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Melatonin modulates daytime-dependent synaptic plasticity and learning efficiency

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Abstract

Mechanisms of hippocampus-related memory formation are time-of-day-dependent. While the circadian system and clock genes are related to timing of hippocampal mnemonic processes (acquisition, consolidation, and retrieval of long-term memory [LTM]) and long-term potentiation (LTP), little is known about temporal gating mechanisms. Here, the role of the neurohormone melatonin as a circadian time cue for hippocampal signaling and memory formation was investigated in C3H/He wildtype (WT) and melatonin-receptor-knockout ($MT_{1/2}^{-/-}$) mice. Immunohistochemical and immunoblot analyses revealed the presence of melatonin receptors on mouse hippocampal neurons. Temporal patterns of time-of-day-dependent clock gene protein levels were profoundly altered in $MT_{1/2}^{-/-}$ mice compared to WT animals. On the behavioral level, WT mice displayed better spatial learning efficiency during daytime as compared to nighttime. In contrast, high error scores were observed in $MT_{1/2}^{-/-}$ mice during both, daytime and nighttime acquisition. Day-night difference in LTP, as observed in WT mice, was absent in $MT_{1/2}^{-/-}$ mice and in WT animals, in which the sympathetic innervation of the pineal gland was surgically removed to erase rhythmic melatonin synthesis. In addition, treatment of melatonin-deficient C57BL/6 mice with melatonin at nighttime significantly improved their working memory performance at daytime. These results illustrate that melatonin shapes time-of-day-dependent learning efficiency in parallel to consolidating expression patterns of clock genes in the mouse hippocampus. Our data suggest that melatonin imprints a time cue on mouse hippocampal signaling and gene expression to foster better learning during daytime.

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Author's contribution: AJ, AS, AZ and JHS conceived the project. AJ, PB, AS, AR and AZ conducted experiments. JHS wrote the manuscript. MD, TL and GT gave critical input to the manuscript. All authors read and approved the final version of the manuscript.

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Keywords

LTP; CREB; clock gene; ganglionectomy; SCN; radial arm; behavior; circadian

Introduction

Almost every reoccurring aspect in physiology and behavior has a temporal facet that is of circadian (*circa*: about; *dies*: day) nature. Since the ability to anticipate and control circadian events represents a striking evolutionary advantage, endogenous clocks have evolved in parallel with life. Mechanistically, these circadian clocks constitute a transcriptional/posttranslational feedback loop[1]. In mammals, the expression of the clock genes *Period* (*Per1–3*) and *Cryptochrome* (*Cry1,2*) is driven by binding of homo/heterodimers of the clock gene protein products BMAL1 and CLOCK to E-box promoter elements on *Per* and *Cry* genes[1]. The circadian master oscillator, located in the hypothalamic suprachiasmatic nucleus (SCN)[2], adjusts remote, light-blind cells, tissues, or organs via neuronal and/or humoral output. Thereby, body functions are temporally coordinated and physiological incompatible events are separated within the omnipresent circadian environment.

Since 1973, learning and memory have been known to be modulated through time-of-day dependent mechanisms[3]. Ever since, the presence of a circadian rhythm in memory acquisition, consolidation and retrieval has been demonstrated in numerous species, ranging from invertebrates (*Aplysia*:[4]; fruit fly:[5]), to vertebrates (Zebrafish:[6]; rodents:[3,7]) including the human[8] (for review see:[9,10]). However, despite these widespread and common observations in circadian gating of memory formation, the mechanisms underlying time-of-day-dependent dynamics in learning efficiency are far from being understood.

In mammals, a central structure for long-term memory (LTM) formation is the hippocampus[11–14], with the plasticity of long-term-potentiation (LTP) mirroring hippocampal LTM[13,15]. It is well described that hippocampal learning efficiency depends on the time-of-day[10,16–19]. Notably, this temporal coordination depends on the integrity of the SCN[20] that shows a higher activity during daytime, independent of whether an animal is day- or night-active[21]. Despite this, in night-active laboratory mice, disparate findings for the time-of-day window for eased learning are reported[10]. This raises the question, if additional factors are involved in shaping the time-of-day dependent efficiency in memory formation.

Molecular mechanisms that efficiently drive and execute the encryption of memory traces into LTM are common and evolutionary highly conserved in both, fish and mammals[9]. In zebrafish, the pineal hormone melatonin, which is secreted exclusively during nighttime, is of critical importance for the circadian modulation in retention of LTM. Zebrafish display enhanced performance, if the task is acquired during daytime, coinciding with the animal's active phase[6]. Disrupting melatonin synthesis in zebrafish by pinealectomy, abolishes this rhythm in day-night learning[6].

In mammals, little is known about the role of melatonin within principal mechanisms of time-of-day-dependent modulation of LTM formation and/or retrieval. We therefore

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attempted to pinpoint the importance of circadian timing for learning efficiency and elucidate the role of melatonin in dynamic mnemonic processes in the hippocampus. Our experimental approach was based on previously published evidence that

- **i.** transcripts for melatonin receptors (MT_1 and MT_2) are present in rat hippocampus [22],
- **ii.** in rat brain, both, MT_1 and MT_2 antibodies labelled all hippocampal subfields [23],
- **iii.** day-night difference in LTP is absent in both, $MT_{1,2}^{-/-}$ mice [24], and melatonin-deficient C57BL/6 mice [25],
- **iv.** melatonin inhibits the cAMP-signaling pathway [16,26], notably also in the hippocampus [27], and (v) WT mice display a higher cocaine-induced conditioned place preference during daytime, than during nighttime, with the difference being abolished following pinealectomy [28].

Our results indicate that decoding of the nighttime melatonin signal is required for both, the maintenance of a proper phase-relationship in hippocampal signaling and for an efficient time-of-day-dependent gating of memory processing. Moreover, our data support that the circadian clock in the SCN gates time-of-day-dependent learning via the night-time-restricted impact of melatonin on hippocampal circuity.

Materials and methods

Animals.

All animal experiments were conducted as approved by the Policy on the Use of Animals in Neuroscience Research, the Policy on Ethics of the Society for Neuroscience, the Federal Guidelines and the European Communities Council Directive (89/609/EEC), and the local veterinary administration (approval file number: FU/1045). $MT_1^{-/-}$-, $MT_2^{-/-}$-, and $MT_{1/2}^{-/-}$-mice (all knockout mice were a kind gift from Dr. D.R. Weaver, Worcester, USA), and wildtype (WT) littermates were bred back onto a melatonin proficient C3H/He background[16]. C3H/He wildtype (WT) mice, C57BL/6 mice, and WT mice that were subjected to a bilateral surgical removal of their superior cervical ganglia (WT-SCGX) were purchased from Charles Rivers (Sulzbach, Germany).

The here additionally used C57BL/6 mice lack an appreciable endogenous melatonin rhythm[29,30], due to a nucleotide difference in an intron that creates a new splice acceptor, and incorporation of a pseudoexon, leading to a stop codon and truncation of the protein of the rate-limiting enzyme of the melatonin synthesis, arylalkylamine *N*-acetyltransferase (AANAT)[31]. However, MT receptors in C57BL/6 mice are functional as melatonin inhibits (i) hippocampal LTP[27], and (ii) CREB phosphorylation in the SCN[32], and also (iii) shifts the activity phase of this strain[33]. Thus, C57BL/6 mice endow a retained capacity to interpret the melatonin signal and were therefore used in some additional experiments in the presence of an artificially simulated nocturnal melatonin surge.

All animals were aged 8–12 weeks during experimentation. Mice were maintained under a standard 12:12 light/dark (LD) cycle, with 12 h light (daytime: 250 lux; onset [“ON”]

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defined as Zeitgeber Time [ZT] 0) and 12 h darkness (nighttime: dim red light <10 lux, >680 nm). Animals were kept under constant room temperature with food and water available ad libitum. Tissue sampling was carried out with animals sacrificed under deep anesthesia at ZT 2, 6, 8, 10, 12, 14, 18, and 22 (3–5 animals per time point, unless indicated otherwise), as previously described[18,34].

Immunohistochemistry.

Immunohistochemical analyses were performed in the mouse hippocampus and SCN as described previously[34]. In brief, deeply anesthetized mice (Ketamine 100 mg/kg, Xylazine 10 mg/kg) were flushed transcardially with saline, followed by perfusion with a paraformaldehyde solution (4% in 0.02 M phosphate buffered saline). Brains were post-fixed and sliced into 12 μm thick sections in the coronal plane on a freezing microtome. Sections were incubated overnight at 4°C with according antibodies (Tab.1). Immunoreactions were visualized with standard ExtrAvidin-biotin labelling method (Vector Laboratories, Peterborough, UK) using 0.05% 3.3´-diaminobenzidine. Analyses of clock gene protein levels were performed as described[34]. Briefly, cryo-protected brains were cut into 40-μm-thick sections in the coronal plane on a freezing microtome. To reduce nonspecific labeling in the immunohistochemical analyses, free floating sections were incubated for 1 h at room temperature in 0.01 M phosphate buffered saline, containing 0.3% triton, 1% bovine serum albumin, and 2% normal goat serum prior to an overnight incubation at 48°C with clock gene protein antibodies (Tab.1). Immunoreactions were visualized with a standard ExtrAvidin-biotin labeling method (Vector Laboratories, Peterborough, UK, USA), using 0.05% 3.3-diaminobenzidine, as described earlier[34].

For immunofluorescence analyses a protocol was adapted as described[18,19,34]. Briefly, tissue slices were pre-incubated for 1 h at room temperature in PBS, 5% NGS (Sigma), and primary antibodies were applied at 4°C for 24 h in PBS plus 5% normal goat serum. Alexa Fluor 488 goat anti-mouse secondary antibody was used at a 1:200 dilution (Molecular Probes, Göttingen, Germany; Tab.1). Adjacent sections, not treated with the primary antibody, were run for each animal in parallel. For immunofluorescence double labelling, sections were incubated overnight at 4°C in the appropriate antibody cocktail containing 1% bovine serum albumin, 0.1% Triton X-100 in 0.1 M PBS. After 3 washes in 0.1 M PBS, sections were incubated with Alexa 488- or 568-conjugated anti-mouse IgG (2 h, room temperature, in 0.1 M PBS), washed again, and incubated with Alexa 568- or 488-conjugated anti-rabbit IgG (2 h, room temperature, in 0.1 M PBS), respectively. After rinsing with 0.1 M PBS, the sections were mounted in fluorescent mounting medium (Dako, Hamburg, Germany). Fluorescent images were acquired using an Axio-Cam digital camera mounted on a Zeiss microscope (Carl Zeiss, Jena, Germany). Single fluorescent images of the same section were digitally superimposed.

For semiquantitative densitometric analyses of the immunoreactions, images were digitized with an Axiocam system (Zeiss, München, Germany; 1,030 × 1,030 pixel, 8-bit color depth), using NIH ImageJ software (Image Processing and Analysis in Java, developer Wayne Rasband), as described previously[18,19,34]. Briefly, background staining was defined as the lower threshold, and was kept constant for all sections processed with an antibody in a single

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experiment. The relative intensity of the nuclear and the cytoplasmic immunoreaction in the entire hippocampal formation was assessed separately as gray scale units above background. Staining was time- and genotype dependent (see Results). In addition, sub-regions of the hippocampal formation (CA1, CA3, and dentate gyrus [DG]) were selected and analyzed individually, as described[34], and the relative optical density (rel. O.D.) to background staining was measured within selected areas. In immunofluorescent images the corrected total cell fluorescence (CTCF = Integrated Density of cell ROI – [Area of ROI x Mean fluorescence background]) was measured[35,36]. Subsequently, values were averaged from three to four sections per animal.

Immunoblotting.

Western blots of extracts taken from excised mice hippocampi, or punched out SCN, respectively, were performed with slight modifications to a previous protocol[37]. Briefly, tissue samples 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 and separated electrophoretically 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 Semi-Dry Blotting System (Invitrogen, Carlsbad, USA). Prior to incubation with primary antibodies (Tab. 1), membranes were blocked with RotiBlock® (Roth, Karlsruhe, Germany) for 1 hour at room temperature. Subsequently, membranes were incubated with secondary antibodies (Tab. 1) for 1 hour at room temperature. Signals were detected using Immobilon Western Chemoluminescent HRP Substrate (Millipore, Billerica, USA), digitized using the ChemiDoc XRS System (BioRad, München, Germany) and analyzed using a luminescence system (Quantity One, ChemiDoc XRS, Bio-Rad, Hercules, CA, USA). The optical intensity of target signals on a given Western blot was 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.). In separate control experiments with MT antibodies, membranes were preincubated for 1hour with corresponding blocking peptides (Santa Cruz, Heidelberg, Germany).

Real-Time PCR.

Whole hippocampus samples and SCN tissue punches from WT, $MT_1^{-/-}$-, $MT_2^{-/-}$-, and $MT_{1/2}^{-/-}$-mice were rapidly isolated from deeply anaesthetized mice upon decapitation and frozen on dry ice. Subsequently, total RNA was extracted using minispin columns Absolutely RNA miniprep kit (Stratagene, La Jolla, USA) as described earlier[38], and amplified using primers for *mPer1*, *mPer2*, *mCry1*, *mCry2*, *mClock*, *mBmal1*, and *mHprt*, as described earlier[34]. Primers for MT_1- and MT_2- melatonin receptors were designed, according to reported mouse sequences (GeneBank accession number given in brackets):

mMtnr1a (MT_1) [NM_008639.2]

forward : 5'-CTC AAT GCC ACT CAG CA-3' (25–41)

reverse: 5'-GAG CTT CTT GTT GCG GTA-3'(183–166)

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mMtnr1b (MT_2) [NM_145712.2]

forward : 5'-ATC CCT AAC TGC TGT GA-3' (115–131)

reverse: 5'-AGC TTG CGG TTC CTG A-3' (308–293)

In brief, Real-Time PCR was performed using a LightCycler 1.5 (Roche Diagnostics GmbH, Mannheim, Germany) and a LightCycler FastStart DNA MasterPLUS SYBRGreen I kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the instructions of the manufacturer, and as described previously[34]. A 10 min initial denaturation step at 95°C was followed by 40 amplification cycles of 10 sec denaturation at 95°C, 10 sec of elongation at 60°C, and 10s elongation at 75°C. To ensure the specificity of the PCR amplicon, a temperature controlled melting curve analysis was performed at the end of the PCR reaction. As expected, each melting curve exhibited a single peak, corresponding to the expected specific amplification product. To confirm the specificity of the PCR reaction products were separated on ethidium bromide stained agarose gel (2% w/v). For technical reasons, clock gene mRNA expression could not be analyzed in $MT_{1/2}^{-/-}$ mice.

Behavioral analyses.

Locomotor activity of animals was analyzed routinely by an infrared sensor system, as described[34] (see technical description at www.infra-emotion.de). Accumulated averaged activity of a given animal was analyzed within 1 hour time bins.

An 8-arm radial maze task was used to test for day/night differences of a specialized form of short-term memory (STM), known as working memory[39], in addition to the long-term-memory (LTM) component of the reference memory. STM refers to a temporary storage of information from an animal's environment, to enhance performance in a given task in the near future. STM can be improved by repetitive exercise over several days, as measurable by the decreasing number of errors in successive trials. This improvement reflects a hippocampus-dependent learning process that results in the long-term storage of strategic information as a reference memory. Upon retrieval of the latter STM performance improves during task exposure[40].

To assess LTM performance, WT, $MT_1^{-/-}$, $MT_2^{-/-}$, $MT_{1/2}^{-/-}$, WT-SCGX, C57BL/6 and melatonin-treated C57BL/6 mice were trained in a food-baited 8-arm radial maze testing procedure, as described previously[34]. Briefly, food-deprived animals were trained for 5 consecutive days (1 trial/day), starting either at ZT2 or at ZT14, for daytime and nighttime training, respectively. A trial was completed, when the animal had eaten all rewards, or after 15 minutes, whichever came first. An entry to an arm was counted, when the mouse had entered with all four paws. An error was recorded, when an animal had either re-entered an arm it had visited previously, or if it did not eat the food pellet. Errors were plotted as percentage of the maximum error score. Experiments during the dark phase were performed under dim red light. Experimenters were blinded to the mouse`s genotype during the behavioral testing.

For the melatonin substitution experiments, C57BL/6 mice were treated with melatonin (10 mg/100 ml water; dose according to calculations elaborated in[41], added to their drinking

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water at nighttime only, starting 3 days before behavioral experiments and lasting throughout the food-rewarded radial arm maze test.

Anxiety was analyzed at ZT2 in the elevated plus test as described earlier[34,42], by measuring the time an animal spent in the open arm.

Mice can react extremely sensitive to changes of the handling persons, particularly to changes in odorant stimuli[43,44]. This may explain why the number of errors, particularly on the initial day of training differs between different experiments. In order to still be able to compare individual experiments, some of the obtained data are presented in a different context as percent of the maximal mean error value.

Electrophysiology.

Acute hippocampal slices were prepared 2 hours prior to LTP recordings at ZT2, or ZT14, respectively, according to previously described procedures[45,46]. The entire duration of the LTP experiment from slice preparation to the final recording lasted strictly between ZT12-ZT16, or ZT22-ZT2, respectively. In brief, concentric bipolar microelectrodes were used to stimulate the Schaffer collaterals at a frequency of 0.033 Hz. Stimulation was adjusted to elicit a fEPSP with a slope of ~40–50% of maximum for LTP recordings. After 20 minutes of baseline stimulation, LTP was induced by applying theta-burst stimulation (TBS), consisting of 4 pulses at 100 Hz per burst, which were repeated 10 times in a 200-ms interval (5 Hz). Three such trains served to induce LTP. Basic synaptic transmission and presynaptic properties were analyzed via input-output (IO) measurements and paired pulse facilitation by applying a pair of two stimuli at an interstimulus interval (ISI) of 40 ms. The IO measurements were performed by application of a defined value of current (0–100 μA in steps of 10 μA).

Statistics.

Statistical analyses were performed using OriginPro 7.5 SR4 (OriginLab Corporation, Northampton, MA, USA). Group means within each genotype were compared with One-way analysis of variance (ANOVA) with Bonferroni´s multiple comparison test, to estimate differences between examined time points. The impact of the genotype in the behavioral experiments was assessed by with a Two-way ANOVA with Bonferroni´s multiple comparison test. An unpaired t-test was performed to compare two different conditions at a distinct time point.

Results

MT receptor expression in the mouse hippocampus.

We initially analyzed MT receptor mRNA expression and MT receptor protein levels in mouse hippocampus (Fig. 1, Suppl. Fig. 1). MT_1 and MT_2 receptor transcripts could be demonstrated by RT-PCR (Suppl. Fig. 1 A,B), lining up to data previously obtained in the rat[22,47]. In addition, we show that with RT-PCR for the MT_1 or the MT_2 receptors, signals were absent in hippocampal extracts derived from $MT_1^{-/-}$ and $MT_2^{-/-}$ mice, respectively (Suppl. Fig. 1A,B).

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Notably, melatonin acts on the level of the expressed receptor proteins. During the course of experiments, 3 different MT receptor antibodies were used (Tab.1). Using antibodies from Santa Cruz, both MT receptors could be detected by immunofluorescence in hippocampal subfields of WT mice (Fig.1 A,C). Co-localization with the nuclear marker DAPI confirmed the presence of high-affinity G-protein-coupled MT receptors exclusively outside the nucleus, presumably on membranes of mouse hippocampal principal neurons (Fig.1 A,C). Signals were notably absent in $MT_{1,2}^{-/-}$ mice hippocampus (Fig. 1A,C). As these antibodies were discontinued, we had to switch during the course of experiments to antibodies purchased from Alomone (Tab. 1; Suppl. Fig. 2). While these antibodies gave a similar signal as obtained with Santa Cruz antibodies, they labelled next to hippocampal subfields also the meninges (Suppl. Fig. 2). The additionally seen positive signals in $MT_{1,2}^{-/-}$ mice hippocampus is explained by us and others to be due to the fact that these mice are functional knockout animals[48,49], with only a replacement of exon 1 of both the MTs. Exon 1 encodes the 5´untranslated region and the coding region through the first cytoplasmic loop of MTs. Exon 2 of both, the MT_1 and the MT_2, encode the rest of the coding region and the 3´untranslated region remained unaltered in here used $MT_{1,2}^{-/-}$ mice. Thus, it is possible that the expression of exon 2-based truncated MT receptor proteins, which notably have no biological activity[48,49], still leads to false positive immunohistochemical signals in the $MT_{1,2}^{-/-}$ mice, as the antibodies may be raised against a peptide, translated from this exon.

In addition, a MT_1 receptor antibody, previously validated in mouse retina[50], yielded a specific signal in WT mouse hippocampus. MT_1 receptor localized within the hippocampal formation in CA1, CA3 and the DG (Suppl. Fig. 1C). Co-localization with the neuronal marker MAP2 confirmed the presence of the MT receptors exclusively outside the nucleus on membranes of mouse hippocampal principal neurons. Notably, using this MT receptor antibody, signals were absent in $MT_{1/2}^{-/-}$ mice (Suppl. Fig. 1C).

Using the Santa Cruz MT receptor antibodies, Western blots of lysates derived from WT mouse hippocampus (Fig.1B,D, lane 1,3), revealed bands corresponding to the predicted molecular weights for MT_1 (Fig. 1B: about 37 kDa[47], indicated by arrows) and for MT_2 (Fig.1D: about 37 kDa[26], indicated by arrows). Bands of identical size as observed in hippocampal extracts were detected in a tissue, with reported high density of MTs, the SCN[51] (Fig. 1B,D, lane 2). No signals were detected in Western blots with MT_1 and MT_2 receptor antibodies when they were pre-incubated with the corresponding antigenic peptides (Fig. 1B,D, lane 4). With both MT receptor antibodies, additional sized bands were evident in Western blots. While these bands are possibly unspecific, the band sized 48 kDa may likely representing a glycosylated form of the receptor[52] (Fig. 1B,D, lanes 1–3). The reason for the very low background signals, present in the immunofluorescence images of $MT_{1/2}^{-/-}$ mice hippocampus (Fig. 1A,C) may be caused by unspecific binding of Santa Cruz MT receptor antibodies (see additional bands in Western blots in Fig. 1 B,D).

Dynamics in hippocampal clock gene expression in WT and $MT^{-/-}$ mice.

In WT mouse hippocampus and DG, the temporal patterns of clock gene protein levels for PER1, PER2, CRY2, CLOCK and BMAL1, and of corresponding clock gene mRNA expression levels, respectively, showed a highly dynamic time-of-day-dependent pattern

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(Fig. 2A,B; Fig. 3; Suppl. Fig. 3,4; Suppl. Tab. 1, 2) confirming our earlier data[34]. In $MT_{1/2}^{-/-}$ mice, clock gene protein levels showed remarkable differences in temporal dynamics, as compared to WT mice (Fig. 2 B,C; Suppl. Fig. 3,4; Suppl. Tab 1,2), with the notable exception of PER1 (Figs. 2,3, Suppl. Fig. 3).

PER2 expression in $MT_{1/2}^{-/-}$ mice was rhythmic, with a peak during the early day (ZT2) and a trough during the first half of the night (ZT14-ZT18), in contrast to a constant expression level of PER2 in WT animals (Figs. 2,3, Suppl. Fig. 3). The amplitude of CRY2 expression levels was significantly higher in $MT_{1/2}^{-/-}$ mice compared to WT mice where the peak expression phase advanced from early night (ZT14) to early day (ZT2) (Figs. 2,3, Suppl. Fig. 3). Likewise, the peak expression level of CLOCK protein was dampened and phase-advanced in $MT_{1/2}^{-/-}$ mice from mid-night (ZT18) to early-day (ZT4) as compared to WT animals (Figs. 2, 3, Suppl. Fig. 3). Rhythmic BMAL1 expression was greatly reduced in $MT_{1/2}^{-/-}$ mice, as compared to WT, with the expression peak shifted from mid-night (ZT18) in WT to early day (ZT2) in $MT_{1/2}^{-/-}$ mice (Figs. 2,3, Suppl. Fig. 3).

The comparison of immunohistochemical signal intensities in the hippocampus of WT, $MT_1^{-/-}$, $MT_2^{-/-}$-, and $MT_{1/2}^{-/-}$ mice showed no significant difference between hippocampal subregions by visual inspection (Suppl. Fig. 3). However, while overall rhythms in subregions showed the same time-phase relationship in all mice strains analysed, statistical analyses revealed some significant differences between subregions in WT (PER2: $P < 0.01$; CRY2: $P < 0.01$) and in $MT_{1/2}^{-/-}$ mice (PER2: 0.05; CRY2: $P < 0.01$; BMAL1: $P < 0.0001$) (see also Suppl. Tab. 10), which we account to subtle amplitude differences at individual time-points investigated.

Comparing expression of clock genes *mPer2*, *mCry2*, *mClock*, and *mBmal1* mRNA in mouse hippocampus revealed that the phase relationship for peak values was greatly altered when only one of the melatonin receptors was knocked out compared to patterns in WT animals ($MT_1^{-/-}$, $MT_2^{-/-}$; Suppl. Fig. 4). The notable exception was the clock gene *mPer1*, where transcriptional dynamics remained unaltered in $MT_1^{-/-}$ and $MT_2^{-/-}$ mice compared to WT animals (Suppl. Fig. 4). This indicates that both melatonin receptors may be likely involved in the time-of-day-dependent modulation of clock gene expression in mouse hippocampus.

Our analyses revealed peak and trough values for clock gene protein levels for PER1, PER2, CRY2, CLOCK, and BMAL1 in the SCN of WT mice as reported earlier[1,53,54] (Suppl. Fig. 5A). Dynamics in clock gene protein levels in $MT_{1/2}^{-/-}$ mice was similar to WT animals (Suppl. Fig. 5B). Transcription dynamics of clock gene mRNAs in WT mouse SCN (Suppl. Fig. 5C) was as reported earlier[1,53,54]. Notably, the day/night pattern in clock gene mRNA expression in the SCN of both $MT_1^{-/-}$ and $MT_2^{-/-}$ mice was identical to WT (Suppl. Fig. 5C; for technical reasons, clock gene mRNA expression could not be analyzed in the SCN of $MT_{1/2}^{-/-}$ mice).

In summary, the absence of melatonin receptor signaling is correlated with a major disturbance in patterns of clock gene mRNA expression and clock gene protein levels in the mouse hippocampus (Figs. 2,3, Suppl. Figs. 3,4; Suppl. Tab. 1,2). Notably, in mouse SCN

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the lack of MT receptors does not affect patterns of clock gene mRNA expression and clock gene protein levels (Suppl. Figs. 5).

Melatonin determines time-of-day-dependent magnitude in LTP.

Hippocampal LTP, a correlate of learning and memory[13,15,45] has a higher magnitude in WT mice at nighttime compared to daytime[25]. This day/night difference in synaptic weight is absent in melatonin-receptor deficient mice[27] and in the melatonin-deficient C57BL/6 strain[25]. We initially confirmed the fundamental presence of a day-night difference in LTP magnitude in WT mice (Fig. 4A; ZT2, LTP elevation 224±6.8%; ZT14: LTP elevation 161±15.7%) and its absence in $MT_{1/2}^{-/-}$ mice (Fig. 4B; ZT2, LTP elevation 167±10.6%; ZT14, LTP elevation 156±13.7%). Next, we measured LTP in WT-SCGX mice, which do not synthetize melatonin[55]. Successful SCGX treatment was confirmed by documentation of ptosis, miosis and enophthalmus (Suppl. Fig. 6). In WT-SCGX mice, the day-night difference in LTP magnitude between ZT2 and ZT14 recordings was abrogated (Fig. 4C; ZT2: LTP elevation 168±9.7%; ZT14: LTP elevation 188±4.8%). These observations underline a functional significance for melatonin in circadian synaptic weight in mouse hippocampus. There was no significant difference between ZT2 and ZT14 in input-output strength or paired pulse facilitation (Suppl. Fig. 7), suggesting that melatonin does not interfere with baseline synaptic function, but only with activity-dependent synaptic plasticity.

Endogenous CREB phosphorylation in the hippocampus of WT and $MT^{-/-}$ mice.

Semiquantitative densitometric analysis demonstrated increased CREB phosphorylation in WT mouse hippocampus and DG as the night progresses from trough levels at ZT 18 to reach peak values at midday (ZT06) (Fig. 5A,C, Suppl. Tab. 3; ZT2 vs ZT10, ZT14, $P < 0.05$; ZT2 vs ZT18, $P < 0.01$; ZT06 vs ZT10, ZT14: $P < 0.05$; ZT06 vs ZT18: $P < 0.01$; ZT18 vs ZT22: $P < 0.05$; 1-Way ANOVA with Bonferroni´s multiple comparison test), substantiating our previously published Western Blot data[18]. A distinct rhythm was present in hippocampal CREB phosphorylation of $MT_{1/2}^{-/-}$ mice (Fig. 5B,C; Suppl. Tab.3; ZT06 vs ZT10, ZT14, ZT18, $P < 0.05$, One-Way ANOVA), which differed significantly from the rhythm observed in WT animals (Suppl. Tab. 3; genotype: $P < 0.05$; time: $P < 0.0001$, Two-Way ANOVA). Hippocampal pCREB immunoreaction of C57/BL/6 mice was evidently diverged between ZT10 and ZT18 (Fig. 5C, Suppl. Tab. 3; $P < 0.05$; One-way ANOVA with Bonferroni´s multiple comparison test). Notably, in C57BL/6 mice peak levels in pCREB were phase delayed by 4 hours as compared to WT animals (Suppl. Fig. 8).

Melatonin impacts hippocampus-dependent learning.

In the hippocampus-dependent spatial learning test of the food-baited 8-arm radial maze test, WT animals acquired the task independently of whether they were trained during the day or during the night (Figs. 6,7 and Suppl. Tab. 4). In line with earlier studies[18,34], improved learning occurred during daytime (Fig. 6A). In all three MT receptor deficient mouse lines ($MT_1^{-/-}$, $MT_2^{-/-}$, $MT_{1/2}^{-/-}$, respectively; Fig. 6D; Suppl. Fig. 9; Suppl. Tabs. 5–7) as well as in melatonin-deficient C57BL/6 mice (Suppl. Fig. 10; Suppl. Tab. 8), daytime learning was severely compromised compared to WT mice. Of note, the performance of mouse strains tested in the radial maze did not depend on their overall activity since no differences

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were observed between animals when locomotor activity was analyzed in 1 hour time bins under the current experimental conditions (Suppl. Fig. 11).

WT mice.

The number of errors during daytime learning (ZT2) decreased in WT mice progressively from day 1 to day 5 of training (Fig. 6A; day 1 vs. days 2 and 3. $P < 0.01$; day 1 vs. days 4, 5: $P < 0.0001$). A significant difference in the radial arm reward task was evident between day 1 and day 5 of training within nighttime performance (Fig. 6A; $P < 0.01$; Suppl. Tab. 4) and between daytime and nighttime learning (Fig. 6A, Suppl. Tab. 4; $P < 0.05$; Two-Way ANOVA). In conclusion, memory consolidation in WT mice occurs during both, daytime and nighttime with better retrieval of reference memory into STM in WT mice during daytime training (Fig. 6A), which is in line with earlier reports[18,34].

$MT_{1/2}^{-/-}$ mice did not show any improvement in STM during nighttime testing throughout the 5 days of training sessions (Fig. 6D; Suppl. Tab. 7). In daytime performance, STM improvement was evident between day 1 and days 2–5 (in all cases $P < 0.05$; One-Way ANOVA with Bonferroni´s multiple comparison test). However, no further amelioration was evident after day 2 of training (Fig. 6D; Suppl. Tab. 7). Furthermore, no difference between daytime and nighttime learning was evident in $MT_{1/2}^{-/-}$ mice (Fig. 6 E,F) on day 3 and day 5 of learning. Remarkably, the number of errors observed in $MT_{1/2}^{-/-}$ mice at ZT2 and at ZT14 of training day 5 resembled results obtained in WT mice trained at ZT14 (compare Fig. 6A with 6D) and was significantly different from results obtained in WT mice trained at ZT2 (Fig. 6A; Suppl. Tab. 7).

In **$MT_1^{-/-}$ mice**, STM did not improve with training during daytime (Suppl. Fig. 9A, Suppl. Tab. 8). In contrast, during nighttime training, STM improved progressively (Suppl. Fig. 9A; day 1 vs. day 3: $P < 0.05$; day 1 vs day 4, 5: $P < 0.001$; day 2 vs. day 4, 5: $P < 0.05$; 1-Way ANOVA with Bonferroni´s multiple comparison test; Suppl. Tab. 5). While daytime learning was significantly altered from nighttime learning at day 1 and day 2 ($P < 0.0001$, $P < 0.05$, respectively; Two-way ANOVA; Suppl. Tab. 5), animals performed equivalent during day and night at training day 4 and 5 (Suppl. Fig. 9A; Suppl. Tab. 5).

In **$MT_2^{-/-}$ mice**, STM performance during daytime was significantly different between day 1 and day 4 of learning (Suppl. Fig. 9B; day 1 vs day 4: $P < 0.05$; One-Way ANOVA with Bonferroni´s multiple comparison test; Suppl. Tab. 6). For nighttime training, a significant increase in STM performance became apparent from the second day of training onwards (day 1 vs. day 2: $P < 0.001$; day 1 vs day 3–5: $P < 0.0001$; day 2 vs day 3: $P < 0.05$; day 2 vs day 4, 5: $P < 0.001$; One-Way ANOVA with Bonferroni´s multiple comparison test; Suppl. Fig. 9B; Suppl. Tab. 6). While daytime learning differed significantly from nighttime learning at day 1 ($P < 0.05$, Two-way ANOVA; Suppl. Tab. 6), no apparent difference in performance was observed after day 2 (Suppl. Fig. 9B; Suppl. Tab. 6).

C57BL/6 mice.

Learning during daytime did not improve in melatonin-deficient C57BL/6 mice (Suppl. Fig. 10; 1-Way ANOVA with Bonferroni´s multiple comparison test, Suppl. Tab. 8). Of note,

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after day 5 of training, the number of errors in C57BL/6 mice was similar to that of $MT_{1/2}^{-/-}$, and WT-SCGX mice (Fig. 7).

Melatonin-treated C57BL/6 mice.

In in C57BL/6 mice, which were treated with melatonin during nighttime, significantly improved daytime learning was witnessed in comparison to untreated animals, (Suppl. Fig. 10; $P < 0.05$, Two-Way ANOVA; Suppl. Tab. 8). On the final day of training, melatonin treated C57BL/6 mice showed similarly low number of errors as WT mice (Fig. 7).

In the elevated plus maze test conducted at ZT2, no significant differences were observed between WT, $MT_1^{-/-}$, $MT_2^{-/-}$ and $MT_{1/2}^{-/-}$ mice (Suppl. Tab. 9). Hence, we conclude that results obtained in the food-rewarded radial arm maze test are not associated with significant changes in anxiety-related behavior.

Taken together, these results show that on the final day of training, no day/night differences in learning were seen in all three melatonin receptor-deficient mouse lines ($MT_1^{-/-}$, $MT_2^{-/-}$, $MT_{1/2}^{-/-}$), as well as in WT-SCGX mice and in C57BL/6 mice. Notably, $MT_{1/2}^{-/-}$, WT-SCGX and C57BL/6 mice made significantly more errors compared to intact melatonin-proficient WT and compared to melatonin-treated C57BL/6 mice (Fig. 7).

Discussion

We tested the effects of melatonin on time-of-day-dependent synaptic plasticity and learning. Our results suggest that melatonin receptor signaling shapes time-of-day-dependent learning efficiency and affects underlying events such as hippocampal gene transcription and CREB phosphorylation, which are associated with learning.

In particular, we show that

- **i.** MT_1 and MT_2 melatonin receptors are expressed in WT mouse brain structures that are critically involved in learning and memory,
- **ii.** the rhythmic expression of clock genes in WT hippocampus and in the DG is greatly altered in $MT_{1/2}^{-/-}$ mice,
- **iii.** day/night difference in hippocampal LTP in WT mice is absent in $MT_{1/2}^{-/-}$ and in WT-SCGX animals,
- **iv.** time-of-day-dependent deflections in hippocampal pCREB levels are significantly different in the absence of a melatonin signal,
- **v.** day/night difference in learning in WT animals is absent in $MT_{1/2}^{-/-}$ mice and in WT-SCGX mice,
- **vi.** substitution of melatonin in melatonin-deficient C57BL/6 mice improves daytime learning.

On the basis of our data, we hypothesize that melatonin influences signaling events in hippocampal cells, which underlie mechanisms of time-of-day-dependent learning efficiency in mice.

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i. Various specific effects of melatonin on hippocampal signaling have been described in rodents in the past [27,56–58], suggesting a fundamental role of MTs in the hippocampus. While ^{125}I-melatonin receptorautoradiography did not detect melatonin binding sites in the hippocampus of various species [51,59,60], MT receptor mRNA has been detected in rat hippocampus [22,61]. Using different MT-antibodies and sensitive immunofluorescence, we show the presence of MTs on WT mouse hippocampal cells, confirming earlier data obtained in the rat [23]. These data can potentially provide a structural basis for the effects of melatonin on hippocampal signaling and learning as outlined below.

ii. Clock gene mRNA expression and clock gene protein levels become greatly disturbed in mouse hippocampus with the inability to interpret the nocturnal melatonin message in $MT_{1/2}^{-/-}$ mice compared to WT animals. On the contrary, neither clock gene protein levels in the SCN, nor SCN-driven day/night activity patterns of locomotor activity (Suppl. Fig. 11) were different in $MT_{1/2}^{-/-}$ animals compared to WT animals. This indicates a direct mechanistic impact of melatonin on hippocampal cells via both G-protein-coupled melatonin receptors, the MT_1 and the MT_2 receptor, respectively. Interestingly, it was shown in the Siberian hamster that melatonin can rapidly increase *mPer1* and *mBmal1* mRNA expression [62], despite lacking a functional MT2 receptor [63]. How hippocampal cells dissociate between the earlier shown distinct MT_2-mediated effect on LTP [27], and MT_1-mediated effects on hippocampal clock gene expression and learning, is yet unknown. To this end, a rhythmic and temporally coordinated clock gene expression was reported to be indispensable for proper hippocampal functioning [18–20,34].

The suggested possible link between time-of-day-dependent learning efficiency, clock genes and melatonin is greatly substantiated by our findings that the altered day/night pattern in clock gene protein levels in the hippocampus of $MT_{1/2}^{-/-}$ mice is correlated to deficits in memory processing. More specifically, these data show that it is not only the presence (or absence) of a given clock gene that is important for a coordinated cognitive performance, but also the overall phase relation of the rhythmic clock gene protein levels, i.e. the phase of peaks and troughs. This work shows a 12 hour phase-shift in peak values for PER2, CRY2, CLOCK, and BMAL1 into the daytime period in $MT_{1/2}^{-/-}$ mice, annihilating at the same time differences between day- and nighttime performances in the radial arm maze.

We therefore hypothesize that the temporally restricted impact of melatonin on hippocampal signaling during nighttime functions as a temporal conductor for rhythmic clock gene expression, which in turn is a prerequisite for the improved learning efficiency during daytime as compared to nighttime (Fig. 8).

iii. WT mice exhibited a clear day/night difference in LTP. Melatonin profile in C3H mice rises during the second half of the dark period, peaks prior to the end of the dark period and then drops rapidly to low daytime values [30,64]. As melatonin can inflict on LTP [27], it is likely that LTP levels alter dependent on the relative time

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of slice preparation and recordings in relation to the melatonin cycle. Still, independent of experimental design, day/night differences in hippocampal excitability vanished in $MT_{1/2}^{-/-}$ mice as shown in previous reports [27] and confirmed in the present study. Our novel data from WT-SCGX mice provide additional supportive evidence that melatonin is involved in the time-of-day-dependent variations in LTP magnitude in the mouse hippocampus.

iv. Phosphorylation of the 'memory molecule' CREB is a critical element for efficient memory formation [11,15,65–67]. Time-of-day-dependent fluctuations in CREB phosphorylation are directly linked to cycling memory reconsolidation and maintenance [17,19]. The fact that the endogenous rhythm in CREB phosphorylation in $MT_{1/2}^{-/-}$ mice hippocampus remained almost unaltered in phase compared to the rhythm in WT animals implies that melatonin does not directly affect the time when this important transcription factor for memory formation is phosphorylated. However, the overall amplitude of the pCREB rhythm in $MT_{1/2}^{-/-}$ mice is significantly dampened as compared to WT animals. It is well known that melatonin acutely inhibits cAMP accumulation [68] and CREB phosphorylation [16,69]. As an additional effect, chronic melatonin exposure (>4h) leads to a time-dependent sensitization of the adenylyl cyclase in pars tuberalis cells [16,68,69]. In WT mouse hippocampus, CREB phosphorylation rises as the nocturnally elevated melatonin surge progresses, reaching peak values only several hours after the onset of daytime. Consequently, the persistent nocturnal melatonin impact on mouse hippocampal cells may lead - likewise as observed in pars tuberalis cells [16] - to a sensitization of the adenylyl cyclase. We hypothesize that the less prominent pCREB rhythm in $MT_{1/2}^{-/-}$ mice compared to WT animals may be the reason for a weakened memory-related gene expression: in WT animals, pCREB-induced hippocampal gene expression would be enhanced by the melatonin-initiated sensitization of the adenylyl cyclase, exactly at times, when animals learn better, i.e. during daytime.

In C57BL/6 mice, the pCREB rhythm is phase-advanced by approximately 4 hours compared to WT and $MT_{1/2}^{-/-}$ mice. As C57BL/6 mice do not produce considerable amounts of melatonin [30,64], the suggested sensitization of hippocampal adenylyl cyclase by melatonin may not take place, which potentially explains why spatial learning during daytime is poor in C57BL/6 mice. A likewise explanation may be valid for low daytime learning in $MT_{1/2}^{-/-}$ and in WT-SCGX mice. Our suggestion is supported by the finding that substitution of melatonin to C57BL/6 mice significantly improved learning, up to the level seen in WT animals (Fig. 7).

v. Several reports demonstrated that performance of mice in acquisition, recall, and extinction of hippocampus-based memory is better during daytime than nighttime, even under conditions of constant darkness [18,70] (for review see: [10]). The rhythm in hippocampal spatial learning was severely affected in $MT_{1/2}^{-/-}$ mice as they lack the WT-specific improvement of memory formation during daytime. Both MT receptors are involved in this effect, as already mice with a

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knockout of only a single MT receptor, the $MT_1^{-/-}$ or the $MT_2^{-/-}$, show compromised daytime learning.

When training was performed during daytime (at ZT2), no significant amelioration in STM was observed over the 5 consecutive days of testing in C57BL/6 mice. This behavior was similar to the daytime memory performance observed in $MT_{1/2}^{-/-}$ mice. However, when C57BL/6 mice were treated with melatonin during nighttime, daytime learning was significantly improved. Since C57BL/6 mice lack melatonin and therefore also N-acetylserotonin (NAS) but behaved similar to NAS-proficient $MT_{1/2}^{-/-}$ mice, this observed effect of the pineal hormone is not related to the melatonin precursor NAS, which is known to enhance cognition via a circadian activation of TrkB receptors[71]. Moreover, diminished learning in C57BL/6 mice was rescued by the sole substitution of melatonin and was therefore independent of NAS.

It still has to be delineated, if melatonin unfolds its impact on memory processes in mice by inhibiting STM during the second half of the night or if the hormone promotes better retrieval during daytime, or possibly both. It may well be that better learning during daytime in night-active animals is linked to the well-documented enhanced memory consolidation during sleep[72,73]. Sleep is characterized by cyclic occurrence of rapid-eye-movement (REM) and non-REM sleep. The latter includes slow-wave sleep (SWS). SWS prevails in the first part of the night (early sleep), whereas REM sleep dominates the second half (late sleep), which is critical for memory consolidation[74,75]. As long as REM sleep can be established, learning can even be rescued from interference[76]. Thus, interference with the REM phase will likely lead to declined learning. Reiteration during the early night on the other hand can improve learning, possibly via use- or experience-dependent processes or enhancement[72,73]. In addition, better learning during daytime in night-active animals is also linked to a temporally gated role of PER1 within memory formation[18,19,34].

Across phylogeny the achievement of an optimal cognitive performance is vital for survival. Within the molecular mechanisms that regulate the circadian nature of memory formation, circadian clock gene expression[18,34] and the MAPK/pCREB cascade[17,19] are central players in mouse hippocampus. Here, we add melatonin to these modulatory impacts on hippocampal circuity (Fig. 8). The pineal hormone seems to affect hippocampal gene expression, LTP and time-of-day-dependent differences in memory performance. In an evolutionary conserved manner, melatonin seems to be involved in mental performance in mice, similar to Zebrafish[6]. Clarifying the role of melatonin within hippocampus-dependent learning and memory will foster our understanding of circadian rhythm organization in cognition. Lastly, it was shown that disturbance of the hippocampal circuity severely affects sleep and cognition in fish[77] and in humans[78]. In the light of an increased awareness of the link between sleep-wake disturbances in a 24-h society and cognitive and neurodegenerative disorders in humans[9,79], a better understanding of how the circadian clock modulates hippocampal circuity and the role of melatonin within this mechanism have great merit.

Supplementary Material

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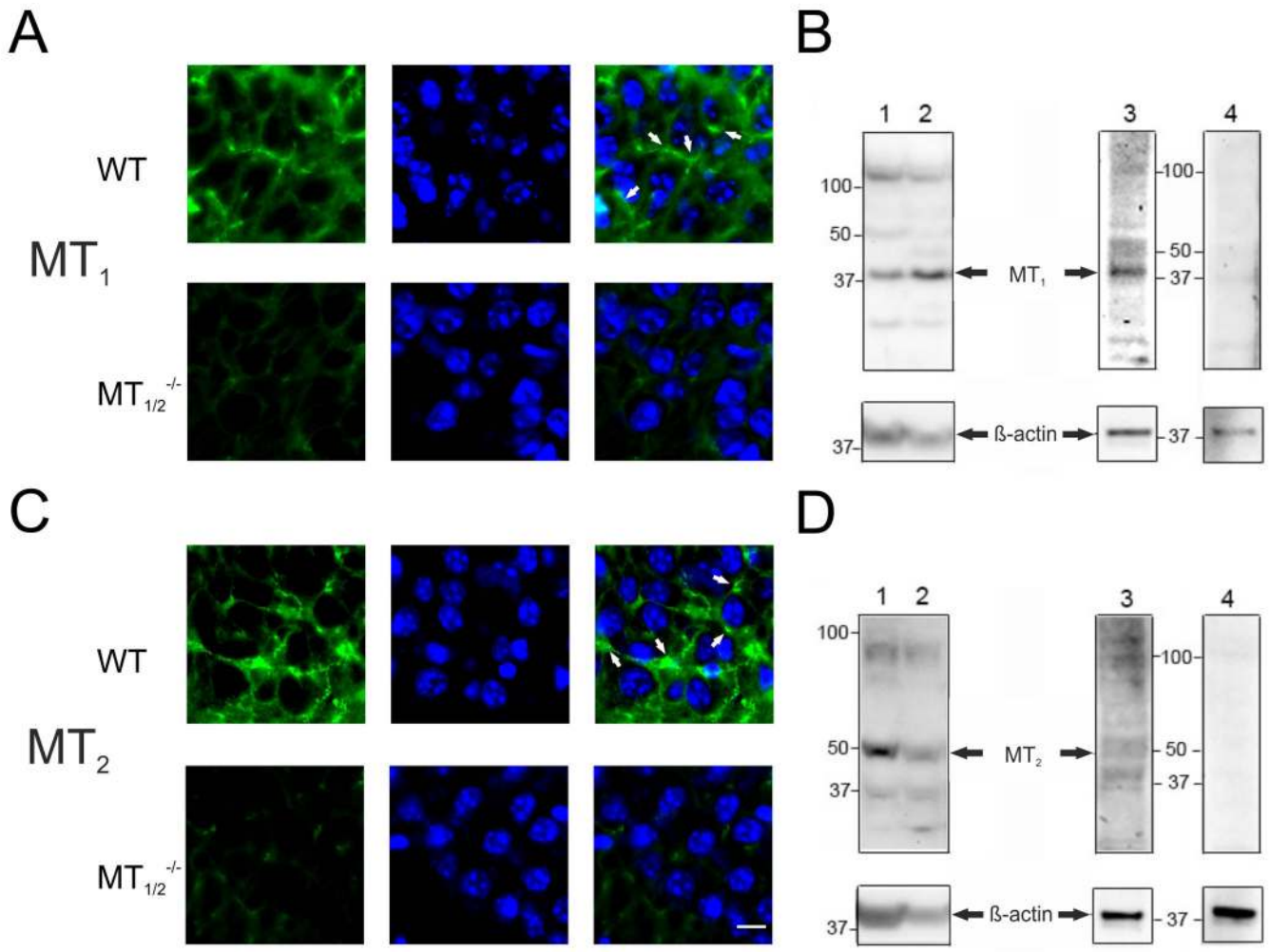

Fig. 1:

Melatonin-receptors expression in the hippocampus of WT mice. Representative immunohistochemical staining for melatonin-receptors (green, left column) and DAPI (blue, middle column) and merged images (right column: white arrows exemplify the membranous MT receptor signal) in the hippocampus of WT (A) and $MT_{1/2}^{-/-}$ (C) mice. Western blots (B, D) show the expression of melatonin-receptor proteins in the mouse hippocampus (lanes 1,3) and SCN (lanes 2). The arrows point to the protein bands corresponding to the size of MT_1 (B: ~37kDa), or MT_2 (D: ~37kDa). The prominent band, sized ~48 kDa may be the glycosylated form of the receptor[52]. Signals in hippocampal extracts were abolished upon preincubation of MT antibodies with corresponding antigenic peptides (lanes 4). β-actin served as loading control. Scale bar in lower right picture: 10 μm. Used antibodies SC13186 and SC13177 (see Tab. 1).

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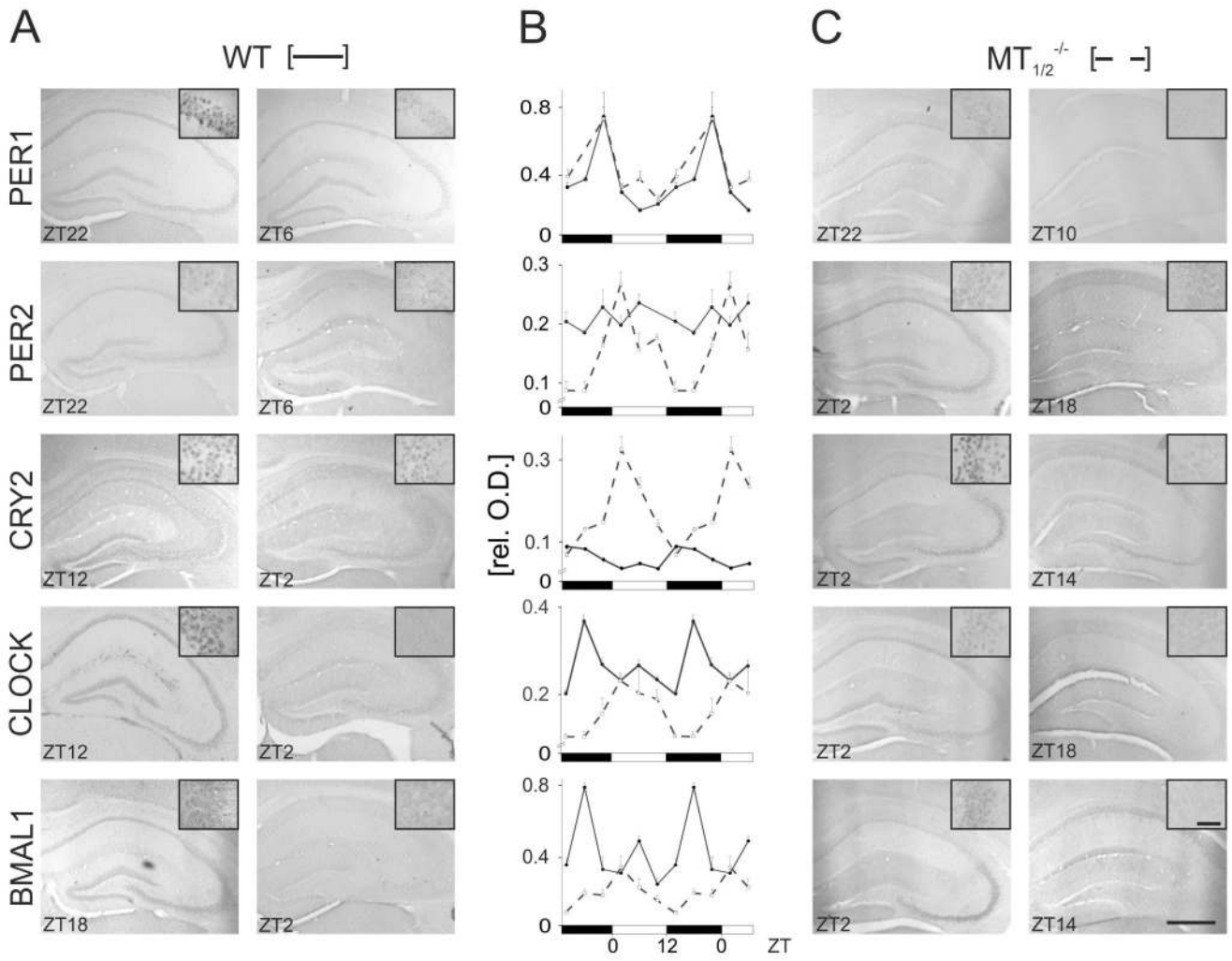

Fig. 2:

Clock-gene protein levels in the hippocampus of WT and $MT_{1/2}^{-/-}$ mice. Representative immunohistochemical images of peak (left columns) and trough (right columns) clock protein expression in coronal hippocampal sections for both, WT (A) and $MT_{1/2}^{-/-}$ mice (C). Time points are indicated as ZT values. Insets are exemplary images from the CA3 region taken with a higher magnification. Scale bars in lower right picture: 200 μm, in inset 10 μm. (B) Semiquantitative densitometric analysis of immunohistochemical signals compiled form DG, CA1 and CA3 region, expressed as mean relative optical densities (rel. O.D.) of all three regions together for the time-of-day-dependent levels of clock gene proteins, PER1, PER2, CRY2, CLOCK, and BMAL1, in the hippocampus of WT (●, solid lines) and $MT_{1/2}^{-/-}$ mice (□, dashed lines). Values (n = 3/group with 3 sections/animal) are expressed as mean ± SEM. For significances see text and Suppl. Tabs. 1–3. Data in B are double-plotted against Zeitgeber time (ZT) for clarity reasons.

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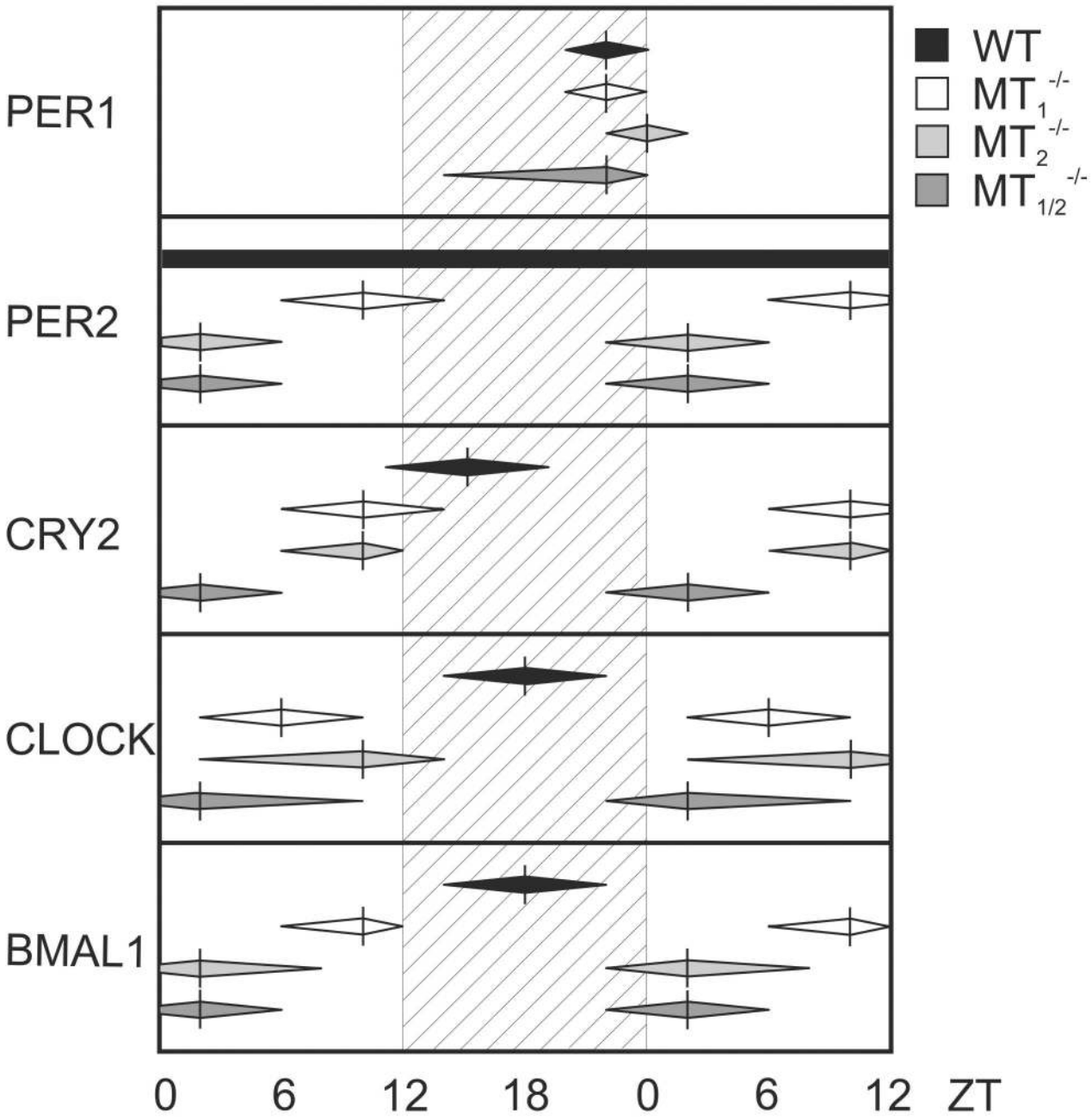

Fig. 3:

Schematic overview of the phase-relationship of peak expression of clock gene proteins in the hippocampus of WT, $MT_1^{-/-}$, $MT_2^{-/-}$, and $MT_{1/2}^{-/-}$ mice. Vertical bars represent times of peak levels, with approximate times of the rise to, and the decline from maximal clock gene protein levels estimated by eye fitting on the basis of the original data sets and indicated by the horizontal extend of the deltoids.

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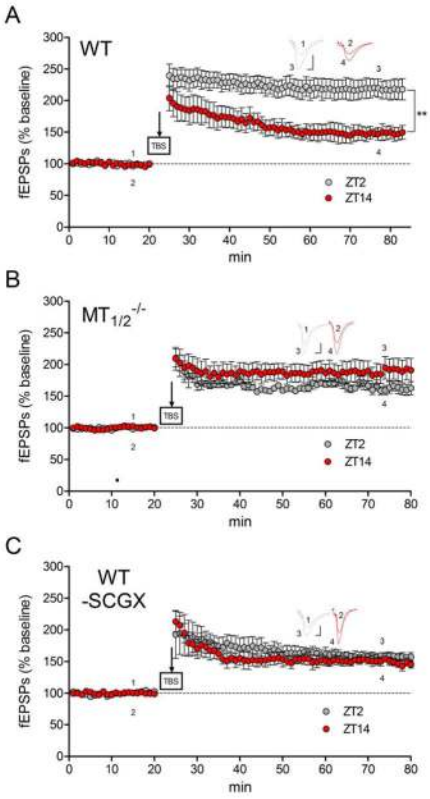

Fig. 4:

Daytime-dependent hippocampal synaptic plasticity in WT, $MT_{1/2}^{-/-}$ and WT-SCGX mice. Long-term potentiation (LTP) was induced in mouse hippocampal brain slices at ZT2 (gray circles) and ZT14 (red circles). (A) In WT mice, a significant difference was observed between ZT2 (n=8) and ZT14 (n=6) (**= p=0.038; t-test). This change was abolished in $MT_{1/2}^{-/-}$ mice (B; p ≥0.05; t-test; n=3 at ZT2 and ZT14, respectively) and in WT-SCGX mice (C; p ≥0.05; t-test; n=3 at ZT2 and ZT14, respectively). Insets in A-C show representative field potential waveforms, indicated by numbers at the respective time points, scale bars: 0.5mV, 5ms.

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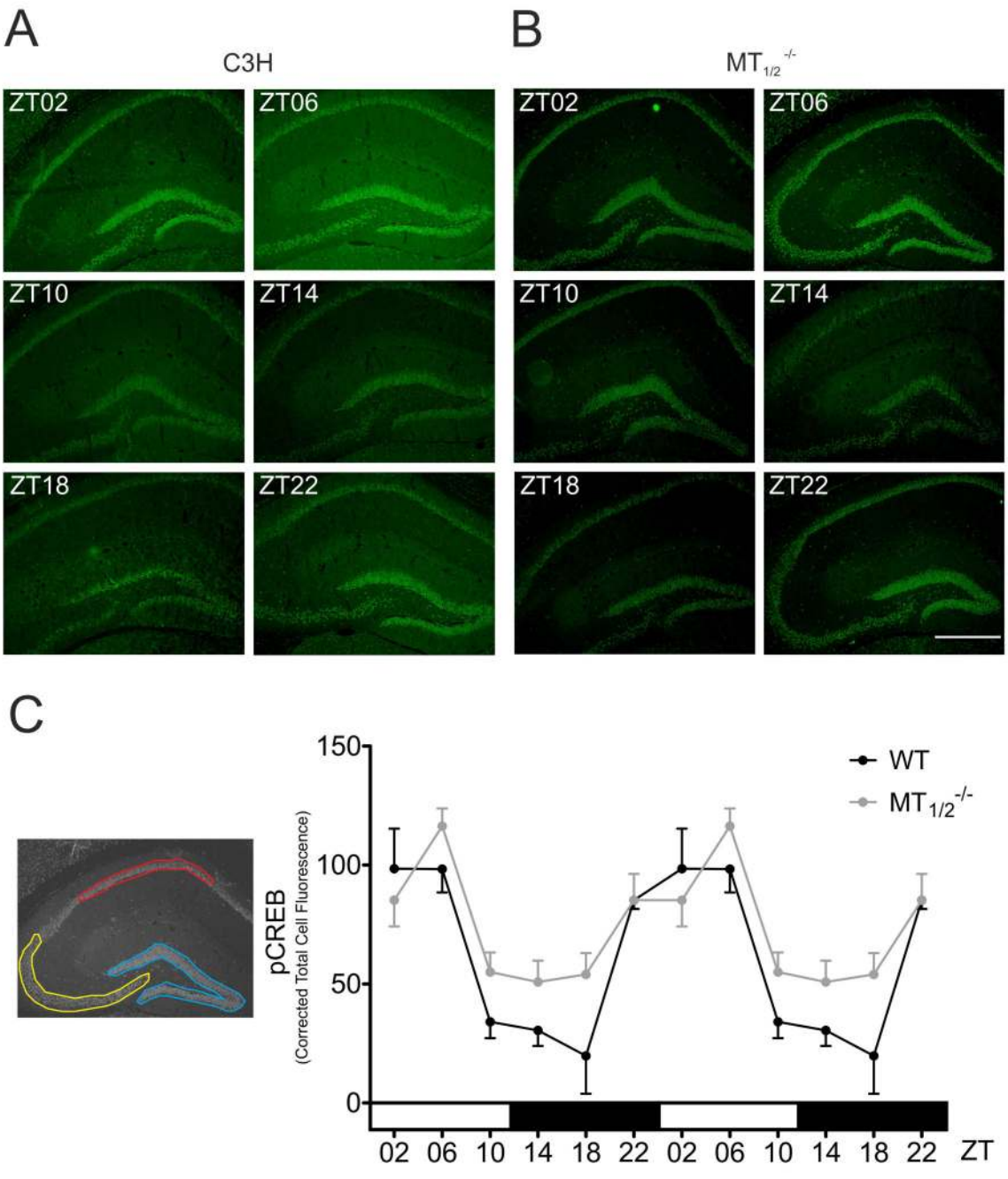

Fig. 5:

Time-of-day-dependent CREB phosphorylation in mouse hippocampus. Representative immunohistochemical images of pCREB signal in the hippocampus of WT (A) and $MT_{1/2}^{-/-}$ mice (B) at indicated ZTs. Scale bar: 500μm. (C) Semiquantitative densitometric analysis of immunohistochemical signals pooled over hippocampal subregions DG (blue), CA1 (red) and CA3 (yellow), expressed as Corrected Total Cell Fluorescence (CTCF) in WT and $MT_{1/2}^{-/-}$ (all n = 3) mice. Both genotypes show a significant time-of-day-dependent rhythm in CREB phosphorylation (WT: $P \leq 0.001$; $MT_{1/2}^{-/-}$: $P \leq 0.01$) and are

significantly different to each other (*: P<0.05, Two-way ANOVA). Values are expressed as mean ± SEM. For clarity reasons, data are double-blotted against Zeitgeber time (ZT).

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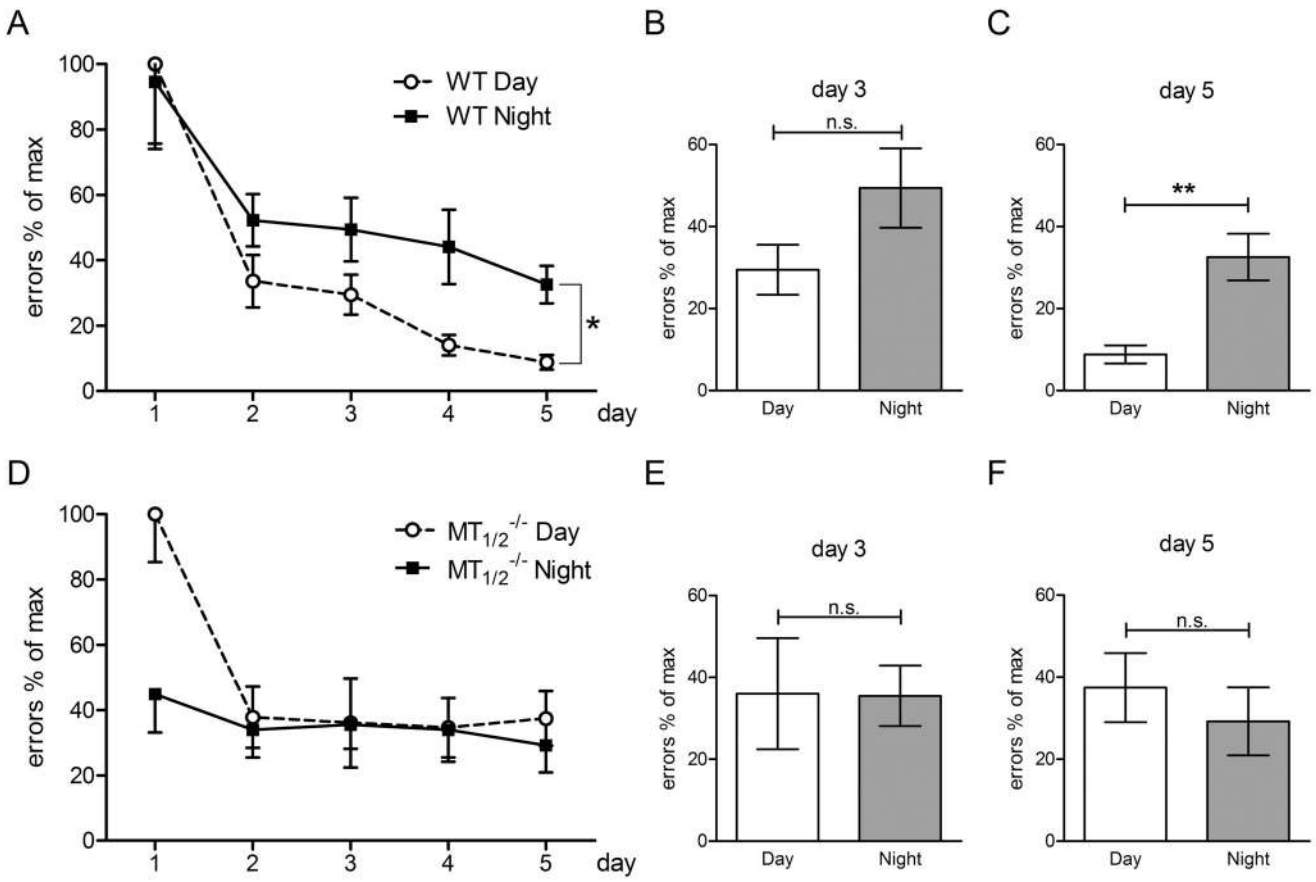

Fig. 6:

Spatial learning ability of WT (A, B, C) and $MT_{1/2}^{-/-}$ (D, E ,F) mice. Illustrated is the number of errors (mean ± SEM) of WT (A) and $MT_{1/2}^{-/-}$ mice (B) in the 8-arm radial maze test over 5 consecutive days, tested during daytime (Day) and nighttime (Night)(WT n = 9; $MT_{1/2}^{-/-}$ n = 9; *= P ≤0.05 Two-way ANOVA). Comparison of the radial arm maze performance between daytime and nighttime at day 3 and day 5 of WT (B, C) and $MT_{1/2}^{-/-}$ (E, F) mice (** = P ≤0.01; t-test unpaired). Values are expressed as mean ± SEM.

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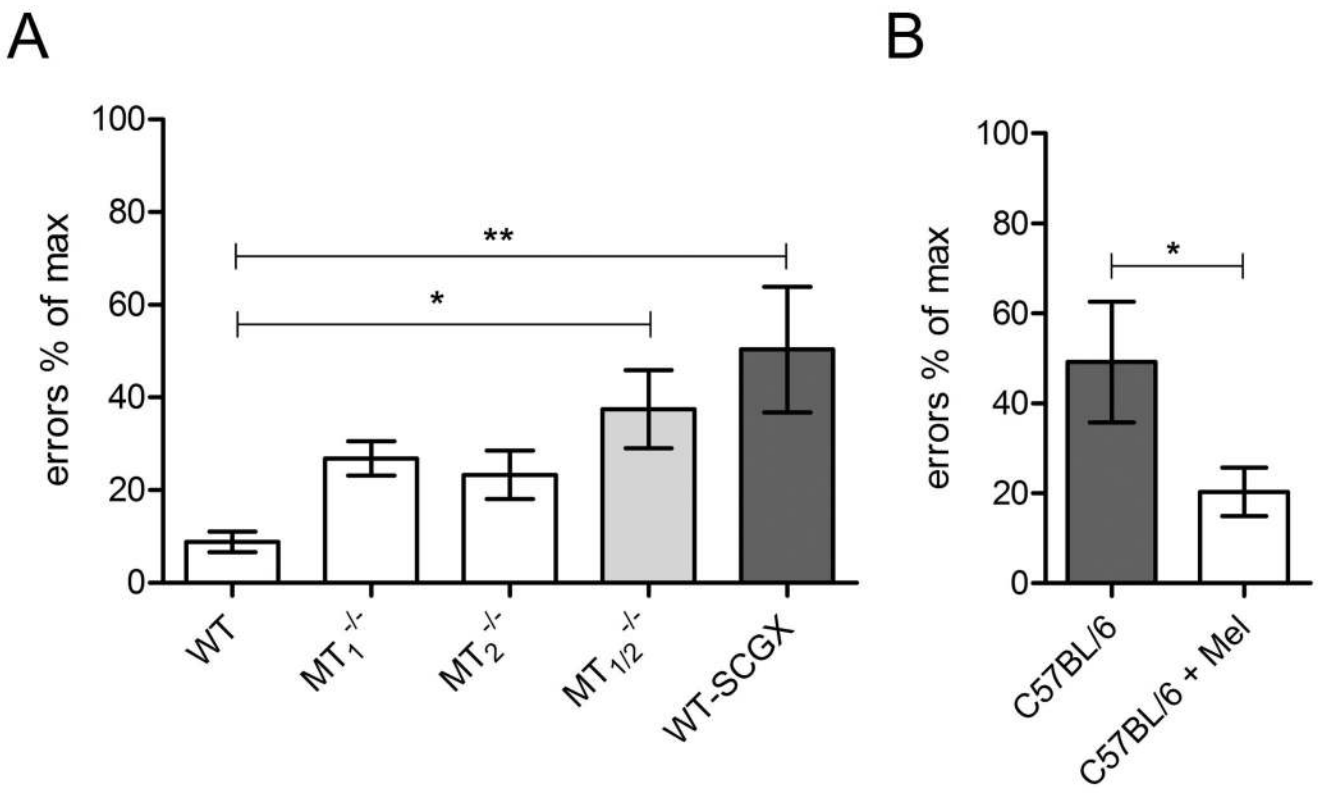

Fig. 7:

Comparative analysis of errors investigated across mouse strains. Illustrated is the number of errors of (A) WT mice (WT, $MT_1^{-/-}$, $MT_2^{-/-}$, $MT_{1/2}^{-/-}$, WT-SCGX) and (B) C57BL/6 mice ± melatonin during daytime training in the food-rewarded 8-arm radial maze test on day 5. (*= P ≤0.05; ** = P ≤0.01; One-way ANOVA with Dunnett´s Post-Test; unpaired t-test; WT: n = 9; $MT_1^{-/-}$: n = 10; $MT_2^{-/-}$: n = 8; $MT_{1/2}^{-/-}$: n = 8; C57BL/6 n = 7; C57BL/6 + Melatonin: n = 7; WT-SCGX: n = 8). Note that nighttime melatonin treatment increased the performance of C57BL/6 mice (B) in the 8-arm radial maze test. Values are expressed as mean ± SEM. Datasets of the radial arm maze performance during daytime on day 5 (see Figs. 6, Suppl. Figs. 8,9) were replotted here as % of maximal number of errors for a comparison across genotypes.

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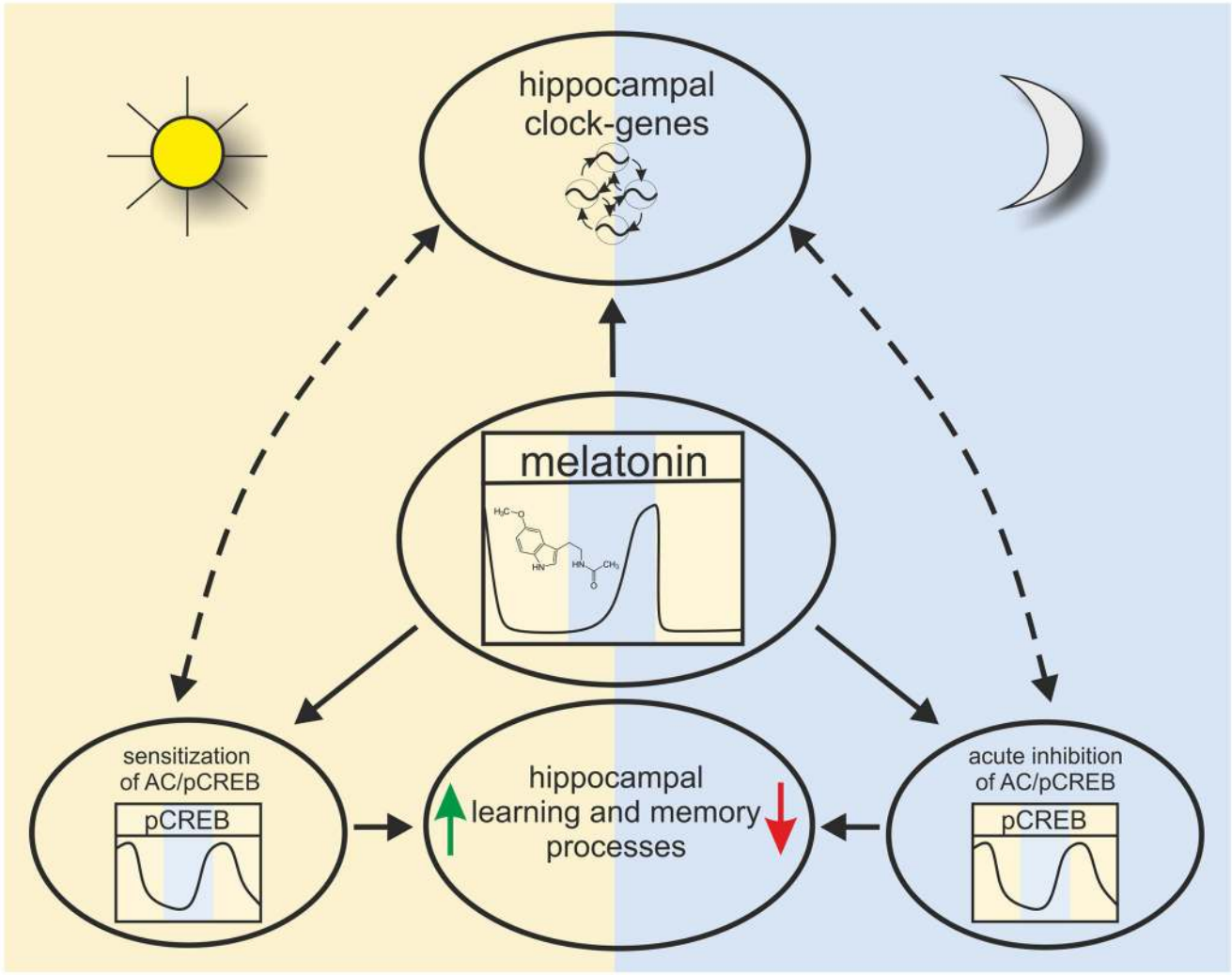

Fig. 8:
Working model for the possible mechanistic role of melatonin within hippocampal learning and memory processes.

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Tab. 1:

List of used antibodies

Antibody		Host	Company	IHC	WB
Primary antibody	MT_1	rabbit	Sengupta et al., 2011	1:500	-
	MEL-1-A-R (MT_1)	goat	Santa Cruz #SC13186	1:200	1:500
	MEL-1-B-R (MT_2)	goat	Santa Cruz #SC13177	1:200	1:500
	MT_1	rabbit	Alomone #AMR-031	1:250	-
	MT_2	rabbit	Alomone #AMR-032	1:250	-
	MAP2	mouse	BD Biosciences #566320	1:500	-
	ß-Actin	mouse	Sigma-Aldrich #A5316	-	1:40000
	pCREB(Ser133)	rabbit	Millipore #06519	1:1000	-
	PER1	rabbit	kind gift from S.M. Reppert, University of Massachusetts Medical School, Worchester, USA	1:5000	-
	PER2	rabbit	Alpha Diagnostic #PER21-A	1:250	-
	CRY1	rabbit	Alpha Diagnostic #CRY11-A	1:50	-
	CRY2	rabbit	Alpha Diagnostic #CRY21-A	1:250	-
	CLOCK	rabbit	Affinity BioReagents #PA1–520	1:500	-
	BMAL1	rabbit	Affinity BioReagents #PA1–523	1:500	-
Secondary antibody	Alexa Fluor 488 anti-rabbit/mouse	goat	Molecular Probes #A-11008/A-11001	1:200	-
	Alexa Fluor 568 anti-rabbit/mouse	goat	Molecular Probes #A-11011/A-11004	1:200	-
	Anti-rabbit biotinylated antibody	goat	Vector #BP910050	1:600	-
	Anti-mouse IgG HRP	goat	Dako #P0447	-	1:40000
	Anti-rabbit IgG HRP	goat	Santa Cruz #SC2054	-	1:40000