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Period1 gates the circadian modulation of memory-relevant signaling in mouse hippocampus by regulating the nuclear shuttling of the CREB kinase pP90RSK

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Running Title
Hippocampal PERIOD1, a local circadian pacemaker

Keywords
mitogen-activated protein kinase (MAPK), extracellular regulated kinase (ERK), learning, clock, long-term memory (LTM)

Abbreviations
AC, adenylyl cyclase; AHS, acute hippocampal slices; CRE, cyclic AMP-responsive element; CREM, cAMP responsive element modulator; DD, constant darkness; ERK, extracellular regulated kinase; FSK, forskolin; LD, light-dark; LTM, long-term memory; MSK1, mitogen- and stress-activated protein kinase 1; pCREB, phosphorylated cyclic-AMP response element binding-protein; PER1, PERIOD1; *Per1*$^{-/-}$, Per1 knockout; PHC, primary hippocampal cultures; PKA, protein kinase A; PKC, protein kinase C; pMAPK, phosphorylated mitogen-activated protein kinase; pP90RSK, pMAPK activated ribosomal S6 kinase; s.e.m., standard error of the

mean; *Sp*-cAMPS, Sp-diastereomer of cyclic AMP; STM, short-term memory; ZT, zeitgeber time.

Abstract

Memory performance varies over a 24h day/night cycle. While the detailed underlying mechanisms are yet unknown, recent evidence suggests that in the mouse hippocampus, rhythmic phosphorylation of mitogen-activated protein kinase (MAPK) and cyclic adenosine monophosphate response element-binding protein (CREB) are central to the circadian (~24 h) regulation of learning and memory. We recently identified the clock protein PERIOD1 (PER1) as a vehicle that translates information encoding time-of-day to hippocampal plasticity. We here elaborate how PER1 may gate the sensitivity of memory-relevant hippocampal signaling pathways. We found that in wildtype mice (WT), spatial learning triggers CREB phosphorylation only during the daytime, and that this effect depends on the presence of PER1. The time-of-day-dependent induction of CREB phosphorylation can be reproduced pharmacologically in acute hippocampal slices prepared from WT mice, but is absent in preparations made from Per1-knockout mice. We showed that the PER1-dependent CREB phosphorylation is regulated downstream of MAPK. Stimulation of WT hippocampal neurons triggered co-translocation of PER1 and the CREB kinase pP90RSK (pMAPK activated ribosomal S6 kinase) into the nucleus. In hippocampal neurons from *Per1*$^{-/-}$-mice, however, pP90RSK remained perinuclear. A co-immunoprecipitation assay confirmed a high affinity interaction between PER1 and pP90RSK. Knocking down endogenous PER1 in hippocampal cells inhibited adenylyl cyclase-dependent CREB activation. Taken together, the PER1-dependent modulation of cytoplasmic-to-nuclear signaling in the murine hippocampus provides a molecular explanation for how the circadian

system potentially shapes a temporal framework for memory performance dependent on time-of-day, and adds a novel facet to the versatility of the clock gene protein PER1.

Introduction

In contrast to short-term memory (STM), long-term memory (LTM), involves protein synthesis and is modulated by the circadian (~24 h) system across species (Gerstner *et al.* 2009). This temporal modulation of LTM processing is contingent upon the chronobiological regulation of learning-dependent signaling events. Studies in the sea slug *Aplysia* show that training at different day and night phases induces a characteristic molecular signature within neurons involved in the learning paradigms (Lyons *et al.* 2006). Furthermore, post-translational modifications of molecular signals that are essential for LTM formation (Bourtchuladze *et al.* 1994, Dash *et al.* 1990, Davis & Squire 1984, Tully *et al.* 1994) oscillate with a circadian rhythm in brain centers involved in memory processing (Eckel-Mahan *et al.* 2008, Lyons *et al*. 2006, Rawashdeh *et al.* 2014). Although such findings implicate the presence of an intricate multi-systemic interplay between the circadian clock and memory formation, the mechanistic link between the two systems remains obscure. Potential candidates are the circadian clockwork protein PERIOD1 (PER1) and phosphorylated CREB (pCREB), a molecular marker of memory processing in the hippocampus (Mizuno *et al.* 2002, Kandel 2001), as these two elements are common constituents to both the circadian and the memory system (Lee *et al.* 2010, Frank & Greenberg 1994, Motzkus *et al.* 2000).

Canonical circadian clock genes are not only essential to maintain the accuracy and plasticity of biological clocks (Albrecht *et al.* 1997, Brown *et al.* 2005, Shigeyoshi *et al.* 1997, Travnickova-Bendova *et al.* 2002), but also affect behavior (Jilg *et al.* 2010, Rawashdeh *et al*. 2014). In mice,

PER influences traced fear conditioning (Wang *et al.* 2009), spatial memory performance (Rawashdeh *et al*. 2014), social behavior (Bechstein *et al.* 2014), and behavioral sensitization associated with drugs of addiction (Abarca *et al.* 2002, Andretic *et al.* 1999). This suggests that elements of a circadian pacemaker are likely also integral to circadian modulation of LTM processing. Like PER1, pCREB plays a central role in LTM consolidation (Bourtchuladze *et al*. 1994), and transduces "clock-input" signals (Belvin *et al.* 1999, Obrietan *et al.* 1999). Expression of the *Per1* gene depends on pCREB binding to the cAMP-response elements (CRE) promoter region (Zmrzljak *et al.* 2013, Travnickova-Bendova *et al*. 2002, Hida *et al.* 2000, Motzkus *et al*. 2000) and *Per1* gene expression is maximally affected in mice deficient for the CREB-related transcription factor cAMP responsive element modulator (CREM) (Zmrzljak *et al*. 2013). Likewise, in *Per1*-knockout ($Per1^{-/-}$) mice, hippocampal pCREB dynamics are significantly altered (Rawashdeh *et al*. 2014).

Expanding on our previous finding that PER1 accounts for daily variations in spatial memory performance (Rawashdeh *et al*. 2014), our hypothesis for the underlying mechanism is that PER1 modulates hippocampus-dependent memory by gating the sensitivity of incoming signals.

Using *ex vivo* and *in vitro* techniques, we assessed communication between PER1 and CREB in the mouse hippocampus. This is the first demonstration that the tight coupling of CREB activation to *Per1* gene expression is in fact bidirectional, since PER1 effectively influences the sensitivity of memory-relevant pathways that feed back to CREB phosphorylation in the hippocampus. On a mechanistic level, we show that PER1-dependent signaling affects the trafficking of phosphorylated MAPK activated ribosomal S6 kinase (pP90RSK) into the nucleus,

where it modulates the phosphorylation of its target CREB. This mechanism thus represents a functional link to encode time-of-day and translate it into the circadian modulation of LTM.

Material and Methods

Animals and tissue sampling

All animal experiments were approved by the local veterinary office (permission#: V54-19c20/15-FU/1045) and the European Communities Council Directive (89/609/EEC). Male *Per1*$^{-/-}$-mice (kindly provided by Dr. D.R. Weaver, University of Massachusetts Medical School, Worchester, USA; bred on a C3H/HeN background [von Gall *et al.* 2002]) and wild-type controls, aged 8-15 weeks were bred in-house for use in this study. Animals were kept for at least 2 weeks prior to experiments under a standard light-dark (LD) cycle, with 12 h light (daytime: 250 lux; onset ("ON") defined as Zeitgeber Time [ZT] 0) and 12 h darkness (nighttime: dim red light <10 lux, >680 nm), or under constant darkness (DD) (dim red light <10 lux, >680 nm), at constant room temperature and with food and water available *ad libitum*. Animals were anesthetized and sacrificed for removal of the hippocampus at indicated time points (n=3-6 animals per time point and experiment unless stated otherwise).

Behavioral analysis

To assess spatial memory performance in mice we used an 8-arm radial arm maze as described before (Rawashdeh *et al*. 2014, Jilg *et al*. 2010). In brief, animals received daily adjusted meal sizes to maintain their body weights at 85-90% of their pre-train values for the duration of the experiment, habituated for two days to the maze and the reward, and trained on the third day (single trial). A trial was terminated once all rewards (dry breadcrumbs; Brandt Backwaren

Vertriebs GmbH, Hagen/Germany) were consumed, or 15 min after the animalentered the maze. An "entry" into an arm was recorded when all four paws were within the arm. An "error" was recorded if an animal re-entered a previously visited arm. Animals were placed in the 8-arm radial arm maze at ZT 02 or ZT 14, and sacrificed either 30 min or 6 h post training. Hippocampi were removed and stored at -80°C until further use. Importantly, to differentiate between training-induced changes in pCREB levels and cycling endogenous pCREB, we included control (naïve) groups of mice for both time points (ZT 02 or ZT 14). Naïve mice were handled identically to the experimental group, without, however, the training session.

Signaling analyses using acute hippocampal slices

Acute hippocampal slices (AHS) were prepared as described for electrophysiological studies (Selbach *et al.* 2004) at ZT 02, 06, 10, 14, 18 and 22. In brief, hippocampi were cut into 350 µm thick transverse sections. Hippocampal slices from a given animal were randomly assigned to incubation chambers. One set of slices served as control, while other sets were stimulated with forskolin (50 µM; Merck KG, Germany) or *Sp*-cAMPS (300 µM; BioLog Life Science Institute, Bremen, Germany), respectively, for 1 h. To study differences between genotypes with regard to the pCREB and pMAPK induction dynamics in response to forskolin (50 µM) or phorbol-12-myristate-13-acetate (PMA;1 µM), hippocampal slices were stimulated at the time, when endogenous pCREB levels were shown to be maximal in WT mice (ZT 10; (Rawashdeh *et al.* 2014) for 30 min, 1 h, or 2 hrs, respectively. Another set of slices prepared from WT mice was pre-incubated with the MEK1/2 inhibitor U0126 (10 µM) (Cell Signaling Technology Inc, Beverly, USA) for 30 minutes, followed by the addition of forskolin (50 µM) for 1 h. At the end

of the experiments, slices were transferred into 500 µl of ice-cold sample buffer (Invitrogen, Carlsbad, USA), frozen and stored at -80°C until further processing.

Primary hippocampal cell cultures

Primary hippocampal cultures (PHC) were prepared as described (Benz *et al.* 2010).WT and *Per1*$^{-/-}$-mice were sacrificed at postnatal day 3, hippocampi were removed and kept in DMEM at 4°C. Hippocampi were triturated in a papain-rich medium at 37°C and cells were seeded onto glass coverslips coated with poly-l-lysine (Sigma-Aldrich, Germany), containing Minimal Essential Medium (Invitrogen, Germany), supplemented with 1% (vol/vol) penicillin-streptomycin (Invitrogen, Germany), 1% GlutaMax (vol/vol) (Invitrogen, Germany), 1% glucose (vol/vol) (Sigma-Aldrich, Germany), 10% fetal bovine serum (vol/vol), and B-27 (Invitrogen, Germany). After exchange for Neurobasal-A medium (Invitrogen, Germany) supplemented with 1% (vol/vol) penicillin–streptomycin mix, 1% GlutaMax (vol/vol) and B-27 supplement (Invitrogen, Germany), the cells were maintained for 14 days under atmospheric conditions (95% CO_2 and 5% O_2) at 37°C prior to further experimentation. PHC from both, WT and *Per1*$^{-/-}$-mice, were treated for various time periods (0 to 180 minutes) with forskolin (10 µM), with or without the addition of the inhibitor of pP90RSK, BRD-7389 (10µM; 1-[(2-Phenylethyl)amino]-3*H*-naphto[1,2,3-de]quinoline-2,7-dione, TOCRIS Bioscience, Wiesbaden, Germany), or U0126 (Cell Signaling Technology, Leiden, Netherlands), dissolved in DMSO. Experiments were terminated by transferring culture plates on ice, removing the medium from the cells, and adding 50 µl of sample buffer (Invitrogen, Germany). The samples were sonicated and heated to 70°C for 10 min prior to immunochemical analyses.

Immunochemistry

Immunoblotting

Western blots of hippocampal extracts were performed with slight modifications to previous protocols (Maronde *et al.* 1999a). Hippocampi were sonicated in NuPAGE® LDS sample buffer (Invitrogen, Carlsbad, USA) (10% Glycerol, 141 mM Tris Base, 106 mM Tris HCl, 2% LDS, 0.51 mM EDTA, 0.22 mM SERVA® Blue G250, 0.175 mM Phenol Red, 100 mM DTT, pH 8.5) and proteins were denatured by heating. Proteins were separated using NuPAGE® Novex 4-12% Bis-Tris Gels according to the manufacturer's instructions (Invitrogen, Carlsbad, USA), and transferred onto a PVDF membrane using the iBlotTM Dry Blotting System (Invitrogen, Carlsbad, USA). Prior to incubation with primary antibodies (Supplemental Table 1), membranes were blocked with RotiBlock® (Roth, Karlsruhe, Germany) for 1 h at room temperature. Subsequently, membranes were incubated with secondary antibodies (Table 1) for 1 h at room temperature. Signals were detected using Immobilon Western Chemoluminiscent HRP Substrate (Millipore, Billerica, USA), digitalized using the ChemiDoc XRS System (BioRad, München, Germany) and analyzed using a luminescence system (Quantity One, ChemiDoc XRS, Bio-Rad, Hercules, CA, USA). For experiments investigating the effects of forskolin on hippocampal tissue across a 24h day/night cycle, only experimental samples for a given time point and their respective control were loaded onto a single gel. Forskolin-induced CREB phosphorylation was quantified for a single time point rather than across different membranes, and for comparability, expressed as percent change of pCREB levels. The optical intensity of all target signals on any given Western blot was always normalized to the optical intensity of the actin signal on the same blot. The normalized signal intensities were then expressed as relative signal intensities (rel. O.D.), or as percent (%) change.

Immunofluorescence

Forskolin-stimulated (1 h) hippocampal primary cell cultures were fixed post treatment with paraformaldehyde 4% (wt/vol) in 0.1 M phosphate buffer. Immunofluorescence staining was performed as described (Benz *et al*. 2010). In brief, fixed cells were washed with phosphate-buffered saline (PBS) and blocked at room temperature for 45 min with 3% bovine serum albumin (BSA; Sigma-Aldrich, Taufkirchen, Germany) in 0.1 M PBS. Cells were incubated overnight at 4°C with primary antibodies dissolved in PBS containing 1% (wt/vol) BSA (Sigma-Aldrich, Taufkirchen, Germany). Cells were washed with PBS and incubated with fluorochrome-coupled secondary antibodies in PBS for 1 h. Coverslips were washed three times with PBS and mounted on slides using Vectashield mounting medium (Vector Laboratories, Inc. Burlingame, CA, USA) containing DAPI (4',6-diamidino-2-phenylindole) to stain nuclei. Fluorescent images were acquired using an Axio-Cam digital camera mounted on a Zeiss microscope (Carl Zeiss, Oberkochen, Germany) with a 20x objective lens. Fluorescent images were digitally merged to superimpose the different signals. ImageJ was used to quantify the nuclear and/or cytoplasmic fluorescence signal as described previously (Burgess *et al.* 2010).

Co-Immunoprecipitation

To assess whether PER1 interacts with pP90RSK, we used a co-immunoprecipitation (Co-IP) approach, following manufacturer's instructions (PierceTM Crosslink Magnetic IP/Co-IP Kit, Pierce Biotechnology, Rockford, Il, USA). Hippocampi, isolated at ZT 23 (peak of PER1 protein levels; Rawashdeh *et al*., 2014), were homogenized in 500 µl lysis buffer. The supernatant was incubated with magnetic beads covalently bound to the rabbit anti-PER1 antiserum (Fahrenkrug *et al*., 2006), or with the beads only, washed and eluted. Three eluates ("unbound", eluate 1 and

eluate 2) were mixed with 2x sample buffer, sonified, heated to 70°C for 10 min, chilled on ice, loaded onto a 12% NuPage gel with MES running buffer (both from Invitrogen, Waltham, MA, USA) and separated for 60 minutes. The gel was transferred to PVDF membrane using an iBlot (Invitrogen, Waltham, MA, USA). After blotting and washing, the membrane was blocked for one hour in 1x Rotiblock (Roth, Karlsruhe, Germany) at room temperature, incubated with rabbit anti-pP90RSK antisera (1:1000; Cell Signaling, Bad Nauheim, Germany). After washing and incubation with a secondary antibody (1:50000; goat anti-rabbit-HRP, Santa Cruz) the signals were detected using a Vilbert Fusion CCD camera (PeqLab, Erlangen, Germany).

CRE-Luc reporter assay

For transient transfection experiments, HT22 hippocampal neuronal cells (kindly provided by Dr. David Schubert, The Salk Institute for Biological Sciences, La Jolla, CA, USA) were seeded on 6-well plates with 200,000 cells per well and incubated overnight in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% fetal bovine serum (vol/vol), 1% Pen/Strep (vol/vol), and 1% GlutaMax (vol/vol), as described (Benz *et al*. 2010). On the following day cells were transferred into DMEM without serum and transfected with a CRE-Luc reporter plasmid (Promega, USA), and either an equal mix of four *Per1*-directed short hairpin RNAs (sh-*Per1*) (OriGene Technologies, Inc., USA), or scrambled RNA, using the FuGene HD kit (Promega, Mannheim, Germany). Cells were re-seeded at equal numbers onto a 96-well plate in Leibovitz L15-medium, containing 1 mM luciferin and 1% fetal calf serum (FCS). HT22 cells were stimulated the next day with forskolin (10 μM) or DMSO (1%). Luminescence was measured (96-well luminometer, Centro LNB, Berthold Technologies GMBH & Co. KG, Wildbad Germany) at 1 h intervals for 0.1 seconds per well, and is expressed as relative light units (RLU).

In addition, *Per1* mRNA was quantified using real-time PCR, as described previously (Jilg *et al*. 2010).

Statistical analyses

All statistical analyses were performed using GraphPad software 5.0 and 6.0 (La Jolla, California, USA). Group means between the various time points, and within each genotype, were compared by 1-way analysis of variance (ANOVA, $P<0.05$ as criterion of significance) followed by Holm-Sidak's and Bonferroni posthoc multiple comparison tests. Potential differences and interaction terms between genotype and treatment conditions were detected by a two-way ANOVA, and a Student's t-test for pairwise comparison of these variables ($P<0.05$ as criterion of significance). Data are presented as mean $\pm$ standard error of the mean (s.e.m.). For all *in vitro* primary culture experiments, the sample size "n" refers to the total number of independent experimental repeats.

Results

Time-of-day-dependent differences in spatial-learning-induced CREB phosphorylation is dependent on PERIOD1

Among the various hippocampal rhythmic-signaling molecules we identified so far, CREB is the one that is most significantly influenced by the absence of PER1 (Suppl. Fig. 1; Rawashdeh *et al*. 2014). To confirm the role of PER1-dependent CREB phosphorylation in memory processing, we examined whether PER1 regulates temporal differences in learning-induced CREB activation as a functional gateway to daytime-dependent spatial learning efficiency. After one day of spatial memory training, hippocampal CREB phosphorylation exhibited rapid and persistent induction

dynamics that were limited to WT mice and daytime (ZT02) only (ZT02: trained vs. naïve; WT-mice [0.5 h and 6 h], $P<0.05$, *Per1*$^{-/-}$-mice [0.5 h and 6 h], $P>0.4$). Interestingly, nighttime training (ZT14) induced a significant decline in pCREB levels by 6 h post-training as compared to the naïve control mice in both genotypes (ZT 14: trained vs. naïve; WT-mice and *Per1*$^{-/-}$-mice [0.5 h], $P>0.3$ and for [6 h], $P<0.05$, *t*-test; Fig. 1A, B).

PERIOD1 modulates time-of-day-dependent inducibility of the AC-PKA-CREB signaling pathway

Having confirmed the essential role of PER1 in diurnal learning-induced CREB activation (Fig. 1A, B), we aimed to identify those memory-relevant pathways that (a) signal to trigger CREB phosphorylation in the hippocampus, (b) are temporally modulated, and (c) are influenced by PER1. To that effect, we used pathway-specific stimulants to target defined molecular cascades known to signal to CREB in acute hippocampal slices (AHS). To factor in time as a variable into our *in vitro* system, AHS were prepared at 4 h intervals (ZT 02, 06, 10, 14, 18 or 22) from WT and *Per1*$^{-/-}$-mice kept under a 12:12 h LD cycle. Only the forskolin stimulated AHS prepared from WT-mice during daytime (ZT 02, 06 and 10) showed pronounced CREB phosphorylation in response to specific activation of adenylyl cyclase (AC)/protein kinase A (PKA)-dependent signaling pathways (WT-AHS; ± FSK [ZT 02-10], $P<0.05$, and [ZT 14-22], $P>0.67$, *t* test, Fig. 1C). The temporal restriction of CREB phosphorylation to daytime in *in vitro* AHS was thus congruent with *in vivo* observations in the WT mice that had undergone hippocampal spatial memory training during the daytime. In line with our hypothesis, CREB phosphorylation could not be induced in AHS prepared from *Per1*$^{-/-}$-mice at any of the assessed time points (*Per1*$^{-/-}$-AHS; ± FSK [ZT02-22], $P>0.1$, *t* test, Fig. 1C), even after prolonged stimulation (120min) with

forskolin at ZT10 (pCREB in *Per1*$^{-/-}$-AHS; ± FSK, $P>0.05$ for all time points tested, *t* test, Fig. 1D).

Silencing *Per1* inhibits CREB-dependent gene expression

To confirm that the clock protein PER1 is directly involved in the intracellular regulation of CREB activation, we knocked down endogenous PER1 expression in mouse hippocampal HT22 cells, transiently transfected with a luciferase reporter for CREB activity (CRE-Luc). Knocking down endogenous PER1 with small hairpin *Per1*-RNA (sh-*Per1*) significantly reduced the forskolin-induced CRE-Luc signal as compared to the controls treated with scrambled RNA of either vehicle or forskolin-stimulated cells ($P<0.05$, 2-way ANOVA, Fig. 2). These data confirm PER1 as a modulator of cAMP-dependent CREB activation.

PERIOD1 regulates cAMP-mediated CREB activation downstream of MAPK/ERK

cAMP signaling in the hippocampus can activate CREB either directly via PKA, or indirectly through more complex cascades involving PKA-Ras-Raf-MAPK/ERK signaling (Gonzalez & Montminy 1989, Impey *et al.* 1998). This constitutes an array of possible targets for PER1 to exert its modulatory effects on signaling to CREB phosphorylation. Inhibiting the PKA-Ras-Raf-MAPK/ERK pathway at the level of the dominant ERK kinase MEK1/2 with its antagonist U0126 (Thomas & Huganir 2004) completely blocked forskolin-induced MAPK/ERK and CREB phosphorylation in WT AHS prepared during daytime (Fig. 3A, B). This indicates that forskolin triggers CREB phosphorylation predominantly via the indirect PKA-Ras-Raf-MAPK/ERK pathway.

We next assessed if PER1 regulates this pathway upstream or downstream of MAPK/ERK. Similar to stimulation by forskolin, the direct downstream activation of PKA by *Sp*-cAMPS led to phosphorylation of MAPK/ERK and CREB in WT AHS (Fig. 3A, C), whereas U0126 inhibited *Sp*-cAMPS-induced MAPK/ERK activation (Fig. 3A-C). Importantly, in the absence of PER1, *Sp*-cAMPS also phosphorylates MAPK/ERK, in line with observations in WT AHS (Fig. 3A, C). Since PER1 regulates signaling to CREB downstream of MAPK/ERK, activation of the alternative protein kinase C (PKC) signaling pathway (PKC-MAPK/ERK-CREB) is also expected to be affected by the lack of PER1. Indeed, stimulating PKC with PMA in AHS from *Per1*$^{-/-}$-mice failed to induce CREB phosphorylation despite MAPK/ERK activation (Fig. 3D, E), further supporting that PER1 enhances signaling downstream of MAPK/ERK (Fig. 3F).

It is not surprising that U0126 abolished even baseline ERK phosphorylation in WT hippocampal slice homogenates (Fig. 3A; compare lane 1 vs. lanes 3-6). Notably, in hippocampal neurons, the PKA signaling pathway coupled to CREB phosphorylation is ERK1/2-dependent (Eckel-Mahan *et al*. 2008). By contrast, U0126 altered basal levels of CREB phosphorylation only marginally (Fig. 1B, lane 1). This is plausible since various kinases may activate CREB, and thus contribute to its basal phosphorylation level. The observed differences in the induction dynamics of pERK and pCREB across stimulants like PKC and PMA (Fig. 3D, E), are likely linked to differences in target localization, drug permeability, and activation dynamics among other kinetics.

PERIOD1 modulates the induction of CREB phosphorylation by targeting pP90RSK

To further narrow down the point at which PER1 affects CREB activation, we focused on the downstream targets of MAPK/ERK, specifically on the CREB kinases MSK-1 (mitogen- and stress-activated protein kinase 1) and pP90RSK (Sindreu *et al.* 2007, Xing *et al.* 1996), using primary hippocampal cultures (PHC). PHC are a simplified experimental system suitable to investigate signaling pathways that are relevant for memory consolidation, particularly the cAMP/PKA/CREB pathway (Deisseroth *et al.* 1996, Deisseroth *et al.* 1998).

The cAMP-dependent molecular cascade that signals to CREB phosphorylation in response to forskolin is the same across the different *in vitro* models. Furthermore, while forskolin stimulates ERK phosphorylation in AHS and PHC prepared from PER1$^{-/-}$-mice, there is no induction of CREB phosphorylation in either *in vitro* system (compare Fig. 4A, C [PHC] vs. Figs. 1D, 3A [AHS]). At the cellular level of individual hippocampal neurons, the modulation of the PKA/PKC-MAPK/ERK pathway by PER1 happens downstream of pMAPK/pERK, which is remarkably similar to the observation in AHS (Fig. 3A-C). Notably, phosphorylation of MAPK/ERK can be induced via AC/PKA signaling in both, WT and *Per1*$^{-/-}$-mice AHS (Fig. 4A, B). We furthermore demonstrated that PER1-dependent CREB phosphorylation is intrinsic to hippocampal neurons (Fig. 4C, D), which is in line with the data obtained using AHS.

In addition to CREB phosphorylation, forskolin also induces phosphorylation of P90RSK (pP90RSK) in a MAPK/ERK-dependent manner in WT PHC (pP90RSK; FSK ± U0126 [0min – 30min]; $P<0.0001$, 2-way ANOVA, Fig. 5A). To confirm, whether pP90RSK mediates CREB phosphorylation, we inhibited P90RSK prior to forskolin stimulation in WT PHC. The RSK

inhibitor BRD-7389 antagonized the effect of forskolin on CREB phosphorylation (pCREB; FSK ± BRD-7389 [0min – 30min]; $P<0.0001$, 2-way ANOVA, Fig. 5B, C). The CREB kinase MSK1 (mitogen- and stress-activated protein kinase 1), a direct target of pMAPK/pERK, was activated by forskolin in parallel with pP90RSK (forskolin vs. vehicle [15 min – 60 min]; $P<0.05$, ANOVA, and forskolin+BRD-7389 [30 min – 60 min]; $P<0.05$, ANOVA and Holm-Sidak's multiple comparison test for posthoc analysis, Fig. 5D, E). Importantly, forskolin stimulated the pMAPK/pERK-dependent translocation and nuclear phosphorylation of MSK1 even in the absence of PER1 (MSK1; FSK ± U0126 [15min – 60min]; $P<0.0001$, 2-way ANOVA, Fig. 5F). This observation confirms our earlier conclusion that pMAPK/pERK is not targeted by PER1, and suggests that PER1-dependent temporal regulation of CREB phosphorylation does not involve MSK1 in hippocampal neurons. The broad spectrum across which the effects associated with the lack of *Per1* expression can be observed, reconfirms the suitability of PHC to investigate PER1-dependent signaling, and also shows that under artificial *in vitro* conditions, PER1 remains linked to dynamics in CREB phosphorylation.

PERIOD1 modulates CREB activation by regulating the nucleo-cytoplasmic shuttling of pP90RSK

P90RSK is phosphorylated and activated by pMAPK/pERK in the cytoplasm (Chang & Karin 2001, Dalby *et al.* 1998); it thereafter translocates to the nucleus (Frodin & Gammeltoft 1999, Gao *et al.* 2012) to target CREB phosphorylation (Impey *et al*. 1998). Thus far, our results imply that PER1 regulates CREB phosphorylation by modulating either the cytoplasmic activation of P90RSK or its nuclear translocation. Immunofluorescence analyses of the intracellular distribution of pP90RSK following forskolin stimulation show that cytoplasmic activation of

P90RSK does not rely on the presence of PER1. Surprisingly, however, and in contrast to MSK1, pP90RSK remains perinuclear in forskolin-stimulated PHC prepared from *Per1*$^{-/-}$-mice (Fig. 6A). Additionally, we show for the first time that in PHC from WT mice, endogenous PER1 accumulates in the nucleus in response to AC/cAMP signaling, with kinetics similar to that of P90RSK activation and translocation, and importantly also in parallel to CREB phosphorylation (PER1; ± FSK [0min – 30min]; $P<0.0001$, ANOVA, and $P<0.001$, Holm-Sidak's multiple comparison test for posthoc analysis; pCREB; ± FSK [0min – 30min]; $P<0.0001$, ANOVA and $P<0.05$, Holm-Sidak's multiple comparison test for posthoc analysis, Fig. 6B).

To confirm the suggested interaction of PER1 with pP90RSK, we tested whether immunoprecipitating PER1 would co-precipitate pP90RSK. In Western blots using antisera against pP90RSK (Fig. 6C), both, the crude extract of hippocampal lysate (lanes 2) and the PER1-bound eluted fraction (lanes 3) yielded bands at the expected molecular weight of 90 kDa (Fig. 6C, indicated by arrow). The 90 kDa signal is absent in both washouts, in supernatants incubated with beads only (lanes 1), or with anti-Per1 antibody bound-beads (lanes 4), respectively.

Discussion

In mouse hippocampus, circadian rhythmicity in the basal phosphorylation of essential memory-related molecules such as pCREB and pMAPK/pERK, as well as in the expression of the clock gene protein PER1, are well documented (Rawashdeh *et al*. 2014, Eckel-Mahan *et al*. 2008, Jilg *et al*. 2010). The absence of PER1 impairs spatial memory and hippocampal rhythms in memory-relevant molecules including epigenetic modifications (Rawashdeh *et al*. 2014). Collectively,

this implicates an interaction and interdependence of systemically common signaling molecules like CREB and MAPK/ERK with the clockwork component PER1. Our study demonstrates that the time-of-day and learning-dependent activation of MAPK/ERK and CREB signaling relies on the presence of PER1 in hippocampal neurons. Additionally, by pinpointing the modulatory effect of PER1 on pCREB to the nuclear translocation of the CREB kinase pP90RSK, we provide a key to understanding how learning-induced hippocampal memory consolidation may vary rhythmically between day- and nighttime.

Pharmacological stimulation of the cAMP/PKA-signaling pathways *ex vivo* showed an induction of MAPK/ERK and CREB that was restricted to daytime and early nighttime. Behavioral training-induced LTM and *ex vivo* electrophysiological stimulation-induced plasticity depend on the activation of PKA, PKC and Ca^{2+}-dependent pathways, all of which converge to phosphorylate MAPK/ERK and CREB (Roberson *et al.* 1999, Bonini *et al.* 2007, Bourtchouladze *et al.* 1998, Davis *et al.* 2000, Sweatt 2004, Xing *et al*. 1996). Importantly, the responsiveness of the PER1-dependent cAMP/PKA-signaling pathway to behavioral and pharmacological stimuli suggests that PER1 functions in temporally structuring memory processing. The link between CREB phosphorylation and PER1 is further strengthened by observations that elevated hippocampal sensitivity during the daytime temporally coincides with the phase of endogenously high PER1 expression (Jilg *et al*. 2010).

The preparation of acute brain slices can trigger a transient phosphorylation and dephosphorylation of memory-relevant signaling molecules such as calcium/calmodulin-dependent kinase II (CaMKII) and ERK (Ho *et al.* 2004). Therefore, the interpretation of *ex vivo*

dynamics reported here bears the caveat that results might be subject to a systematic error if phosphorylation levels were distorted by injury during slice preparation. However, this would not affect the general message of our study, namely that the hippocampus responds to stimuli in a time-of-day-dependent manner. This is even more so as in the study by Ho *et al*. (2004), changes in ERK activation returned to baseline within 30-60 minutes, thus within a time frame well before our hippocampal slices were pharmacologically stimulated. Even if injury caused a substantial ERK phosphorylation, the amount of unphosphorylated ERK molecules left to be activated seems to be sufficient to allow detection of a rhythm in ERK induction that is not only very robust, but also exhibits a phosphorylation profile similar to that of CREB.

The preserved responsiveness of MAPK/ERK to PKA- and PKC-signaling in the absence of PER1 suggests that the post-translational modification-dependent STM (Sweatt 2004) likely remained intact. Our study verifies that signaling upstream of MAPK/ERK is unaltered in the absence of PER1. It is intriguing and mechanistically plausible that a PER1-dependent temporal gate for memory processing, located downstream of MAPK/ERK, may specifically impact the gene-expression-dependent LTM (Gerstner *et al*. 2009, Lyons *et al.* 2005, Rawashdeh *et al.* 2007).

The use of constitutive *Per1*$^{-/-}$-mice makes it difficult to discriminate hippocampus-specific PER1 signaling from its effects on remote brain structures that are synaptically coupled to this limbic component. However, the data presented in this study strongly suggest that signaling to CREB in hippocampal neurons is primarily and locally regulated by PER1. Behavioral deficiencies in spatial memory of *Per1*$^{-/-}$-mice (Rawashdeh et al., 2014) are associated with

impaired working memory-induced CREB activation in the hippocampus (this study). Notably, *Per1*$^{-/-}$-mice do learn and form long-term spatial memory but the change in behavior is less dramatic across repetitive training sessions as compared to WT-mice (Rawashdeh et al. 2014). This difference in learning efficiency likely reflects the impairment of signaling to pCREB, as detected here in *Per1*$^{-/-}$-mice.

Furthermore, we confirmed the regulatory role of PER1 in hippocampal signaling by demonstrating that knocking down *Per1* expression *in vitro* significantly inhibits forskolin-induced pCREB-dependent gene expression. Additional support for PER1 as a local hippocampal modulator of memory-relevant signaling pathways directed at pCREB is provided as we and others have shown earlier that the SCN appears functionally intact in *Per1*$^{-/-}$-mice (Bae *et al.* 2001, Jilg *et al.* 2010, Liu *et al.* 2007), yet hippocampal rhythms in clock gene expression and epigenetic modifications are altered in the absence of PER1 (Jilg *et al*. 2010, Rawashdeh *et al*. 2014), and learning and memory is affected in *Per1*$^{-/-}$-mice (Jilg *et al*. 2010, Rawashdeh *et al*. 2014). Thus, our studies establish that PER1 endows a local Zeitgeber function in mouse hippocampus.

CREB plays an important role in SCN clock physiology, particularly in mediating external and internal entrainment cues to the master circadian clock (Lee *et al*. 2010). Beyond the well-known pCREB-dependent *Per1* gene expression (Naruse *et al.* 2004, Tischkau *et al.* 2003, von Gall *et al.* 2001), we here provide novel molecular evidence for a bidirectional interaction between these two molecules, in which PER1 feeds back to signaling pathways coupled to CREB phosphorylation, similar to the autoregulatory feedback loop described for mammalian circadian

clockwork models (Shearman *et al.* 2000). Furthermore, this represents the first direct interactive link between the circadian clock and signaling relevant to memory processing in the hippocampus. Our observations on the bidirectional interaction between CREB and PER1 in the mouse hippocampus may also apply to the SCN, which would imply a novel role for *Per1* beyond the well-established function as an immediate-early gene in SCN photic-transduction (Albrecht *et al.* 1997, Shearman *et al*. 2000). Studies to test this interesting hypothesis are currently underway.

The impairment of hippocampal PKA and PKC signaling to CREB phosphorylation in *Per1*$^{-/-}$-mice hippocampus cannot be generalized. In the pineal gland for instance, a model system for cAMP signaling (Stehle *et al.* 1993, Maronde *et al*. 1999a), we found that PKA activation readily phosphorylates CREB also in *Per1*$^{-/-}$-mice (data not shown). Notably, in contrast to our findings in the hippocampus, *ex vivo* forskolin-stimulated pineal cAMP signaling is not influenced by PER1, since pineal PKA is directly coupled to pCREB, and not indirectly linked via the PKA-Ras-Raf-MAPK/ERK pathway (Maronde *et al.* 1999b, Maronde *et al.* 1999c). Nevertheless, *in vivo* nighttime cAMP-dependent melatonin synthesis and the transcription of its rate-limiting enzyme, the arylalkylamine *N*-acetyltransferase (AANAT), are disinhibited by the absence of PER1 (Christ *et al.* 2010). While the authors attribute this to a liberation of the CRE in the AANAT promoter from the inhibitory impact of PER1, our results imply that the molecular impact of PER1 is tissue-specific.

An intriguing aspect of our data is that the modulatory action of PER1 on signaling to pCREB is linked to the nuclear translocation of pP90RSK. Our Co-IP data clearly demonstrate that PER1 binds pP90RSK at least in the phosphorylated form, and, thus, confirms the interaction of these proteins. Thus, pP90RSK may be part of the previously described PER1-protein complex (Brown *et al.* 2005) to facilitate cytoplasmic-nuclear shuttling. A mutation in the gene *RPS6KA3,* coding for the human pP90RSK, is associated with the Coffin-Lowry syndrome, an X-linked disorder (Trivier *et al.* 1996). Markedly, cognitive deficits are among the primary symptoms associated with the more than 100 mutations discovered thus far in the *RPS6KA3* gene (Merienne *et al.* 1999), which are linked to an affected CREB phosphorylation at serine-133 (Harum *et al.* 2001). These observations are in agreement with the memory-relevant molecular deficits reported here in signaling to CREB *in vivo* and *in vitro* as well as with our previously reported behavioral data on working-memory impairment in *Per1*$^{-/-}$-mice (Rawashdeh *et al.* 2014).

The ability to convert transient stimuli into long-term changes of brain function is central to the capacity of an animal to adapt to, and to learn from, a changing environment. Coping with periodically re-occurring harmful or rewarding stimuli requires an efficient molecular time-management machinery capable to associate, retain, and recall temporal information. Such molecular clockworks were originally detected in neurons of the SCN (Reppert & Weaver 2002, O'Neill *et al.* 2008). On the basis of the results presented here and in conjunction with our earlier observations (Jilg et al., 2010; Rawashdeh et al., 2014), we propose that within the molecular mechanisms in hippocampal memory processing, the clock gene product PER1 is an important element for locally disseminating temporal extra-hippocampal cues (Phan *et al.* 2011, Rawashdeh *et al*. 2007, Rawashdeh & Maronde 2012) by influencing cAMP-signaling and the

epigenome (Fig. 7). In accordance with the need for transcription in LTM consolidation, both the total gene expression and chromatin modifications are PER1-regulated and, similar to pCREB, temporally gated (Rawashdeh *et al*. 2014).

Unlike the SCN, the hippocampus does not show circadian rhythmicity *ex vivo* (Abe *et al.* 2002), suggesting that the *in vivo* rhythms observed in the mouse hippocampus are at best the product of a hippocampal slave oscillator. Accordingly, we do not expect an endogenous rhythm in our *in vitro* dissociated PHCs to be rhythmic. Nevertheless, our observations on the differential response of the hippocampus to pharmacological stimulation *ex vivo* in a time-of-day dependent manner, strongly suggests that the temporal physiological status of the hippocampus is maintained *ex vivo*.

Normalization of the phosphorylation state of proteins analyzed here, CREB and ERK, against actin or any other housekeeping gene product is problematic, if total protein levels are rhythmic across a 24h day/night cycle. However, the cycling nature of both CREB and ERK is restricted to their phosphorylation state not the total protein levels. In fact, it has been validated previously that normalization of pCREB against total CREB protein or actin yields the same results in mouse hippocampal protein extracts (Eckel-Mahan *et al*. 2008). This supports the use of actin or any other protein that is not regulated by the circadian system for normalization to detect changes in the phosphorylation state of CREB and ERK.

In conclusion, our study provides compelling evidence that in mouse hippocampal neurons the clock protein PER1 temporally regulate cAMP as well as PKC signaling to CREB. This novel role of PER1 is not only evident on the molecular and cellular levels, but is also reflected on the systemic level in hippocampus-dependent behavior. Such a prominent and important function of PER1 in mnemonic processes may also be linked to phenotypic observations in humans suffering from single-nucleotide polymorphisms within the *Per1* gene (Harum *et al*. 2001, Merienne *et al*. 1999). Last but not least, this study provides novel mechanistic insights in the PER1-CREB connection, showing a bidirectional interaction between both molecules that modulates memory relevant signaling pathways in the hippocampus.

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Conflict of Interest

The authors declare no competing financial interests.

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Figure Legends

Figure 1.

Induction of CREB phosphorylation in the hippocampus is PER1-dependent. Experimental outline is indicated in the time bar on top, with training and sampling times indicated by arrowheads. ***A***, ***B***, Semi-quantitative analysis of pCREB protein levels in the hippocampi from naïve (1-3 and 7-9) and trained (4-6 and 10-12) WT and *Per1*$^{-/-}$-mice following daytime or nighttime spatial learning (n = 3-4 mice for all naïve and post-training time points sampled). **P*<0.05, *t* test when comparing time matched naïve control pCREB levels to post-training experimental pCREB levels. ***C***, Western blot analysis of pCREB levels in AHS, prepared and stimulated with forskolin (FSK) in 4 h intervals during one 24 h day/night cycle (n≥3 for all Zeitgeber times (ZT) sampled, indicated by red arrow heads on the time bar). **P*<0.05, *t* test when compared to time-matched vehicle treated AHS. ***D***, Semi-quantitative analyses of Western blots for samples derived from AHS, prepared at ZT10, of WT and *Per1*$^{-/-}$-mice and stimulated with forskolin. WT-AHS: ■; n≥3; *Per1*$^{-/-}$-AHS: ■; n≥3, **P*<0.05, *t* test against baseline; #*P*<0.05, *t* test between genotypes for every time point. Error bars represent s.e.m.. Representative Western blots are shown below the graphs. The lines between bands signify

different membranes. ZT corresponds to the time (hours) post light onset, with lights ON/OFF corresponding to ZT0 and ZT12, respectively.

Figure 2.

Knockdown of *Per1* inhibits CREB-dependent gene expression. Real-time bioluminescence measurements of luciferase signals (relative light units [RLU]) in CRE-Luc transfected hippocampal HT22 cells, transiently co-transfected with either short-hairpin-*Per1* RNA (sh-*Per1*) or scrambled RNA (scr), treated with either forskolin (FSK) or vehicle (VEH) (n=4; mean ± s.e.m.). Inset shows the quantification of real-time PCR results of *Per1* mRNA in HT22 cells post-transfection with sh-*Per1* or scr RNA (n=3; **P*=0.028 ANOVA).

Figure 3.

PER1 targets PKA-signaling downstream of ERK. ***A***, ***B***, Semi-quantitative analysis of pERK and pCREB induction in AHS prepared from WT-mice at ZT10 followed by stimulation with forskolin (FSK) or *Sp*-cAMPS in the presence and absence of the MEK1/2 inhibitor U0126. #*P*<0.05, ANOVA and Bonferroni multiple comparison for posthoc analysis. ***C***, Comparison of total pERK (pERK1/2) induction in AHS from WT and $Per1^{-/-}$-mice at different intervals post-stimulation with forskolin (FSK) or *Sp*-cAMPS. #*P*<0.05, *t* test between genotypes for every time point. ***D***, ***E***, Western blots analyses of total pERK and pCREB induction in AHS stimulated with PMA for 30-120min. Error bars represent s.e.m.. ***F***, Schematic representation of AC/PKA signaling in the hippocampus is coupled to CREB phosphorylation via the Ras/Raf/ERK signaling cascade, with PER1 regulation of CREB phosphorylation localized downstream of ERK. **P*<0.05, *t* test against baseline in all figures. WT-AHS: ■; n≥3; $Per1^{-/-}$-AHS: ■; n≥3. The

sample size refers to the number of independent repeats. Representative Western blots are shown below all graphs, with lines demarking different membranes. Notably, all pCREB and pERK signal intensities were normalized to their corresponding beta-actin signal intensities within the same blot.

Figure 4.

AC/PKA signaling to CREB (Ser133) phosphorylation is intrinsically regulated in hippocampal neurons. ***A***, Semi-quantitative analysis of ERK induction in WT-PHC by forskolin (FSK) and its complete inhibition by U0126. **P*<0.05, *t* test against baseline. #*P*<0.05, ANOVA). Error bars represent s.e.m.. Representative Western blots are shown below the graph. ***B***, Quantification of pERK immunofluorescence signal (*right*) in NeuN positive primary hippocampal neurons (*left*) of WT and *Per1*$^{-/-}$-PHC. **P*<0.05, *t* test against baseline; #*P*<0.05, *t* test between genotypes. ***C***, Western blots analyses of pCREB levels post forskolin (FSK) stimulation of PHC. **P*<0.05 against individual baselines/time point, ANOVA and Holm-Sidak's multiple comparison test for posthoc analysis. Error bars represent s.e.m.. Representative Western blots for the time response curves are shown below the figure. ***D***, Representative nuclear pCREB immunofluorescence signal in NeuN positive primary hippocampal neurons of WT and *Per1*$^{-/-}$-PHC (*left*), and its quantification (*right*). **P*<0.05, *t* test against baseline; #*P*<0.05, *t* test between genotypes.

Figure 5.

PER1 modulates CREB phosphorylation by regulating pP90RSK. ***A***, Time course for forskolin (FSK) stimulated shuttling of pP90RSK in WT-primary hippocampal neurons (insets; magnification: 63X). Note the inhibition of the nuclear signal by U0126. Quantification is based

on the neuronal nuclear immunofluorescence signal. *:P<0.001, ANOVA and Holm-Sidak's multiple comparison for posthoc analysis. #: P<0.001, 2–way ANOVA. ***B***, Time response curves for forskolin (FSK) -induced CREB phosphorylation in WT-primary hippocampal neurons in the presence and absence of BRD-7389. Quantification is based on the intensity of the nuclear immunofluorescence signal after FSK stimulation (*:P<0.001, ANOVA and Holm-Sidak's multiple comparison for posthoc analysis) and in the presence of BRD-7389 (P>0.05, ANOVA). #:P<0.001 2–way ANOVA. Inset, representative neuronal nuclear pCREB immunofluorescence images (63X). ***C***, Schematic representation of pP90RSK as a downstream target of PKA-signaling and as a CREB kinase in the FSK-stimulated signaling pathway of primary hippocampal neurons. ***D***, Immunochemical analyses of FSK-induced pMSK1 in PHC (n=3) prepared from WT-mice. ***E***, Immunochemical assessment for the specificity of the RSK inhibitor BRD-7389 on FSK-stimulated pMSK1 in WT-PHC (n=3). Representative Western blots for the time response curves are shown below the figures. ***F***, *Left*, representative primary hippocampal neurons prepared from *Per1*$^{-/-}$-mice and stimulated with FSK in the presence and absence of the MEK1/2 inhibitor U0126. Yellow quadrants: representative nuclear pCREB immunofluoresence signal (green); red: neuronal marker NeuN; blue: nuclear marker DAPI. *Right*, corresponding quantification of the nuclear pMSK1 immunofluorescence signal (yellow quadrant) in response to FSK stimulation (solid line) and in the presence of U0126 (hatched line) (*P<0.05, ANOVA and Holm-Sidak's multiple comparison for posthoc analysis). #P<0.05, 2–way ANOVA. Error bars represent s.e.m..

Figure 6.

PER1 is required for the nuclear translocation of pP90RSK. ***A***, *Top*, quantification of neuronal nuclear pP90RSK immunofluorescence signal after forskolin (FSK) stimulation of primary hippocampal neurons from WT- and *Per1*$^{-/-}$-mice (**P*<0.001, ANOVA and Holm-Sidak's multiple comparison for posthoc analysis). *Bottom*, representative immunofluorescence images from neurons of primary hippocampal neurons derived from WT- or *Per1*$^{-/-}$-mice (red; neuronal marker NeuN, green; pP90RSK, blue; DAPI). ***B***, *Top*, comparison of the activation and localization dynamics of nuclear PER1 and pCREB immunofluorescence, following FSK stimulation in primary hippocampal neurons of WT-hippocampi (**P*<0.05, ANOVA and Holm-Sidak's multiple comparison for posthoc analysis). *Bottom*, immunofluorescence images showing the time-dependent nuclear translocation and activation of PER1 (green) and pCREB (red) in primary hippocampal neurons, respectively. Note, the co-localization of PER1 and pCREB immunofluorescence signal (yellow) in the merged images. ***C***, Co-Immunoprecipitation (Co-IP) using the PER1 antibody as bait to determine whether a physical interaction between PER1 and phosphorylated P90RSK at S380 (left blot) and at S359/S363 (right blot) exists. Western blot of eluates from beads without bound PER1 antibody (lanes 1), and from beads with bound PER1 antibody from crude hippocampal lysate (lanes 2), from the eluted fraction (lanes 3), and washout (lanes 4) using pP90RSK antibodies. M: protein size marker.

Figure 7.

Working model, based on the here presented results and our earlier observations (Jilg et al., 2010; Rawashdeh et al., 2014) that delineate how the clockwork component PER1 mediates and disseminates temporal information in the hippocampus. The model predicts that rhythmic PER1

abundance in mouse hippocampus gates time-of-day-dependent memory performance, by temporally regulating memory relevant signaling pathways, essential for LTM formation. Mechanistically, the gating of hippocampal signaling by PER1 reflects a temporal regulation of the nuclear translocation of pP90RSK, and thus the phosphorylation of its downstream target, the transcription factor CREB. Notably, molecular gating of downstream, late signaling events within memory relevant pathways, like the earlier shown PER1-dependent chromatin remodeling (Rawashdeh et al., 2014) may explain how the circadian system can differentially influence the protein synthesis-dependent LTM without affecting STM.

Table 1 Information on antibodies used for Western blotting and Immunofluorescence analyses and the PER1 shRNA sequences.

Antibody	**MW [kDa]**	**Host**	**Source**	**Dilution**	**Use**
PERIOD1		Rabbit	Fahrenkrug (Fahrenkrug *et al.* 2006)	1 : 500	IF
β-actin	42	Mouse	Sigma -Aldrich	1 : 40.000	WB
pCREB	43	Rabbit	Millipore	1 : 1.000	IF
pERK1/2	42/44	Rabbit	Cell Signaling	1 : 5.000	WB
pMSK1	90	Rabbit	Cell Signaling	1 : 500	WB/IF
pP90RSK	90	Rabbit	Cell Signaling	1 : 1.000	WB/IF
Anti-rabbit Alexa Fluor 488 conjugate		Goat	Life Technologies	1 : 500	IF
Anti-mouse Alexa Fluor 568 conjugate		Goat	Life Technologies	1 : 500	IF
Anti- rabbit IgG, HRP-linked		Goat	Cell Signaling	1 : 30.000	WB
Anti- mouse IgG, HRP-linked		Goat	Santa Cruz	1 : 30.000	WB

PER1 shRNA Sequences	ORIGENE (Tube ID)	Species Specificity
CAGTGTAGCTTCAGCT-CCACCATCGTCCA	TG501619A / GI342009	Human, Mouse
TCCTACCAGCAGATCA-ACTGCCTGGACAG	TG501619B / GI342010	Human, Mouse
TGTCCGTCACCAGTCA-GTGTAGCTTCAGC	TG501619C / GI342012	Human, Mouse
AGGCAGAGAGCGTGGTG-TCCGTCACCAGT	TG501619D / GI520261	Mouse

Supplementary Figure 1.

PER1 significantly influences endogenous pCREB levels in mouse hippocampus.

Immunofluorescence images showing endogenous nuclear pCREB signal (green; Alexa Fluor 488) in WT and *Per1*$^{-/-}$-mice hippocampi. Mice were sacrificed at ZT02, thus, when endogenous pCREB levels in mouse hippocampus peaks (Rawashdeh et al., 2014). Images of different hippocampal regions (single plain) were acquired using a Zeiss Fluorescence microscope (Carl Zeiss, Oberkochen, Germany) with a 10X objective lens, and stitched together.

Figure 1

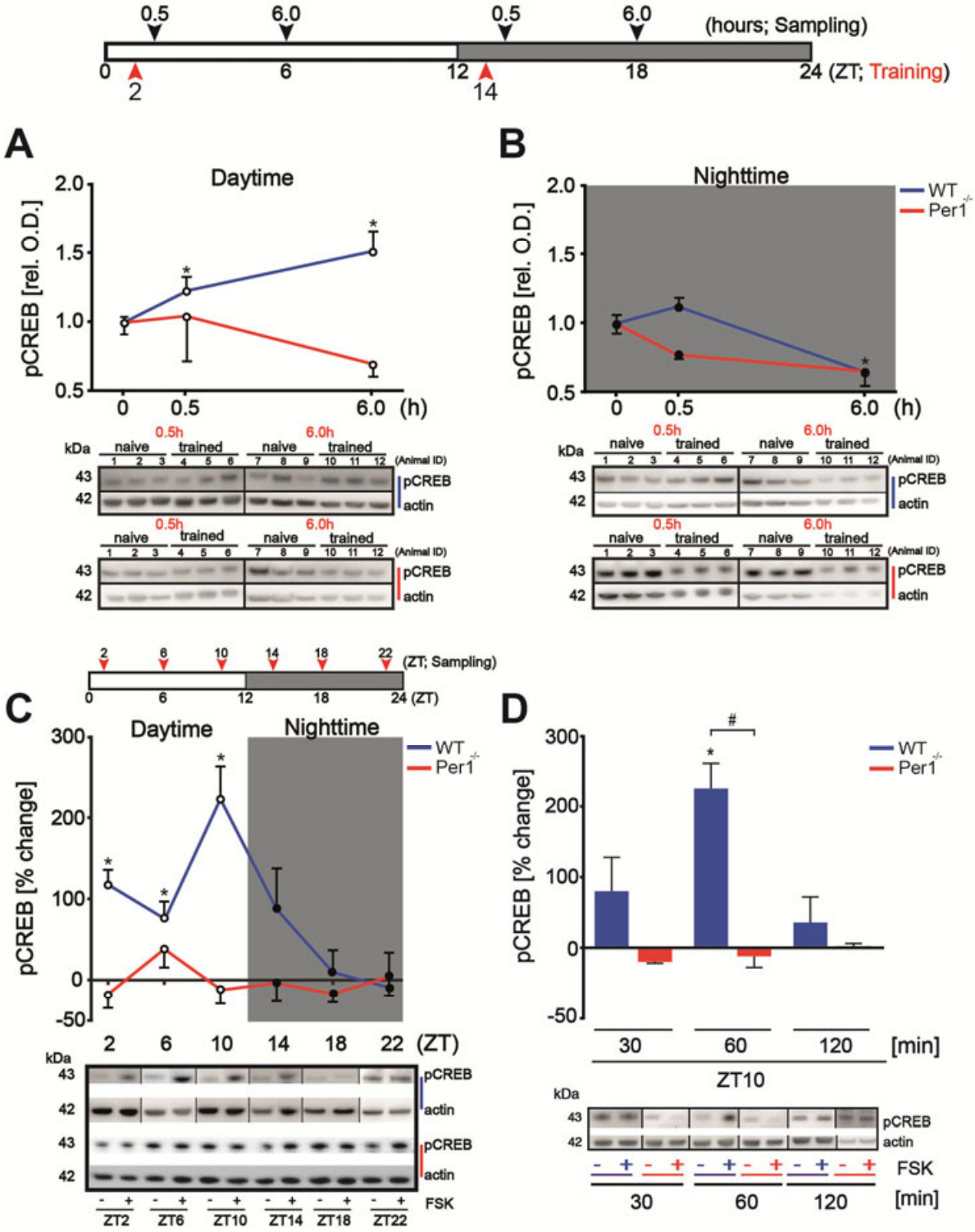

Figure 2

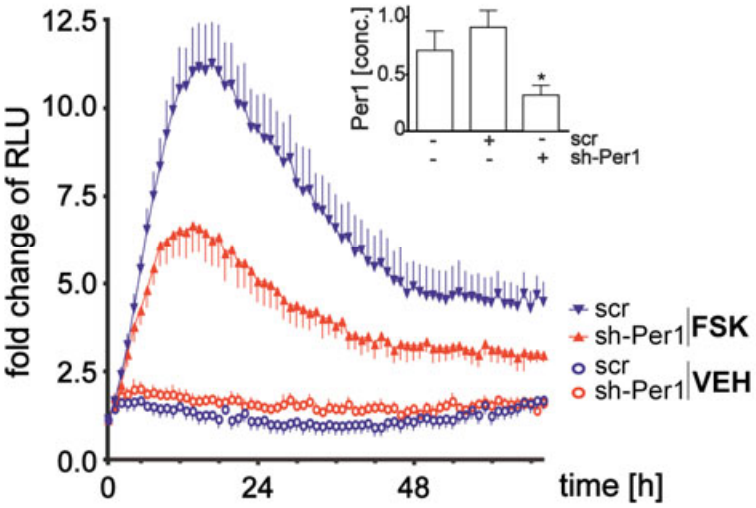

Figure 3

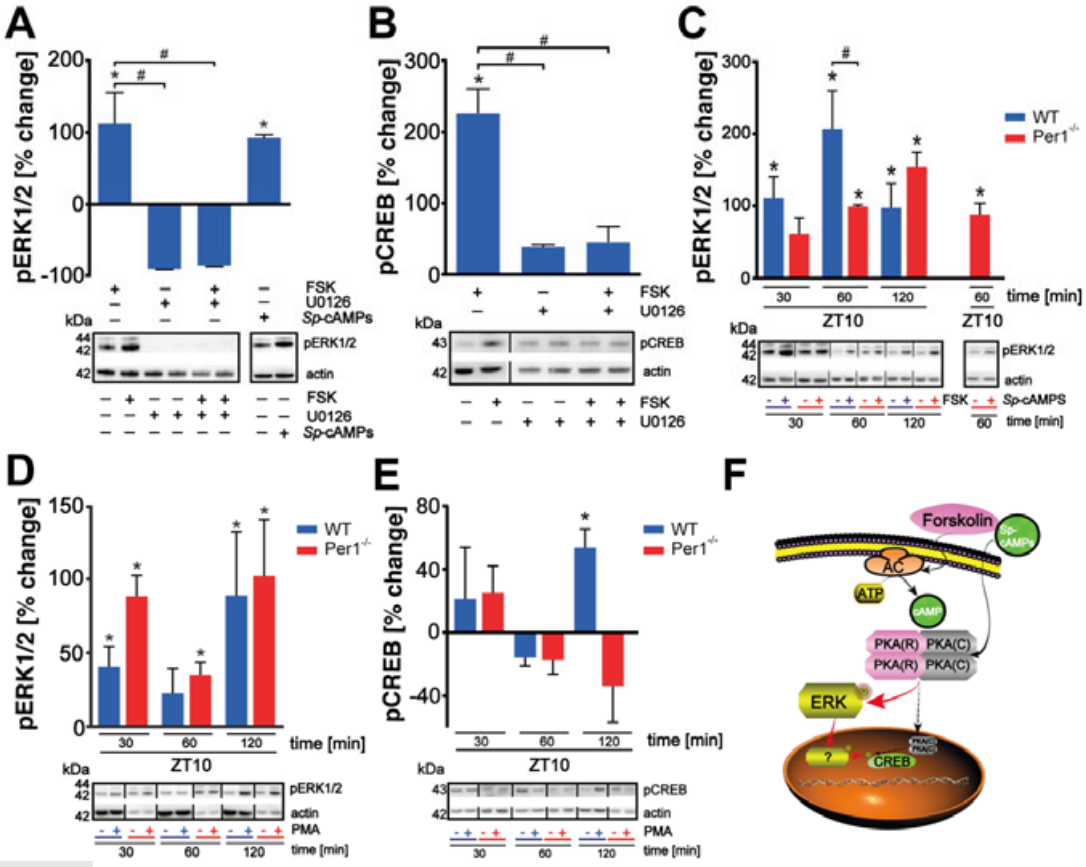

Figure 4

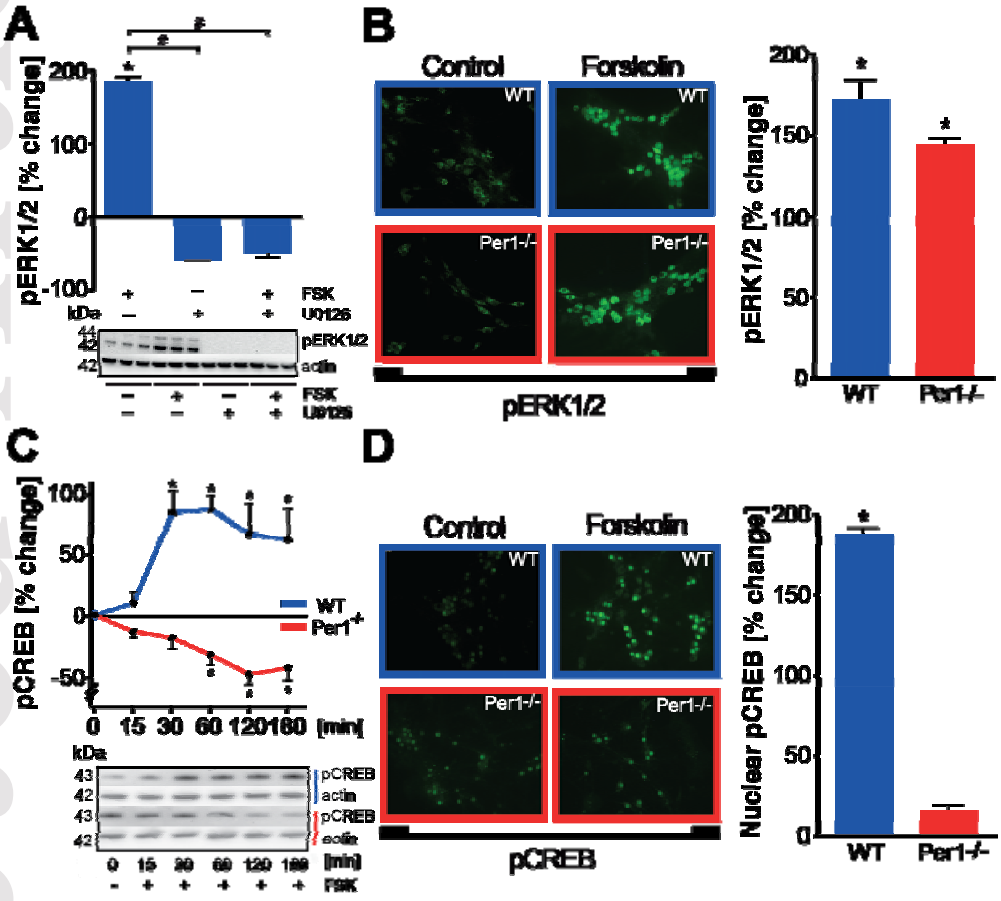

Figure 5

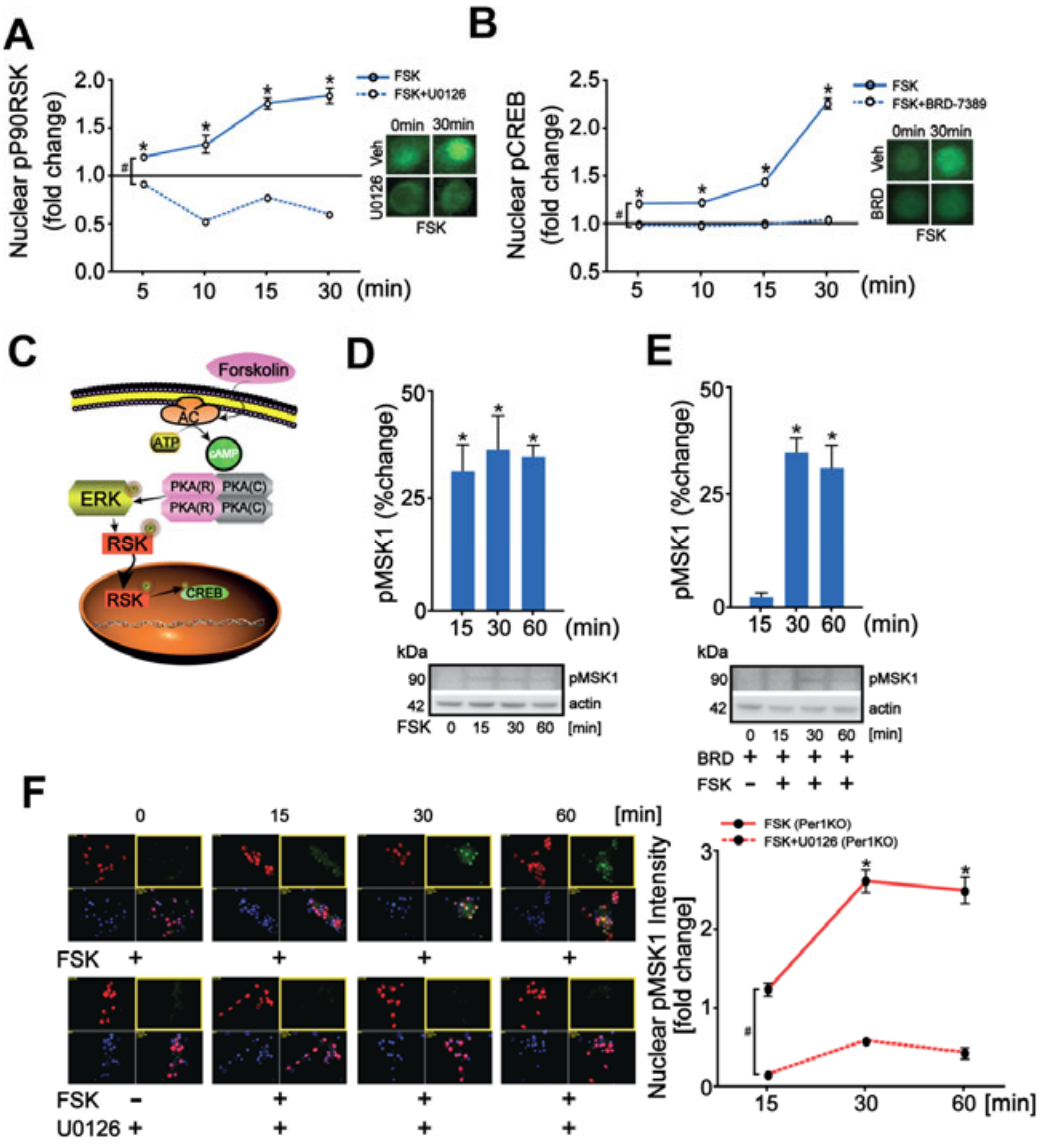

Figure 6

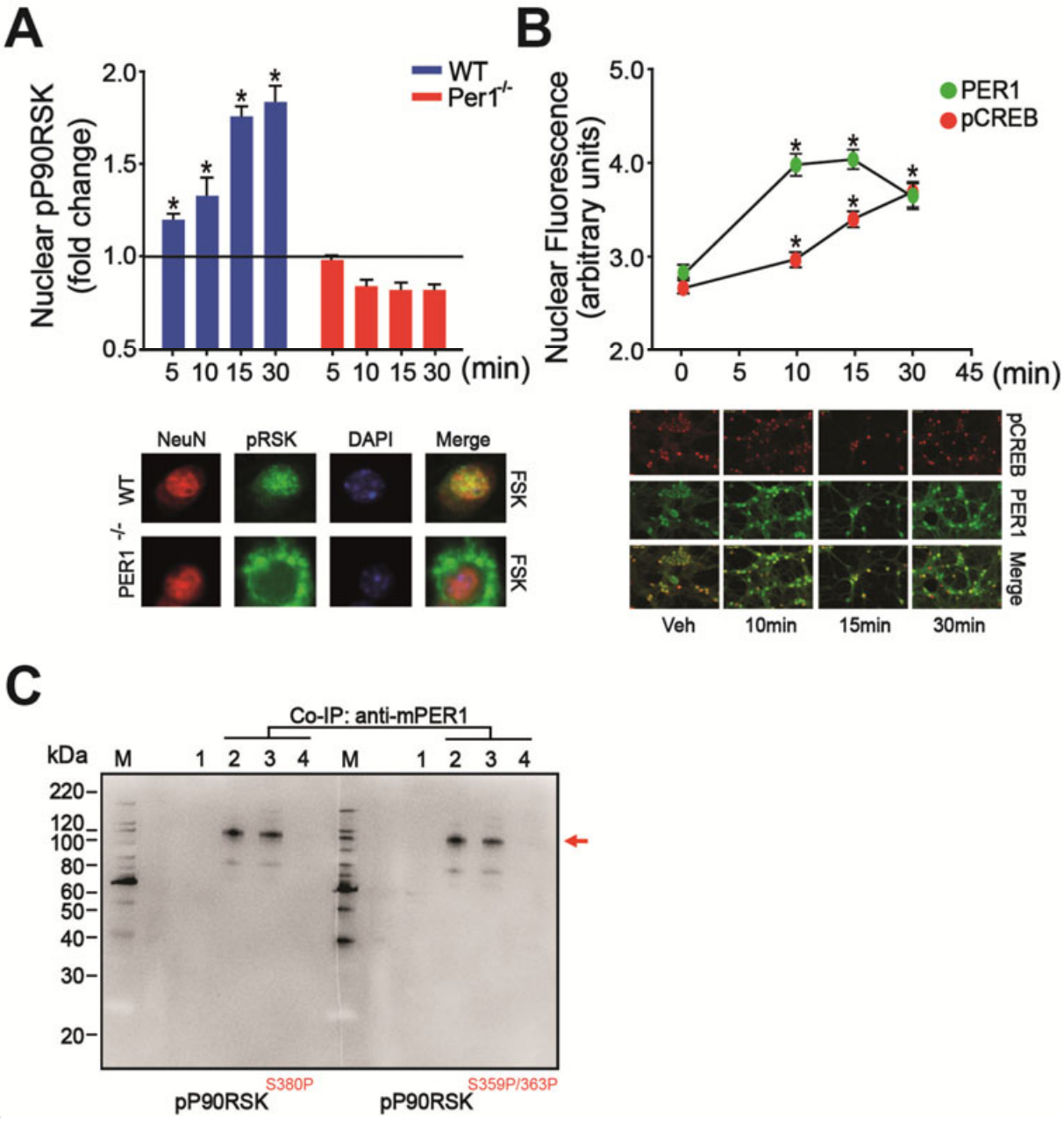

Figure 7