* on the other hand did not show a statistically significant variation between the two time points in control animals. The calculated *Nr1d1/Per2* (N/P) ratios did not show any effect of either conditioning time in the SCN. Out of the examined peripheral brain oscillators only DS and the NAS showed significant changes in clock gene expression after conditioning.

In the DS, the expression of both *Nr1d1* and *Per2* showed significant differences between the two time points in the control group of mice ($p_N=0.0113$ and $p_P=0.0371$). This pattern of clock gene expression levels was reversed compared to the SCN. The differences in *Nr1d1* expression were abolished following conditioning at ZT03, but not at ZT11. The significant difference in *Per2* expression at the two time points was not present in ZT03 conditioned animals and the ZT11 conditioning resulted in its reversal ($p=0.0159$). Corresponding to the two studied clock genes, the calculated N/P ratios also showed a significant difference between ZT03 and ZT11 in control group of animals ($p=0.0029$), with the difference reversed compared to the SCN. In the NAS, the pattern of expression of both clock genes resembled the DS. Similar to the DS results, it was the ZT11 conditioning which showed a difference in the response of *Per2* ($p=0.0016$) and N/P ratio ($p<0.0001$) in animals tested either at ZT3 and ZT11.

Interestingly, none of the rest of the studied brain oscillators showed a response in clock gene expression to the conditioning. In the HPC the expression of *Nr1d1* differed significantly between ZT3 and ZT11 in all three HPC subregions, CA1, CA3 and DG, in control animals ($p=0.0142$, $p=0.0414$ and $p=0.0093$ respectively). This relationship remained unchanged following both ZT3 and ZT11 conditioning in CA1 and persisted at least suggested, if not significant, in CA3 and DG. *Per2* showed no significant difference in expression levels in the studied HPC regions between the two times in either experimental group. The N/P ratio in the HPC regions showed a similar trend, being significantly altered between the two time points in control animals in all three regions, with the significant difference still present in the ZT3 ($p=0.0078$) and ZT11 ($p=0.0004$) conditioning groups in CA1, whereas only staying suggested in CA3 and DG.

The effect of conditioning on Bmal1 expression

The suggested phase shifting of the SCN clock by conditioning was not confirmed by analysis of *Bmal1* expression. We found no differences between the ZT3 and ZT11 timepoints in the expression levels if this gene or the *Bmal1/Per2* (B/P) ratio in the control group of animals, nor was there an effect of conditioning at either time. This indicates the SCN clock was not shifted by the conditioning. CA1 also showed no response in *Bmal1* expression, as expected. In DS however, *Bmal1* expression was decreased following conditioning at both ZT3 and ZT11, but only the effect of ZT3 conditioning reached statistical significance. Furthermore, the calculated B/P ratios reflecting the relationship between *Per2* and *Bmal1* showed an even stronger effect of conditioning.

Glucocorticoid response to conditioning

The conditioned place avoidance (CPA) paradigm which was employed in these experiments uses a stressful event (low intensity electrical foot shock). We therefore analyzed the possible effects of conditioning on GC-associated genes in the extra-SCN brain oscillators responsive to conditioning. The expression of GR gene (*Nr3c1*) was not affected by conditioning procedure in DS or NAS. The amygdala regions (BLA, CeA) were also analyzed, based on their close involvement in fear and anxiety regulation and neither BLA nor CeA showed any significant effects of conditioning. These results were corroborated by an absence of effect of conditioning on expression of genes containing the GRE in their promoter, *Per1* and *Sgk,* in the DS and NAS.

Employment of the corroborative model of clock shifting

To be able to assess the magnitude and direction of a possible phase shift caused by the conditioning at different times in an experiment involving two time points we used the described model. We focused on DS, the region we identified as most likely to shift its clock following the conditioning paradigm. The conditioning at ZT11 caused significant changes in the *Per2* expression and N/P ratio in DS. However, conditioning at ZT3 only changed the N/P ratio, but not the expression of *Per2* itself. To see the relationship between the behavioral performance of individual animals and their clock gene expression we plotted the preference scores of all experimental groups as a function of *Per2* expression. The results show that higher scores in the CPA correlate with higher expression levels of *Per2* ($r=0.397$, $p=0.0446$). The conditioning at matching time is therefore significantly associated with changes in *Per2* expression and N/P ratio in DS. This further supports the role of DS circadian clock shift in time memory formation.

Based on our results we fitted a theoretical *Per2* expression rhythm to the values from control animals at the two timepoints, ZT3 and ZT11. The same method was used in case of the changed *Per2* expression levels in both conditioned groups of animals. The shifted phase of the oscillations fitted to the data from conditioned groups correspond to the 8-hour difference in the time of day when the CPA was carried out in the two conditioned groups.

Discussion

The hippocampal subregions and their synchronization

The HPC, as one of the key brain structures for memory formation, has been widely studied in many contexts, one of them being the potential existence of a circadian oscillator in this tissue. A substantial body of literature has focused on this question, sometimes with somewhat contradictory results. Considerable number of in vivo studies have described circadian rhythms in clock gene and protein expression (Wakamatsu et al., 2001; Lamont et al., 2005; Amir et al., 2006; Segall et al., 2006; Ángeles-Castellanos et al., 2007; Verwey et al., 2007; L. a. Segall et al., 2009; Wang et al., 2009; Wyse and Coogan, 2010; Pantazopoulos et al., 2011; Valnegri et al., 2011; Gilhooley et al., 2011; Ikeno et al., 2013; Duncan et al., 2013; Navigatore-Fonzo et al., 2014; Schnell et al., 2014; Renaud et al., 2015; Tahara et al., 2015; Chun et al., 2015; Shimizu et al., 2016; Ikeno and Yan, 2016; Bellanti et al., 2017; Bering et al., 2017; Besing et al., 2017; Mei et al., 2018; Woodie et al., 2020; Debski et al., 2020); however, several studies did not find rhythmical clock gene expression in vivo (Albrecht et al., 1997; Borgs et al., 2009) or found clock gene expression rhythms which did not correspond to the TTFL model (Jilg et al., 2010), and thus concluded that the HPC was not in fact a functional intrinsic oscillator. Importantly, many of these findings are based on the analysis of gene expression in homogenates from the whole dissected HPC tissue (Jilg et al., 2010; Valnegri et al., 2011; Ikeno et al., 2013; Renaud et al., 2015; Tahara et al., 2015; Bellanti et al., 2017; Bering et al., 2017; Debski et al., 2020; Woodie et al., 2020). This approach, however, fails to distinguish potential differences between the HPC sub-regions, as well as between the functionally distinct ventral and dorsal part of HPC. To take these possible differences into account, in our study we employed more sensitive techniques and analyzed clock gene expression in the CA1, CA3 and DG regions of dorsal HPC separately. This enabled us to compare the clocks in the distinct HPC regions and also to assess the adherence of phases of the chosen clock gene rhythms to the TTFL clock mechanism, providing additional insight into the functioning of the HPC clock in vivo. On top of that, we also present important findings regarding the use of organotypic explants from *mPer2*^{Luc} mice as an in vitro model of HPC clock and moreover, we are the first to establish a viable model, which enabled us to study the HPC sub-regions in vitro.

Both the in vivo and in vitro highly sensitive approaches allowed us to examine the unknown mechanisms of HPC clock synchronization. We explored two plausible candidate synchronizing factors, the GCs, providing tissues with information about the arousal state of the animal, and the GSK3β signaling, connected to memory-related processes in the HPC. Both of these factors also interact with the molecular clock mechanism. Our hypothesis was that both the hormonal GC signal and the GSK3β enzyme activity should influence the clocks in HPC regions. Based on previously published results from other tissue models (reviewed in Spencer et al., 2018) we expected the GC entraining signal to dominate.

HPC clock and its synchronization

Based on the rather contradictory reports concerning the existence of an autonomous HPC clock, we applied sensitive methods to discern between distinct HPC subregions and described the parameters of these brain oscillators in greater detail and in a more reliable way than was done so far by using both in vivo and in vitro approaches. Our results are in agreement with the majority of studies focusing on the HPC clock and support the existence of an intrinsic circadian oscillator in the HPC. We show that CA1, CA3 and DG all exhibit circadian rhythms in the expression of Per1, Per2, Nr1d1 and Bmal1 in vivo, even though with the exception of Nr1d1 these rhythms are quite shallow. The shallowness of the clock gene rhythms however corresponds to previously published reports (Jilg et al., 2010; Duncan et al., 2013; Ikeno et al., 2013; Tahara et al., 2015; Bering et al., 2017; Debski et al., 2020; Woodie et al., 2020) and could possibly result from incomplete synchronization of clocks in individual HPC neurons. This would match the functional propertied of the tissue, where neuronal subpopulations carry out specific functions (Moser and Moser, 1998; Moser, 2011). Importantly, the existence of a functional HPC clock is further supported by the fact that the phasing of clock gene expression rhythms in all three HPC regions corresponds to the TTFL model. In accordance with a large part of published research on HPC clock (Wang et al., 2009; Navigatore-Fonzo et al., 2014; Schnell et al., 2014; Chun et al., 2015; Renaud et al., 2015; Tahara et al., 2015; Shimizu et al., 2016; Debski et al., 2020; Woodie et al., 2020), we show that the phase of these oscillations is delayed compared to the central oscillator in the SCN (Sládek et al., 2012). This is not surprising, as this pattern is also replicated in many peripheral oscillators in the brain and elsewhere in the body (Sládek et al., 2012; Chun et al., 2015; Soták et al., 2016). This consistently reported phase delay of the HPC clock may be the result of the mechanism by which the phase of peripheral clocks is set – by signals coming from the SCN – which may take some time to reach the periphery. Another possibility is that this delay could be functionally

significant in relation to the nocturnal activity of rats and mice, the most commonly used animal models used in the discussed studies, as well as in ours.

Until now, no studies have systematically addressed the important question of HPC clock synchronization to the external environment. We proposed two types of possible synchronizing factors and tested their effect on the HPC clock. We chose GCs, a systemic hormonal signal corresponding to the arousal state of the animal, and an inhibition of GSK3β, an intrinsic kinase, the activation state of which is closely associated with the memory-related processes in the HPC. The potential involvement of glucocorticoid hormones was previously suggested by studies showing that the rhythmical expression of the clock gene Per1 in HPC is abolished in rats with constant levels of corticosterone (Gilhooley et al., 2011) and glucocorticoid pulses cause an induction of Per1 transcription in the HPC (Conway-Campbell et al., 2010).

We are the first to report that endogenous GCs are necessary for the HPC circadian clock to function, and that the lost clock gene rhythmicity in adrenalectomized animals can be rescued by daily DEX injections. Importantly, even though we showed that the GR is expressed homogenously throughout the HPC regions, the synthetic GC analog DEX clearly caused the strongest response in the DG. This shows that input signals for the HPC clock might vary in their effects between the individual HPC regions and highlights the importance of distinguishing between these diverse parts in future studies focusing on the HPC clock synchronization even further. Interestingly, it has already been reported that GCs can have specific effects on the function of the DG region and its excitability (Dana and Martinez, 1984). Surprisingly, even though the DEX injections were administered before lights off to mimic the physiological peak of GC levels in blood, the reinstated rhythms in clock genes produced by DEX were significantly phase shifted compared to the rhythms present in control animals. The reason for this discrepancy could be the lack of ultradian pulses normally present in GC secretion, which were not replicated by the exogenous single dose of DEX. This characteristic secretion pattern was shown to be able to influence the hormones effect in target tissues (Cheifetz, 1971). Another possible explanation for the observed phase change could be the differences in kinetics and potentially also local metabolism of the synthetic GC compared to corticosterone (Miyabe and Herrison, 1983; Alexandrová et al., 1989; Wyrwoll et al., 2011). Additionally, we cannot rule out the contribution of mineralocorticoid receptors (MRs), which can bind corticosterone, but not DEX, or the involvement of other signals produced by the adrenal glands.

To dissect the direct effect of GCs on the HPC clock itself from the other factors potentially influencing the in vivo response of HPC to DEX, we established an in vitro model using organotypic explants from *mPer2*^{Luc} mouse pups. This approach confirmed our findings about the specific sensitivity of DG to the GC signaling and gave us a more detailed information about the changes of clock parameters following DEX administration. Our results show that the GCs can lengthen the period of HPC clock, thus slowing the pace of the oscillator and adjusting the timing of clock controlled processes related to the function of HPC, such as memory formation and other cognitive processes. Taken together, these results show that the GCs have a direct effect on the HPC clock, and we are the first to show that their effect varies between HPC regions, with the DG being the most responsive to GC signaling.

The second potential synchronizing input we tested were the changes in endogenous activity of GSK3β enzyme. The differences between the HPC regions in the activity of this kinase led us to explore its possible involvement in synchronization of clocks in the individual HPC parts. GSK3β is involved in memory-related processes, and it has also been shown to interact with the molecular components of the circadian clock, such as NR1D1, PER2, CLOCK or BMAL1 (Iitaka et al., 2005; Yin et al., 2006; Spengler et al., 2009; Sahar et al., 2010). The inhibition of GSK3β by its specific inhibitor CHIR has been shown to shorten the period and increase the amplitude of circadian oscillations in explants of the SCN and in adult explants containing the HPC, but also other brain regions (Besing et al., 2015, 2017). Using our highly sensitive approach providing cellular-level resolution of the acquired bioluminescence signal, we demonstrate that GSK3β inhibition by CHIR indeed does affect the HPC clock. It shortened the periods and increased the mesors of measured rhythms, however the effects varied between the distinct HPC regions. As we suspected from the region-specific activity pattern of the enzyme, the inhibition of GSK3β had the strongest impact on the clock in DG, where the enzyme was activated in vivo. Here, the application of CHIR dramatically shortened the period of the observed bioluminescence rhythms. CA1 rhythms on the other hand were worsened following the CHIR application. The inhibition of GSK3β we observed in the CA1 under physiological conditions might therefore reflect the insensitivity of this region to this type of signal.

We next compared these two identified physiologically relevant synchronizing signals, affecting the HPC clock, to determine the dominant input in setting the HPC clock. The GCs were repeatedly proven to have a strong synchronizing effects on peripheral oscillators (reviewed in Spencer et al., 2018) and we have shown their essential

role in vivo in the adrenalectomy experiment, therefore, our assumption was that DEX would dominate the cotreatment effect. Nevertheless, the joint application of both DEX and CHIR produced clear CHIR phenotype, shortening the periods and increasing the mesors of PER2-driven bioluminescence rhythms in all three HPC regions. The greater strength of the GSK3β inhibition signal is interesting in the context of the in vivo acute response to DEX administration which we found in HPC of intact rats, where the DEX injection did not affect the expression of clock genes containing GRE (Per1, Per2, Nr1d1 and E4bp4) during the 4 h interval following the injection, even though another GC-induced gene (Gilz) was upregulated. This is surprising compared to findings from other tissues (Balsalobre et al., 2000; Yamamoto et al., 2005; Cheon et al., 2013), however studies focusing on GC effects in HPC have previously reported conflicting results (Conway-Campbell et al., 2010; Bohacek et al., 2015). Our findings suggest that the direct transcription changes in clock gene expression might not be involved in mediating the GC effects on the HPC clock, potentially making the role of GC signaling weaker compared to GSK3β.

The opposite effects of these two signals on the HPC clock parameters: period, amplitude and mesor, suggest these pathways could cooperate to provide the HPC clock, especially DG, with the proper alignment of clock-controlled HPC functions to the external demands. The increased sensitivity of DG to both signals may suggest its possible role as the primary target of synchronization signals reaching the HPC, which would then relay these stimuli to the rest of the HPC formation. Nevertheless, the mechanism of action of both these pathways and their precise function and cooperation in vivo should be studied in more detail in the future.

Comparison of the clock in HPC and non-neuronal oscillator in CP

After characterizing the clock in HPC, we then compared this neuronal brain oscillator to the newly discovered non-neuronal clock located in close proximity to the HPC, the choroid plexus (CP). The CP is responsible for CSF production, but also for the regulation of its composition and for the efficiency of its turnover, crucial for removing metabolites from the brain tissue. Even though the precise function of this strong brain oscillator is not fully understood, it is highly likely that the CP clock is responsible for the previously described daily rhythms in CSF formation, composition, and turnover (Nilsson *et al.*, 1992; Harrington *et al.*, 2010). The proper timing of these processes, like the highest CSF secretion rate during the night (Nilsson *et al.*, 1992), affects tissues throughout the brain, and it could thus be crucial for the proper functioning of the brain as a whole, but particularly the cognition-related, sensitive regions, closely associated with the brain ventricles, such as HPC. This is supported by findings suggesting that deterioration of CP function in aging as well as in various disorders, such as Alzheimer's disease (Silverberg *et al.*, 2001; Wostyn *et al.*, 2011; Chiu *et al.*, 2012; Myung, Wu, *et al.*, 2018), where it might contribute to the cognitive decline associated with these conditions. Additionally, a recent study also shows a worsening of clock gene rhythms with aging and in a mouse model of Alzheimer's disease, implying the worsening of CP function in aging could be connected to the weakening of its clock as well (Furtado *et al.*, 2020). Furthermore, CP paracrine signaling was shown to influence the period of the central oscillator, the SCN, *in vitro* (Myung, Wu, *et al.*, 2018; Yamaguchi *et al.*, 2020). We chose the GCs, crucial hormones, exhibiting a daily rhythm in their plasma levels, tested their role in CP clock entrainment and aimed to compare their effects on the neuronal (HPC) and non-neuronal (CP) brain clocks.

CP clock parameters and their synchronization by GCs

Similar to the HPC regions, we found rhythms in the expression of all four studied clock genes (*Per1, Per2, Nr1d1* and *Bmal1*) in the rat CP. We analyzed expression profiles with sampling every four hours during 24 hours, improving the detection of phase of the CP clock compared to the previous studies (Quintela *et al.*, 2018; Yamaguchi *et al.*, 2020). Interestingly, in contrast to the relatively shallow rhythms in the HPC, the CP clock gene expression rhythms were much more robust. This might be caused by a higher synchronicity of clock gene expression in CP cells, whichall carry out the same function and do not need to be divided into subpopulations with distinct roles. Remarkably, the phase of the clock gene expression rhythms differed between the neuronal and non-neuronal oscillator. The HPC clock gene rhythms were delayed by almost 12 h compared to SCN clock gene rhythms in the same rat strain (Sládek *et al.*, 2012). The CP clock was also delayed compared to the SCN, however, only by approximately four hours. This suggests that even though the two tissues are closely associated in the brain and they both harbor functional circadian clock mechanism their clocks are likely to be synchronized by the SCN to a different phase relative to outside conditions.

The effect of changing levels of these hormones on non-neuronal clocks in the brain has not been shown yet. However, the circulating levels of GCs reflect the arousal state of the animal and would provide a physiologically highly relevant synchronizing signal for CP clock, able to set its phase according to the sleep/wake cycle. The

removal of endogenous GCs significantly decreased the amplitude of both the negative (*Per* genes, *Nr1d1*) and positive (*Bmal1*) elements of the TTFL. Nevertheless, in contrast to the effect of ADX on HPC clock, the expression of all the clock genes apart from *Per1* remained rhythmical. Daily GC rhythm therefore clearly affects the CP clock and maintains its robustness, possibly by increasing the expression rhythm amplitude by synchronizing the population of individual CP epithelial cells to one another. Supporting this, daily DEX injections to ADX animals reinforced the CP clock gene rhythms. Interestingly, similar to the HPC clock, the CP clock gene expression rhythms were shifted in the ADX+DEX group of animals compared to the SHAM controls. It therefore seems that the physiological rhythm properties, such as the gradual buildup of GC levels preceding the active period, the pulsatile quality of the daily rhythm or corticosterone kinetics may be crucial for setting the correct phase of both neuronal and non-neuronal clocks in the brain (Cheifetz, 1971; Wong *et al.*, 1973; Miyabe and Herrison, 1983; Alexandrová *et al.*, 1989).

The effect of *in vitro* application of DEX into the cultivation media of the CP organotypic explants matched our *in vivo* findings, with DEX increasing amplitude of the PER2-driven bioluminescence rhythms. This change of amplitude corresponded to a similar effect observed in HPC explants. However, compared to the HPC clock, the DEX treatment did not have any effect on the period of CP *in vitro*, hinting at possible differences in the mechanism of GC-mediated entrainment of the two oscillators. Additionally, we showed that DEX is able to set the phase of the CP clock. The phase shifting caused by DEX changed in both magnitude and direction depending on the time of its application relative to the PER2-driven bioluminescence rhythm and correspond to the type 0 PRC (Johnson, 1992), with the largest phase shifts up to 12 h. This effect is similar to previously shown effects of GCs on the phase of clocks in other peripheral tissues, such as placenta (Čečmanová *et al.*, 2019) or liver (Balsalobre *et al.*, 2000), however, this is the first time a PRC was constructed for a non-neuronal brain tissue.

Our results show that the two closely associated neuronal and non-neuronal oscillators both exhibit daily rhythms in clock gene expression, however, interestingly with a different phase. Remarkably, the non-neuronal CP clock is more robust *in vivo,* compared to HPC. Both HPC and CP clocks deteriorate in absence of daily GC rhythm, with CP clock gene expression remaining rhythmical and the less robust HPC clock gene oscillations becoming completely arrhythmic. The administration of GCs both *in vivo* and *in vitro* affects both oscillators, but its effects vary, suggesting the possibility of involvement of different mechanisms of GCs action.

Mechanism of CP clock synchronization by GCs

We next explored the mechanisms involved in the observed effect of GCs on the CP clock. The GCs have a wide range of effects on many circadian clock components, which include both activation and repression of clock gene transcription, but also posttranslational modifications of clock proteins and changes in their localization inside the cell or their stability (Torra *et al.*, 2000; Yamamoto *et al.*, 2005; Nader *et al.*, 2009; Lamia *et al.*, 2011; Cheon *et al.*, 2013; Murayama *et al.*, 2019). Remarkably, these effects are tissue specific (Soták *et al.*, 2016). Similar to the HPC tissue, we found that an acute injection of DEX caused induction of *Gilz* expression in the CP. However, we also observed an effect on clock gene expression, namely an upregulation of *Per1* expression, which was not present in case of HPC. This further implies the involvement of a different mechanism in GC-mediated synchronization of the neuronal oscillator in HPC and the non-neuronal CP clock. Surprisingly, even though *Gilz* was already upregulated in CP 1 h following the time of the injection, *Per1* expression only became significantly elevated after 2 h. This delay could be caused by the previously described dual regulation of *Per1* transcription, which is regulated by both GRE and a CRE located in its promoter (Travnickova-Bendova *et al.*, 2002; Yamamoto *et al.*, 2005). The absence of detected upregulation of *Per2* could possibly be the result of a delay in its reaction to the GC signal, which then might not fit into our 4 h time window. The reason for this could be an overlap of GRE with another element, the E-box in its promoter region. It has been shown previously that an interaction of the E-box with BMAL1 protein is necessary for the GC-mediated *Per2*, possibly delaying the process in case of low BMAL1 levels (Cheon *et al.*, 2013).

The suggested involvement of CRE in Per1 transcription induction led us to explore the nongenomic effects of GCs that could be participating in the CP clock resetting. The GCs were previously shown to modulate the PKA and PKC signaling pathways potentially via a membrane receptor (Groeneweg *et al.*, 2012), activating the CREs via downstream ERK1/2 (Impey *et al.*, 1998) and mediating GC effects in the brain (Yoshiya *et al.*, 2013). The interplay between these pathways and GC signaling is even wider, with PKA and downstream MEK and ERK1/2 phosphorylating GR in the cytoplasm, regulating its translocation into the nucleus and cofactor activation (Adcock *et al.*, 2002). Our results show that the phase shifts of the CP clock caused by DEX are partially mediated by the PKA-ERK1/2 cascade. PKC on the other hand does not seem to play a role in this process. This result should

however be interpreted with caution, because the specificity of the used PKC inhibitor chelerythrine has been questioned in literature (Davies *et al.*, 2000).

Additionally, we explored the possible role of changes in turnover of PER2 protein in the GC-mediated resetting of the CP clock. DEX shortened the half-life of PER2 protein following inhibition of proteosynthesis and accelerated the rate of new PER2 protein accumulation in the CP explants. These effects of DEX on the stability of PER2 are in agreement with the PRC type 0, with the DEX administration causing prolonged half-life on the rise of the PER2 levels, delaying the clock, and accelerating the buildup of new protein when DEX is applied on the decline of PER2 levels.

Our findings thus show that the CP clock synchronization by GCs is mediated by induction of *Per1* transcription and changes in the turnover of PER2, and that these effects are partially modulated by the PKA-ERK1/2 signaling pathway. Regarding the comparison of CP clock to that located in the HPC, our results reveal that the two closely associated clocks differ in their strength, with CP remarkably exhibiting higher robustness of clock gene oscillations *in vivo*, and surprisingly also in their phase. The differences in phase of the molecular clock mechanism could reflect the distinct timing of the functions carried out by the two tissues, with the HPC activity coupled to the active phase of the day and the CP on the other hand to the time of rest. Presented findings highlight the importance of the circadian rhythm in GC levels in keeping both the HPC and the CP clock synchronized and fully functional at the right time. Importantly, we also show that the effects of GCs vary depending on tissue type and are likely mediated by different mechanisms in the neuronal and non-neuronal clock.

The relevance of these results however spans beyond this context, because of the wide use of dexamethasone as an immunosuppressant in clinical practice. Our findings indicate that the systemic DEX administration may have so far unrecognized effects on the circadian clock in HPC and other brain oscillators and could potentially alter their function in patients. Both the effect of GCs and the even stronger effect of GSK3β inhibition on circadian clock in HPC could provide a link between circadian disruption and memory deficits it causes and offers potential for future research.

The role of brain clocks in time memory

In the third study we focused on the possible role of the HPC oscillator and other extra-SCN brain clocks in the process of time memory formation. This important mechanism enables animals to associate a physiologically relevant encounter with a particular time of day and predict the recurrence of this potentially vital event. The fact that the acquired memories contains the information about the timing of the event has been shown (Ralph *et al.*, 2002, 2013; Cain *et al.*, 2004), but the mechanism responsible for it is not known. We hypothesized that circadian oscillators present in brain regions implicated in memory formation could provide the timing context for the acquired memory by changes in the phase of the local molecular clockwork. We again focused on the HPC, and also other brain regions harboring circadian oscillators relevant to memory, like dorsal striatum (DS), nucleus accumbens shell and core (NAS, NAC), cingulum (Ci) and basolateral and central amygdala (BLA, CeA).

Behavioral experiments used in the study included conditioned place avoidance protocol carried out at two times of day (ZT3 and ZT11) which confirmed the ability of mice to associate an aversive memory with a particular time of day and to predict the future recurrence of the important event at the right time. Even though the animals successfully formed the time memory of the aversive conditioning, the analysis of clock gene expression in the regions of interest only revealed changes in their expression consistent with clock resetting in the DS and NAS. All the other studied brain regions, including all three examined HPC subregions (CA1, CA3 and DG), only showed several isolated changes in clock gene expression, which did not imply the clock resetting in these extra-SCN oscillators. These results correlate with several previous studies suggesting that the molecular mechanism of the circadian clock is not required for remembering the timing of important inputs in the context of time memory and food anticipation (Ko *et al.*, 2003; Cain and Ralph, 2009; Pendergast *et al.*, 2009; Storch and Weitz, 2009; Cain *et al.*, 2012, 2014; Mulder *et al.*, 2013). On the other hand, our results are in disagreement with a number of studies where the time memory acquisition led to entrainment of the molecular clock in brain regions, which are also studied here (Wakamatsu *et al.*, 2001; Ángeles-Castellanos *et al.*, 2007; Pantazopoulos *et al.*, 2011; Verwey and Amir, 2012; Al-Safadi *et al.*, 2014). The possible explanation of this discrepancy could be the differences in strength of the conditioned stimulus. Whereas the timing of a life-threatening event, such as predation, or the equally essential timing of food availability, might reset the clock gene expression in the whole tissue involved in the acquisition of this memory, a relatively weaker inputs, possibly occurring several times a day, may only affect the molecular clock mechanism in a subpopulation of involved neurons.

DS clock and time memory

Out of the three clock genes chosen for analysis to determine the potential phase shifting of the brain peripheral clocks following time memory acquisition, the most pronounced changes were found in the expression of *Per2* in DS samples from conditioned animals. The pattern of these changes suggested a phase shift of *Per2* expression rhythm in these structures, however, it was not matched by *Nr1d1*. In case of the third analyzed clock gene, *Bmal1*, the low amplitude of the rhythm did not allow a clear interpretation of its involvement in the reaction to conditioning, possibly obscuring the effect.

Implementation of a phase resetting model

Based on these results we focused on *Per2* clock gene and applied the proposed model to predict the possible phase shift of *Per2* in DS of conditioned animals. The model expression profile of *Per2* gene was constructed and fitted to correspond in phase to the values from control groups of animals. This model of DS *Per2* rhythm was then shifted to correspond to the levels of *Per2* expression following conditioning at ZT3 and ZT11. The two constructed phase shifted rhythms then model the predicted phase resetting of *Per2* expression caused by the conditioning. We found that these two rhythms were shifted by 8 h to one another, which reflected the time between the two conditioning times - ZT3 and ZT11. Our results however do not show a simultaneous response of the analyzed clock genes, which would prove a complete reset of the DS clock by conditioning. Nevertheless, the obtained information about the reaction of *Per2* in particular could provide new insight into the mechanisms responsible for acquisition, maintenance, and reactivation of time memory in DS.

Per2 role in DS

The specific changes in *Per2* gene could signify a potentially important role of *Per2* in the function of this brain region. It has been shown previously that some components of the molecular clock network interact with non-circadian processes, thus affecting various cellular processes outside of the circadian clock mechanism (O'Brien *et al.*, 2018; de Lartigue and McDougle, 2019). This type of dual regulation of *Per* genes was found in peripheral tissues in mice (Franken *et al.*, 2007; Kornmann *et al.*, 2007; Ramanathan *et al.*, 2014) and might suggest that conditioning may cause a change in a single specific gene in the DS rather than resetting the phase of the whole clock mechanism tissue wide.

The DS is responsible for the processing of goal-oriented behavior. It acquires appropriate behavioral patterns, fixes them to specific stimuli and implements them under correct circumstances (Balleine and O'Doherty, 2010; Gremel and Costa, 2013; Burton *et al.*, 2015). Together with ventral striatum, HPC and neocortical areas, the DS is involved in reward pathways, decision making and behavioral control. The DS clock could thus potentially provide a time-specific gating of a response to the conditioned stimulus according to the time of its occurrence relative to the phase of the DS clock and consequently enable animals to only generate the desired behavioral response during the right time of day. Our results implicate the specific role of *Per2* gene in this process, possibly acting similarly to an immediate early response gene, orchestrating the desired behavioral response at the right time.

Overall, our results show that a formation of an aversive time memory causes changes in *Per2* clock gene expression in the DS, however, this may not be accompanied by the resetting of circadian clocks in memory-related brain oscillators. We found no effect of conditioning on the molecular circuitry of the HPC circadian clock in our study. Nevertheless, this does not necessarily mean that the HPC clock is not affected. Even though there are daily rhythms in clock gene expression in the HPC, the conditioning might possibly only affect the clock molecular mechanism in a subpopulation of HPC neurons involved in processing the specific recurring event and this change might be obscured when the clock gene expression is performed tissue wide. There is nevertheless still much to be uncovered regarding the mechanism of time memory formation and the possible role of HPC oscillators in it.

Conclusion

Although there is an expanding body of literature concentrating on the HPC oscillator, very little is still known about its regional specifics, synchronization, and function. For that reason, in my PhD thesis, my aim was to address this lack of knowledge and characterize the properties of circadian clocks in distinct HPC parts, their synchronization to the outside environment and their role in memory-related processes.

Due to our sensitive approach, we were able to study individual HPC regions separately and we show that the clocks in these neuronal populations differ in their sensitivity to synchronizing input signals. We are the first to show that the HPC clocks can be synchronized by GCs and GSK3β signaling. Our results demonstrate that these two physiologically relevant signals could cooperate in setting the phase of HPC circadian clocks according to the demands of external conditions and internal signaling processes. Proper setting of the HPC clock is crucial, because it aligns the clock-modulated processes to the optimal time of day, allowing the organism to effectively time the best cognitive performance to the time of day when it is required, thus optimizing energy expenditure, and increasing the chances of survival.

Importantly, we are the first to provide a comparison between neuronal and non-neuronal brain peripheral oscillators showing they differ in their properties and synchronization mechanisms. The relatively lower robustness of clock gene oscillations in the neuronal HPC clock could be explained by existence of functional diverse subpopulations of neurons in the HPC regions, which might not be completely synchronized to one another under physiological in vivo conditions. This finding should be explored in the future, using even more detailed methods. We believe our newly established in vitro protocol using imaging of organotypic HPC explants under luminescence microscope, providing cellular-level resolution could help achieve this goal in the future.

Surprisingly, our results show that the process of time memory acquisition does not require an acute region-wide resetting of the HPC clock. It is however important to note that this result does not exclude the involvement of the clock mechanism in memory-related processes, such as time memory formation, because once again, the effect of used paradigm on the clock gene expression in HPC regions might be obscured by an existence of several neuronal subpopulations, from which only a some may be affected. This further highlights the need for a more precise approaches needed to uncover the detailed mechanism of time memory acquisition in the HPC.

The understanding of HPC circadian clocks, their function and the mechanism of their synchronization is highly relevant to the research focusing on dysfunctions caused by desynchronization of brain clocks. In case of HPC, clock desynchronization is implicated in a number of neuropsychiatric disorders accompanied by a decline of cognitive functions. The identification of synchronizing signals could thus be used in the future development of novel forms of therapy aimed at improving memory and other cognitive processes by providing the desynchronized peripheral clocks with a specific realigning signal.

Our findings showing the strong resetting effect of GCs are important in their own right because of the wide use of systemic GC therapy, mainly in patients suffering from autoimmune disorders, such as multiple sclerosis. We show that the administration of GCs has a widespread effects on both neuronal and non-neuronal brain clocks, which can vary depending on the time of application. It will be important to expand the detail of these new findings in the future research to determine the optimal timing protocol when administering systemic GC therapy, to minimize its potential detrimental effect in this regard on one hand and maximize the potential benefits on the other.

Overall, we succeeded in expanding the knowledge about HPC clock, its properties, synchronization to the external environment, and its role in time memory acquisition. Nevertheless, a lot still remains to be discovered about this important yet enigmatic circadian clock.

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