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A regulatory pathway linking caffeine action, mood and the diurnal clock

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1 Introduction

Circadian rhythms govern a large variety of behavioral, physiological and metabolic functions in virtually all cells of the mammalian body. The circadian clock is a complex molecular machinery that drives a large fraction of circadian rhythms. The core clock mechanism is constituted by transcriptional activators CLOCK and BMAL1, which heterodimerize to drive the expression of a large number of clock-controlled genes. The Circadian Locomotor Output Cycles Kaput (CLOCK) protein is a key player in the circadian system, which is crucial for biological time keeping (1) and the sleep-wake cycle (2). CLOCK is altered in psychiatric conditions that are characterized by changes in circadian rhythms, including intake of the psychostimulant cocaine (3) and mood disorders (4,5).

Caffeine, an antagonist of adenosine receptors, is the most consumed psychostimulant worldwide and has a robust effect on wakefulness. Caffeine can also affect the circadian period of molecular oscillations in vitro (6,7) and alters the effects of sleep deprivation in rodents (8,9). There is accumulating evidence that the psychostimulants cocaine and metamphetamine have an impact on the striatal CLOCK system (10–12). However, it is largely unknown how caffeine affects circadian (around the clock) or diurnal (day versus night) signaling.

Dopamine- and cAMP-regulated phosphoprotein Mr 32,000 (DARPP-32) has been linked to bipolar disorders, antidepressant action and responses to psychostimulants (13). Both, adenosine receptors and DARPP-32 are enriched in the medium spiny neurons of the striatum, a main part of the brain reward system. The striatum has previously been shown to be associated with circadian regulation (14–16). Furthermore, the striatum is involved in a variety of mental illnesses (17–19). Here we describe a previously uncharacterized pathway, which links caffeine response to the diurnal clock system via DARPP-32.

While acute caffeine intake has psychostimulant actions, chronic caffeine affects endophenotypes of depression in mice (20–22). Chronic caffeine intake inversely correlated with depression risk in a large longitudinal study (23) and depressed patients displayed aberrant circadian synchrony (24), with fatigue, mood changes and sleep

disturbances being main symptoms of depression (4,25). However, it is unclear whether stimulating properties of acute caffeine are intertwined with the CLOCK system at the molecular level (26) and whether acute administration of caffeine has an impact on mood as well.

Here we demonstrate that DARPP-32 is relevant for the acute mood-elevating actions of caffeine in mice via altering CLOCK-mediated pathways. Caffeine increases the phosphorylation at Thr75 of DARPP-32 (27–29). T75-DARPP-32 reduces CLOCK:Aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1) binding to chromatin, resulting in caffeine-induced transcriptional changes in the striatum. The effect of DARPP-32 on behavior and chromatin binding of the CLOCK:BMAL1 complex to chromatin is dependent on the light-phase, during which caffeine is administered.

Taken together this study provides a previously unappreciated link between caffeine administration and diurnal signaling and opens new avenues for our understanding of fast-acting effects on mood in the mouse brain.

2 Materials and Methods

2.1 Animals

Mice were housed in accordance with the ethical guidelines (Declaration of Helsinki and NIH, publication no. 85-23, revised 1985; the European Community Guidelines; and the French Agriculture and Forestry Ministry guidelines for handling animals, decree 87849, license A 75-05-22) and approved by the *Institut du Fer à Moulin* ethical committee. Furthermore, experiments and animal maintenance were conducted in compliance with the Federation of Swiss Cantonal Veterinary Office and approved by the Zürich Cantonal Veterinary Office under license ZH241/16. They were also approved by the Thüringer Landesverwaltungsamt, Germany, under License UKJ-18-036. Male C57Bl/6J mice were purchased from Janvier. DARPP-32 T75A knock-in mutants (PPP1R1B-T75A) (30) and DARPP-32-GFP BAC transgenic mice (29) were kindly provided by Paul Greengard, The Rockefeller University, New York. Male and female mice were used at 6-18 weeks of age and pooled in equal numbers for experiments. All behavioral experiments were conducted in a 12D:12L light cycle. Behavioral experiments during the dark phase were conducted in red light.

2.2 Antibodies, drugs and chemicals

Primary antibodies for immunoblotting and immunohistochemistry were: rabbit monoclonal for DARPP-32 (Cell Signaling Technology, Danvers, MA, USA #2306; 1/5,000), rabbit polyclonal for pThr75 (Cell Signaling Technology #2301; 1/2,000); β -ACTIN (Sigma, St Louis, MO, USA #A5316; 1/1,000); MYC-tag (Merck Millipore, Darmstadt, Germany #05-724; 1/500); GFP (Abcam, Cambridge, UK #ab6556; 1/1,000); CLOCK (Abcam #ab93804; 1/1,000). Secondary antibodies comprised of IRDye800-conjugated anti-mouse and anti-rabbit (Rockland, Limerick, PA, USA #610-132-121, #611-132-122). Mice were injected intraperitoneally with caffeine (Sigma #C0750; 7.5 mg kg⁻¹) or vehicle and tested after 40 min or 2 h.

2.3 Cell culture and transfection

COS-7 cells (American Type Culture Collection) were cultured in six-well plates (10⁶ per well) in Dulbecco's minimal essential medium (DMEM, GIBCO by Thermo Fisher Scientific, Waltham, MA, USA #11995065) with 10% fetal bovine serum. Vectors were co-transfected using Lipofectamine 2000 transfection reagent (ThermoFisher Scientific #11668019) in OptiMEM (GIBCO #31985070). All DARPP-32 vectors contained a GFP reporter and a kanamycin resistance, while all CLOCK vectors contained ampicillin. For DARPP-32, full-length, T75A point mutation, as well as the DARPP-32 fragment DARPP-32₉₃₋₂₀₅ were utilized (29,31). The empty GFP and MYC-vectors were used as controls during co-transfection. After 4 h of transfection, the medium was replaced with serum-free DMEM. Cells were treated 24 h later in serum-free DMEM and collected for immunoblot analysis as described (29).

2.4 Co-immunoprecipitation

For co-immunoprecipitation from COS-7 cell lysates, a sequential protocol was used as described (29). Co-immunoprecipitation with DARPP-32-GFP mutants was performed using a GFP-trap kit (Chromotek, Planegg, Germany #gta-20) according to the supplier's instructions as described (29). Pulldowns were processed for immunoblotting against CLOCK. DARPP-32-GFP mutants were sacrificed at ZT12-18.

2.5 Luciferase assay

HEK-296 cells were transfected for 24h with luciferase-reporter containing an E-box regulatory element (5 µg), MYC-CLOCK (5 µg), MYC-BMAL (5 µg) and either one of GFP control vector (1 µg), GFP-DARPP-32-wildtype (2 µg) or GFP-DARPP-32-T75A point mutant (2 µg). The plasmid ratios and concentrations have been optimized prior to the experiment to ensure a stoichiometry of luciferase reporter:CLOCK:BMAL:DARPP-32 expression of approximately 1:1:1:1 by using GFP and MYC-antibodies. Total luminescence was normalized to an actin-signal by immunoblotting to account for variations in sample preparation. A standard curve was generated for the total luminescence signal to determine an optimal sample volume (50 µl). The signal from non-transfected lysate was negligible (total luminescence<1). Fifty µl of cell lysate were taken as reaction volume. Total luminescence was normalized against actin and then the average of Luciferase+CLOCK and Luciferase+BMAL1 were subtracted for each of the three groups from the Luciferase+BMAL1+CLOCK group to eliminate background from unspecific binding. Relative luminescence: The average of Reporter+CLOCK and Reporter+BMAL transfections were subtracted from the Reporter+CLOCK+BMAL groups to eliminate background caused by unspecific binding.

2.6 Behavioral analysis

Tail suspension test was conducted by taping the mice by their tails to a rod using band aid. The rod was placed approximately 50 cm above a table. Immobility time was measured for 5 min. Forced swim test was performed in a transparent beaker filled with 18°C warm water up to 15 cm below the opening. The time spent immobile was assessed during a 6 min time-period. For novelty suppressed feeding, mice were food restricted for 12 h (app. 1g food per mouse). They were then placed alone in a clean home cage with a food pellet located in the middle of the cage. For a maximum of 10 min, the latency until the mouse starts eating the pellet was recorded. Locomotor activity and anxiolytic behavior were assessed in for 1 h in an open field and analyzed using the TSE Multiconditioning system software (TSE Systems). Numbers of male and female mice were balanced between groups. Data separated by sex are listed in Table S1.

Mice did not undergo the same behavioral paradigm twice. At least one week had to pass between experiments and mice were only receiving caffeine once.

2.7 ChIP and quantitative PCR

ChIP was performed essentially as described (32): DNA and proteins were crosslinked using 1% Formaldehyde in PBS for 8 min and the reaction was stopped using 2M glycine. After 4 washing steps with PBS containing EDTA-free protease inhibitor cocktail (Roche, #11873580001), tissue was homogenized by passing it through a 22-gauge needle 30 times. After removing debris (1.230 rcf spin for 5 min, 4°C, discard supernatant), cells were incubated with cell lysis buffer (5mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40, Protease inhibitor cocktail) for 15 min and vortexed every 2-3 min. The cells were spun down for 5 min at 2.650 rcf at 4°C and the pellet was resuspended in nuclear lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS, Protease inhibitor cocktail). They were incubated on ice for 10 min and vortexed every 2-3 min. Next, samples were sonicated in a bioruptor for 2x 12 cycles (30 sec on/off, 5 min break between cycles to avoid overheating). Afterwards, they were spun at 18.000 rcf for 10min at 4°C and the pellet containing chromatin was resuspended in RIPA-buffer (50 mM Tris-HCl, pH 8.0, 5 M NaCl, 2 mM EDTA, pH 8.0, 1% NP-40, 0.5% Sodium-deoxycholate, 0.1% SDS, protease inhibitors). In parallel, the antibody was coupled to magnetic beads (Dynabeads M-280 Sheep anti-rabbit IgG, ThermoFisher, #11203D). Per ChIP-reaction, 2 µg of chromatin were incubated with 2 µg of Bmal antibody (Abcam, #ab93806) overnight at 4°C. The dynabeads containing the antibody-chromatin complex were sequentially washed in various buffers to remove unspecifically bound chromatin fragments (1. Low salt buffer: 0.1% SDS, 1% TX-100, 2 mM EDTA, pH 8.0, 150 mM NaCl, 500 mM Tris-HCl, pH 8.0; High salt buffer: 0.1% SDS, 1% TX-100, 2 mM EDTA, pH 8.0, 500 mM NaCl, 500 mM Tris-HCl, pH 8.0; 3. LiCl buffer: 150 mM LiCl, 1% NP-40, 1% Sodium-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0; 4. TE + 50 mM NaCl: 50 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Beads were collected and chromatin was eluted at 65°C for 30 min (1% SDS, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA). RNA and proteins were removed by digestion with DNase-free RNase (Roche, #11 119 915 001) and Proteinase K (Invitrogen, #25520 049) and DNA was purified

using the MinElute PCR purification kit (Qiagen, #28004). Concentration and shearing efficiency were assessed with the dsDNA HS Assay and Agilent High-Sensitivity DNA kits (Thermo Fisher Scientific, #Q32851; Agilent Technologies, 5067-4626). Data are represented as ChIP/Input. PCR-primers for *Dbp* were: Fwd: 5'-ACA CCC GCA TCC GAT AGC-3'; Rev: 5'-CCA CTT CGG GCC AAT GAG-3'. For quantitative PCR, RNA was converted to cDNA using the GoScript Reverse Transcriptase System (Promega, #A5002). During PCR, SsoFast Evagreen reagents were used (Bio-rad, #1725201). The experiment was run on the CFX96 Real-Time System (settings: 1x: 95.0°C, 5 min; 40x: 95°C, 10 sec; 60.0°C, 10 sec; 72.0°C, 10 sec; 1x: 95.0°C, 1 min; 65.0°C, 5 sec; 95.0°C, 5 sec; 4°C). The *Per1*-signal was normalized with an average of three house keeping genes, *Beta-Actin*, *S18* and *Gapdh*. The following primers were used: *Per1*: Fwd: 5'-ACC AGC GTG TCA TGA TGA CAT AC-3', Rev: 5'-GTG CAC AGC ACC CAG TTC CC-3'; *β-Actin*: Fwd: 5'-TGT TAC CAA CTG GGA CGA CA-3', Rev: 5'-GGG GTG TTG AAG GTC TCA AA-3'; *S18*: Fwd: 5'-TGG CTC ATT AAA TCA GTT ATG GT-3'; 5'-GTC GGC ATG TAT TAG CTC TAG-3'; *Gapdh*: Fwd: 5'-AAC TTT GGC ATT GTG GAA GG-3', Rev 5'-ACA CAT TGG GGG TAG GAA CA-3'.

2.8 Western blotting und Immunofluorescence

Western blots were performed as described (29): Samples were boiled at 98°C for 10 min in Laemmli buffer (100 mM Tris-HCl, pH 6.8, 12% glycerol 40 g x l⁻¹ SDS, 2% v/v β-mercaptoethanol and bromphenol blue). Proteins were blotted using Bio-rad Mini Protean TGX-gels and the Bio-Rad transfer system. Immunofluorescence and quantification for DARPP-32 was performed as described (29,33). Essentially, mice were perfused with 4% paraformaldehyde and brains were postfixed overnight at 4°C. They were then cut into 30-μm sections on a Leica vibratome. Free-floating sections were incubated overnight with anti-DARPP-32 antibody at 4°C. After three rinses with TBS, they were incubated with secondary antibody for 2 h at room temperature. After 3 more rinses with TBS, sections were mounted using Fluoromount-G (Southern Biotech, #0100-01). Photos were taken with a Zeiss LSM880 microscope with a 63x magnification. Per brain area, three to four photos per mouse were analyzed.

2.9 Statistics

Sample sizes were chosen according to variability estimates in previous experiments or in pilot experiments. Data are expressed as means \pm s.e.m. Statistical analysis was performed in GraphPad Prism (version 5). The tests used were two-tailed Student's t-test for comparison of two groups, one-way analysis of variance (ANOVA) when there were more than two groups, two-way ANOVA when two factors were varied and 3-way ANOVA for three factors. When ANOVA was significant, posthoc tests were Newman Keuls-test for one-way ANOVA and Bonferroni test for two-way and three-way ANOVA. If variances were not equal, Dunn's multiple comparison test was used. Three-way ANOVA data are depicted in Tables S2-S4.

3 Results

3.1 Thr75-DARPP-32 interacts with the C-terminal domain of CLOCK

DARPP-32 is a major hub molecule in striatal medium spiny neurons. Previously, LC-MS/MS on striatal lysate pulled down from purified DARPP-32 showed the Acid tolerance regulated protein, which is highly homologous to CLOCK (34), as potential binding partner (29). Hence, we hypothesized that DARPP-32 may be able to bind to CLOCK as well. Therefore, MYC-CLOCK and DARPP-32-GFP were co-expressed in COS7-cells and cell lysates were subsequently incubated on a MYC-affinity column (29). DARPP-32 was pulled down with CLOCK (**Fig. 1**). The experiment was repeated with MYC-BMAL1 and DARPP-32-GFP-CLOCK, however no signal for DARPP-32 was detected in the pulldown.

DARPP-32 is a phospho-protein with a variety of molecular functions that depend on its phosphorylation state on four residues (35). In order to determine the protein domains of DARPP-32 implicated in CLOCK-binding, COS-7 cells were transfected with a CLOCK plasmid and a variety of truncated and mutated forms of DARPP-32. CLOCK-binding was abolished in DARPP-32 Cter1, which lacks the N-terminal amino acids aa1-91 of the protein including the regulatory phosphosite Thr75. Therefore, a T75A mutant form of DARPP-32 was tested for CLOCK binding as well. Remarkably, the T75A mutation prevented DARPP-32 binding to CLOCK (**Fig. 1A-D**).

Next, we sought to identify the domain of CLOCK involved in DARPP-32 binding.

Various CLOCK mutant plasmids were co-transfected with full length DARPP-32 and the pulled down fractions were analyzed by western blot using an anti-DARPP-32 specific antibody. While truncated versions of CLOCK containing aa1-625 and aa1-570 did not impact on CLOCK-DARPP-32 interaction, DARPP-32 binding did not occur on the CLOCK mutant lacking a C-terminus ("DN"; lacking the N-terminal amino acids 1-269) (**Fig. 1F-G**) (34). Taken together, these data suggest that DARPP-32 interacts with CLOCK in its C-terminal region in vitro and that this process is dependent on Thr75-DARPP-32.

To assess whether DARPP-32 and CLOCK interact in vivo, we performed co-immunoprecipitation (Co-IP) using DARPP-32-GFP transgenic mice versus WT littermates and used an anti-CLOCK specific antibody for western blot quantification. GFP-pulldowns from these mice showed a significant enrichment of CLOCK, suggesting that CLOCK can interact with DARPP-32 in the striatum in vivo (**Fig. 1I, J**).

3.2 Thr75-DARPP-32 reduces activation by the CLOCK:BMAL1 complex

The CLOCK:BMAL1 complex activates transcription via binding chromatin at E-box consensus sites (26). In order to determine whether the presence of DARPP-32 may disrupt CLOCK:BMAL1-binding to the E-box, luciferase assays were performed in HEK-293 cells transfected with CLOCK, BMAL1, a luciferase reporter and various forms of DARPP-32 or control (GFP) vectors. The activity of the reporter was much higher when CLOCK and BMAL1 were co-transfected than when either one was transfected alone (**Fig. S1**). Importantly, and in agreement with the Co-IP data (**Fig 1A-D**), DARPP-32 significantly reduced luminescence, suggesting reduced binding of CLOCK:BMAL1 to the E-box (**Fig. 1K**). DARPP-32 reduced luminescence only when both CLOCK and BMAL1 were present. The T75A mutation in DARPP-32 completely abolished this effect. Hence, at least under these artificial in vitro conditions, Thr75-DARPP-32 alters CLOCK:BMAL1 binding to chromatin and subsequent gene expression.

3.3 Caffeine affects CLOCK:BMAL1 binding to chromatin and gene expression via Thr75-DARPP-32 in vivo

DARPP-32 phosphorylation on Thr75 is increased by caffeine (28,29). Since Thr75-

DARPP-32 can reduce CLOCK:BMAL1 binding to chromatin in vitro, we hypothesized that this effect may also be observed in vivo in response to administration of caffeine.

WT mice were IP-injected with caffeine at four time points along the day and night-cycle. Striatal tissue was collected 40 min later when Thr75-phosphorylation on DARPP-32 is known to be at a maximum (28). Striatal lysates were chromatin-immunoprecipitated with an antibody against BMAL1. *D site of albumin promoter binding protein (Dbp)* is a canonical CLOCK:BMAL1 target gene and therefore we measured *Dbp* by PCR and used *Dbp*-pull-down by BMAL1 as a quantitative readout. In both saline and caffeine conditions, diurnal oscillations of pulled-down *Dbp*-levels paralleled those previously reported (36). Importantly, across the entire day and night-cycle, caffeine significantly reduced *Dbp*-pull-down (**Fig. 2A**).

To investigate, whether the effect of caffeine on CLOCK:BMAL1 signaling is dependent on Thr75-DARPP-32, the experiment was repeated with T75A-DARPP-32 knock-in mutant mice, in which caffeine-dependent Thr75-phosphorylation is prevented (29). *Dbp*-pull-down under baseline conditions was not significantly altered in T75A-DARPP-32 knock-in mice (**Fig. 2B**). However, while in WT mice, caffeine reduced CLOCK:BMAL1 binding to *Dbp* in the active phase of the light cycle, this effect was abolished in T75A-DARPP-32 mutants. (**Fig. 2C**) During the light (inactive) phase, the T75A-mutation did not affect binding to the *Dbp*-gene (**Fig. 2D**). Similarly, caffeine reduced gene expression of the circadian regulator *Period 1 (Per1)* 2 h post-injection in WT but not T75A-mutants during the active phase. In agreement with the data above, the mutant effect on caffeine-induced *Per1*-expression did not occur in the light phase (**Fig. 2E, F**).

DARPP-32 can translocate into the nucleus upon phosphorylation of the Ser97-site (31) and may contribute to the presence of DARPP-32 in the vicinity of CLOCK in vivo. Therefore we tested whether DARPP-32 translocation occurred in a diurnal manner. However, this was not the case (**Fig. S2**). Additionally, we did not find evidence for diurnal changes in Thr75-phosphorylation (**Fig. S3**). It is therefore unlikely that the effects of DARPP-32 on CLOCK:BMAL1 binding are due to diurnal fluctuations in nuclear phospho-Thr75-DARPP-32.

3.4 Mood-elevating effects of caffeine are prevented by T75A-DARPP-32 mutation in a diurnal manner

Since chronic caffeine has been shown to have positive effects on depressive-like behaviors in rodents, we asked whether caffeine in non-depressed may positively affect mood-related behaviors as well and whether T75-DARPP-32 might be involved in this effect.

WT mice and T75A mutants were injected with caffeine and assessed for behaviors typically linked to antidepressant responses or mood 40 min and 2 h later. Since neither WT mice nor T75A mutants can be considered depression models under normal circumstances, an improvement in such behaviors may be termed mood-elevating.

Escape behavior was measured in tail suspension and forced swim tests given their temporal specificity. Since the chromatin-IP-data suggested that the light phase is relevant for the effect of the T75A mutation, behavioral tests were conducted in both, light- and dark phases. During the dark phase, 40 min of caffeine efficiently reduced the relative immobility time in WT mice, both in forced swim and tail suspension tests (**Fig. 3A, B**). This effect was abolished in T75A-DARPP-32 mutants. Furthermore, caffeine had similar T75-dependent effects in the novelty-suppressed feeding test (**Fig. 3C**).

During the light phase, 40 min of caffeine significantly reduced escape behavior in the tail suspension test (**Fig. 3D**), while only having a very mild or no effect in the forced swim test and novelty suppressed feeding (**Fig. 3E, F**). Importantly, the effects of caffeine were indistinguishable between WT mice and T75A-DARPP-32 mutants (**Fig. 3D-F**).

When all factors (light phase, drug and genotype) were compared, in the forced swim test, 3-way ANOVA revealed a significant interaction of light phase, drug and genotype (**Table S2**). The Tukey posthoc test showed significant effects of the drug within WT in the dark phase, and of the genotype within the caffeine-group during the dark-phase (**Table S2**). During tail suspension and novelty suppressed feeding tests, 3-way ANOVA did not reveal a significant interaction of circadian time, drug and genotype (**Tables S3, S4**). However, other comparisons were significant such as effects of genotype, and drug, for the tail suspension test and light phase, and drug, for novelty suppressed feeding. For the tail suspension test, the Tukey posthoc test again showed significant

effects of the drug within WT in the dark phase and of the genotype within the caffeine-group during the dark-phase (**Table S3**). For novelty suppressed feeding these measures were not significant, however there were significant effects of light phase within T75A-mutants and of drug within WT. Taken together, these results support the observation that caffeine effects depend on the T75-DARPP-32 mutation and the light phase.

The T75-dependent effects of caffeine in the dark phase measured in tail suspension, forced swim and novelty suppressed feeding tests persisted at the 2 h time point in a new cohort of mice (**Fig.3 G-I**), suggesting that behavioral effects persist at a time point, when gene expression changes occur (**Fig. 2**).

Notably, while caffeine increased the locomotor activity as expected, it did so equally in WT and T75A littermates (**Fig. S4**). Hence, the effects on escape behavior are unlikely to be due to locomotor effects of caffeine. Furthermore, while caffeine increased anxiolytic behavior during the active phase (**Fig. S4**), it did so equally in WT and T75A-mutant mice. Taken together, these data support an interaction between the caffeine-phospho-Thr75-DARPP-32 pathway predominantly in the active phase of the day and night-cycle and suggest effects on molecular and behavioral levels.

4 Discussion

Depression and circadian / diurnal rhythms have been linked. Here we reveal a regulatory pathway which connects caffeine action to CLOCK:BMAL1 signaling via Thr75-DARPP-32. Thr75-DARPP-32 directly binds to CLOCK and disrupts CLOCK:BMAL1 binding to chromatin, subsequent gene expression, and mediates mood-elevating effects of caffeine. This effect is dependent on the light phase, encouraging that future pharmacological studies are being conducted at specific circadian or diurnal time points.

While we show that Thr75 is relevant for the interaction between DARPP-32 and CLOCK, a role of Thr75 phosphorylation is not formally demonstrated. It is a likely explanation but alternatively Thr75 may be necessary for the interaction regardless of phosphorylation state. To test these two scenarios, purified DARPP-32 that has been phosphorylated with cdk5 in vitro (versus control) may be used for the co-

immunoprecipitation experiments in the future. Alternatively, a Threonine to glutamic acid mutation could be used as a phospho-mimetic.

We observed that DARPP-32 binds to the C-terminal domain of CLOCK, which is necessary for heteromerization with BMAL1. There are various scenarios by which DARPP-32 may affect CLOCK, BMAL1 or chromatin, resulting in altered CLOCK:BMAL1 binding: First, DARPP-32 can inhibit protein phosphatase-1 and protein kinase A. In consequence, phosphorylation states of CLOCK and/or BMAL1 may be altered. Second, DARPP-32 may affect histone phosphorylation, for instance on histone 3 Serine 10 (31), which may directly or indirectly affect CLOCK:BMAL1-binding. Third, DARPP-32 may sterically obstruct BMAL1 from CLOCK-binding. Further experiments are warranted to explore the exact mechanisms, by which DARPP-32 affects CLOCK:BMAL1, particularly in vivo. Since T75A-mutation abolished DARPP-32 binding to CLOCK:BMAL1 in luciferase essays (**Fig. 1K**) but did not affect chromatin binding in ChIP with BMAL1-antibodies (**Fig. 2B**), additional binding partners and pathways may be relevant in vivo.

The presented experiments explore the in vivo effects of this pathway mostly from the angle of DARPP-32. More studies on the effect of CLOCK and BMAL1 in this respect may provide further support. This includes a thorough assessment of properties in CLOCK:BMAL1 occupancy on chromatin in the T75A-DARPP-32 mouse model, such as period of oscillation, levels and peak phases of various CLOCK-regulated genes. Since this study was done in a 12D/12L light cycle, further experiments under various other light conditions may exclude that the observed effects are diurnal rather than circadian. Similarly, it should be investigated whether the DARPP-32:CLOCK pathway is also contributing to the effect of caffeine on the circadian period.

The main target of caffeine in the brain are adenosine receptors. These are expressed in various brain regions, including forebrain, striatum and hippocampus. It is well established that Thr75 phosphorylation on DARPP-32 occurs via adenosine A2A-receptors (37,38). Additionally, caffeine has pleiotropic effects beyond striatal adenosine-receptor binding.

Since the T75A-mutation blocks chromatin binding of CLOCK:BMAL1, caffeine likely affects gene expression through the A2A-R:Thr75-DARPP-32 pathway in the striatum. Since A2A-receptors in either forebrain or striatum can affect Thr75 phosphorylation

(38), it is not clear, whether the analysed striatal DARPP-32 is regulated by A2A-receptors within the striatum, outside the striatum and is affected additionally by other caffeine-responsive pathways.

T75A-mutation also blocks behavioral effects induced by caffeine. DARPP-32 is most strongly expressed in the striatum. Hence, it is likely that the observed striatal signaling plays a strong role in the observed mood elevating effects. However, since DARPP-32 is also expressed in other tissues, albeit at lower levels, at this point we cannot exclude that other brain areas have an effect on the behavioral outcome as well.

While we used *Dbp* pulldown and *Per1* gene expression to demonstrate the relevance of DARPP-32 to CLOCK:BMAL1 chromatin binding, in the future, chromatin binding and gene expression profiles should be analyzed in a more unbiased and extensive way to better understand the molecular consequences of the described pathway.

Clinical studies on caffeine in depressed cohorts are scarce and hence, too few data are available to draw conclusions regarding the (fast-acting) antidepressant potential of caffeine in humans. However, in rodent studies caffeine does have ~~reproducible~~ effects on depressive-like behavior or mood in various paradigms. Nevertheless, we would like to emphasize that caffeine was used in this study as a tool to identify novel pathways that may rapidly improve mood. Our aim is not to suggest using caffeine itself as an antidepressant or mood elevator, given its already widespread use in the population as well as potential side effects. The behavioral tests in the study presented here should be repeated in a depression-model such as chronic variable stress (39) to assess, whether the mood-elevating effects of acute caffeine can be beneficial in depressive-like states as well. Moreover, determining a time course of caffeine-induced effects and comparing acute and chronic consequences on molecular and behavioral alterations will be important goals for the future. Nevertheless, ultimately extensions of our study may help identifying potential molecular drug targets for fast-acting antidepressant effects or mood elevation.

5 Conclusions

Caffeine can affect CLOCK:BMAL1 binding to chromatin via Thr75-DARPP-32. This pathway mediates, at least in part, fast-acting mood-elevating effects of caffeine in mice.

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The authors declare no competing interests.

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Author contributions

C.T. and D. B. performed molecular biological experiments. C.A.H. and J-A.G. were mentors and provided financial support. O.E. developed the project idea, performed the majority of experiments and wrote the manuscript.

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Figure legends

Fig. 1 Thr75-DARPP-32 binds to the C-terminus of CLOCK and disrupts E-box binding.

A-H) COS7-cells were co-transfected with MYC-CLOCK and DARPP-32-constructs and cell lysates loaded on a MYC-affinity column. **A-D)** DARPP-32 binding to CLOCK requires Thr75. **A)** DARPP-32 constructs. **B)** Western blot of input for the Co-IP. **C)** Western-blot of the pulldown. **D)** Quantification of the pulldowns. Pulldown was indistinguishable from the GFP-control when the DARPP-32 C-terminus (aa 1-91), including Thr75, was deleted or when Thr75 was mutated to alanine (T75A). n = 7-20 per group, Kruskal-Wallis test: $P < 0.001$, Dunn's Multiple Comparison-Test: WT vs Cter1: $**P < 0.01$, WT vs. T75A: $**P < 0.01$; Cter vs. T75A: $P > 0.05$. **E-H)** CLOCK binds DARPP-32 via its C-terminal domain. **e)** Overview over CLOCK constructs. **F)** Western blot of input for the Co-IP. **G)** Western-blot of the pulldown. **H)** Quantification of the pulldowns. DARPP-32 binding did not occur on the „DN“ mutant, which is lacking the C-terminus of CLOCK. n = 8-20 per group, Kruskal-Wallis test: $P < 0.05$, Dunn's multiple comparison test: DN vs all other groups: $*P < 0.05$; all other comparisons: $P > 0.05$. **I, J)** CLOCK and DARPP-32 bind to one another in vivo. Striatal lysates from DARPP-32-GFP-knock-in mice and WT littermates were immunoprecipitated with GFP antibodies. **I)** Western-blot of the pulldown. **J)** Quantification of the pulldown. n = 10,12 per group, Student's t-test: $t_{(20)} = 2.13$, $*P < 0.05$. **K)** WT-DARPP-32 but not T75A, reduces CLOCK:BMAL1-binding to an E-box reporter in a luciferase-assay. n = 6 per group; One-way ANOVA: $P < 0.0001$, Newman-Keuls posthoc test: DARPP-32 vs. GFP: $***P < 0.001$; DARPP-32 vs. T75A: $***P < 0.001$. SM: size marker. Means +/- s.e.m. and individual data points are shown.

Fig. 2 Caffeine reduces CLOCK:BMAL1 binding to chromatin and gene expression in WT but not T75A-DARPP-32 mutants.

A, C-F) Caffeine, 7.5 mg/kg, was intraperitoneally (IP)-injected at different time points along the circadian cycle. Striatal tissue was extracted 40 min later and chromatin-immunoprecipitated with a BMAL1-antibody. *Dbp*-pull-down was quantified by RT-PCR. **A)** *Dbp*-pull-down is reduced by caffeine. n = 15-16 per group; 2-way ANOVA: effect of time: $F_{(3,116)} = 2.85$, $P < 0.05$; effect of caffeine: $F_{(1,116)} = 13.97$, $***P < 0.001$. **B)** *Dbp*-pull-down is not significantly affected by T75A-mutation under baseline conditions. n = 8-14 per group; 2-way ANOVA: effect of time: $F_{(3,79)} = 2.94$, $P < 0.05$. Bonferroni posthoc test: WT vs. T75A for all time points: $P > 0.05$. **C)** During the dark (active) phase (ZT 12-24), caffeine reduces *Dbp*-pull-down in WT but not T75A-mutants. n = 8-10 per group; 2-way ANOVA: interaction between caffeine and genotype: $F_{(1,33)} = 3.19$, $P = 0.08$; Bonferroni posthoc test: effect of caffeine in WT: $*P < 0.05$, in T75A: $P > 0.05$. **D)** During the light (inactive) phase (ZT 0-12), caffeine-induced reductions in *Dbp*-pull-down are indistinguishable between WT and T75A-mutants. n = 9-14 per group; 2-way ANOVA: interaction between caffeine and genotype: $F_{(1,44)} = 0.14$, $P > 0.05$; Bonferroni posthoc test: effect of caffeine in WT: $P > 0.05$; in T75A: $P > 0.05$. **E)** During the dark phase, caffeine reduces *Per1* gene expression in WT but not T75A-mutants. n = 5-7 per group; 2-way ANOVA: effect of caffeine: $F_{(1,24)} = 6.339$, $P < 0.05$; Bonferroni posthoc test: effect of caffeine in WT: $*P < 0.05$, in T75A: $P > 0.05$. **F)** During the light phase, caffeine-induced reductions in *Per1* expression are indistinguishable between WT and T75A-mutants. n = 5-6 per group; 2-way ANOVA: all measures: $F_{(1,22)} < 1.74$, $P > 0.05$. Means \pm s.e.m. and individual data points are shown. ZT = Zeitgeber time; FC = fold change.

Fig. 3 Mood-elevating effects of caffeine during the active phase are prevented by T75A-DARPP-32 mutation.

A-F). WT mice and T75A mutant were acutely injected with saline vs. caffeine and tested 40 min later in depression-related behavioral paradigm. **A-C)** The percentage of time spent immobile was assessed in the dark phase of the circadian cycle (ZT 12-24). **A)** Tail suspension test: $n = 6-9$; 2-way ANOVA: Effect of genotype: $F(1,26) = 16.05$, $P < 0.05$; Effect of caffeine: $F(1,26) = 17.34$, $P < 0.01$; Bonferroni Posthoc-test: Effect of caffeine in WT: $**P < 0.01$; in T75A: $P > 0.05$. **B)** Forced swim test: $n = 6-8$; 2-way ANOVA: Interaction between genotype and caffeine: $F(1,24) = 8.794$, $P < 0.01$; Bonferroni Posthoc-test: Effect of caffeine in WT: $***P < 0.001$, in T75A: $P > 0.05$. **C)** Novelty suppressed feeding: $n=6-10$; 2-way ANOVA: Effect of caffeine: $F(1,30) = 6.00$, $P < 0.05$; interaction between genotype and caffeine: $F(1,30) = 5.30$, $P < 0.05$; Bonferroni Posthoc-test: Effect of caffeine in WT: $**P < 0.01$, in T75A: $P > 0.05$. **D-F)** Same measures as **A-C** but in light phase (ZT 0-12). **D)** Tail suspension test: $n = 7-12$; 2-way ANOVA: Effect of caffeine: $F(1,32) = 19.70$; Bonferroni Posthoc-test: Effect of caffeine in WT: $**P < 0.01$; in T75A: $**P < 0.01$. **E)** Forced swim test: $n = 7-9$; 2-way ANOVA: Effect of caffeine $F(1,27) = 4.218$; $P < 0.05$; Bonferroni Posthoc-test: Effect of caffeine in WT: $P > 0.05$, in T75A: $P > 0.05$. **C)** Novelty suppressed feeding: $n = 7-9$; 2-way ANOVA: all effects n.s. **G-I)** Behavioral tests were conducted in the dark phase (ZT 12-24) 2 h after injection of saline vs. caffeine. **G)** Tail suspension test: $n = 5-8$; 2-way ANOVA: Effect of drug: $F(1,23) = 8.32$, $P < 0.01$; Interaction between caffeine and genotype: $F(1,23) = 12.22$, $P < 0.01$; Bonferroni Posthoc-test: Effect of caffeine in WT: $***P < 0.001$; in T75A: $P > 0.05$. **H)** Forced swim test: $n = 6-10$; 2-way ANOVA: Effect of drug: $F(1,28) = 4.79$, $P < 0.05$; Bonferroni Posthoc-test: Effect of caffeine in WT: $**P < 0.01$, in T75A: $P > 0.05$. **I)** Novelty suppressed feeding: $n = 12-13$; 2-way ANOVA: Effect of caffeine: $F(1,46) = 4.69$, $P < 0.05$. Interaction between genotype and caffeine: $F(1,46) = 22.29$, $P < 0.001$; Bonferroni Posthoc-test: Effect of caffeine in WT: $*P < 0.05$, in T75A: $P > 0.05$. Means \pm s.e.m. and individual data points are shown.

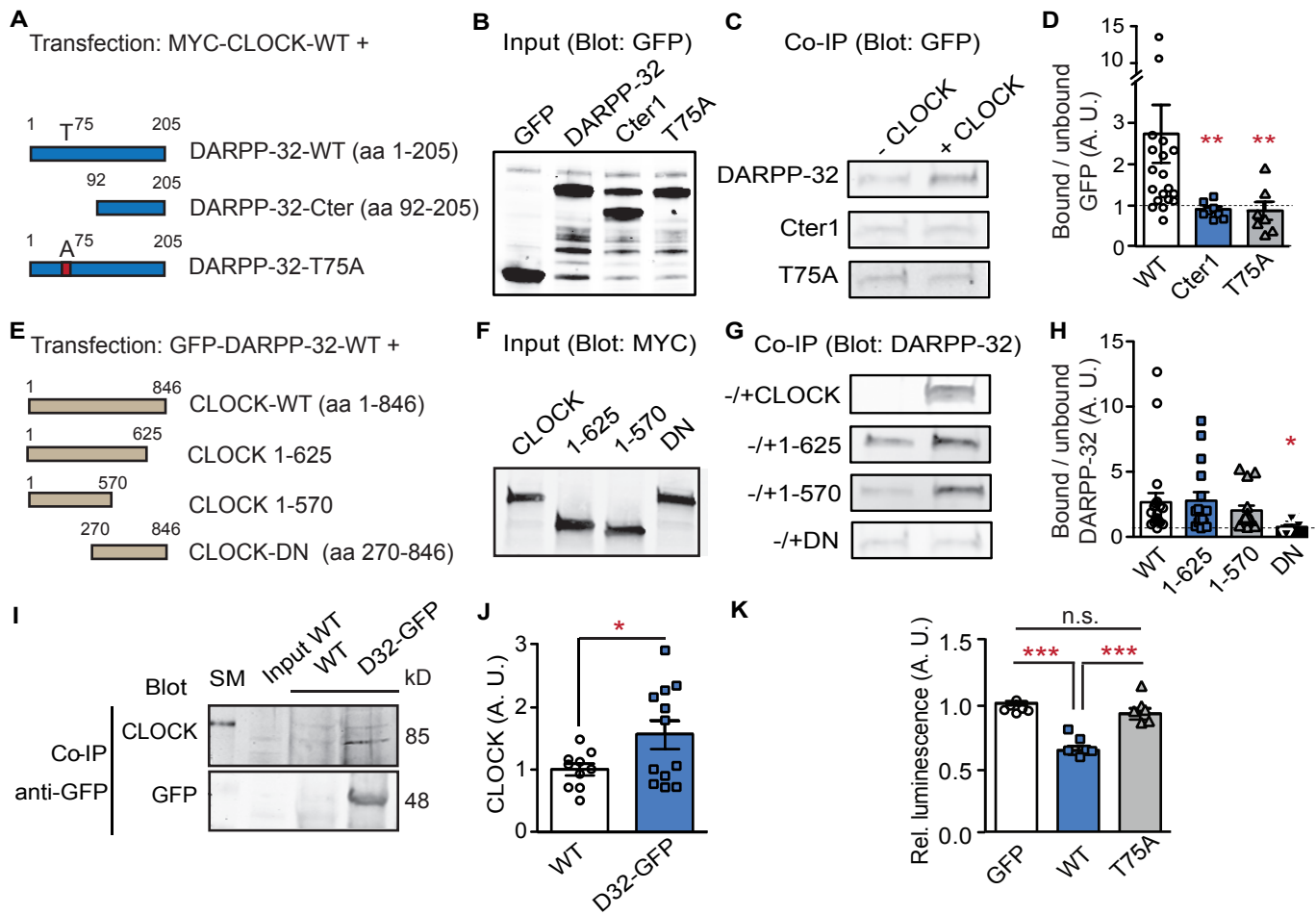


Fig. 1

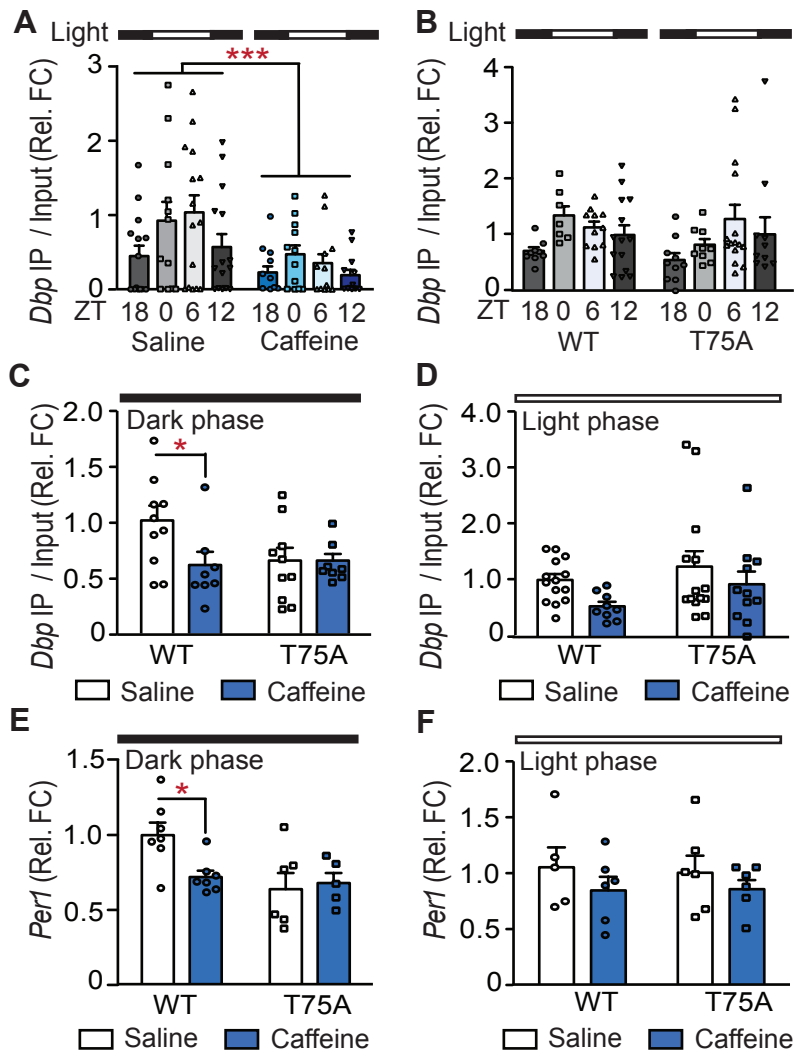


Fig. 2

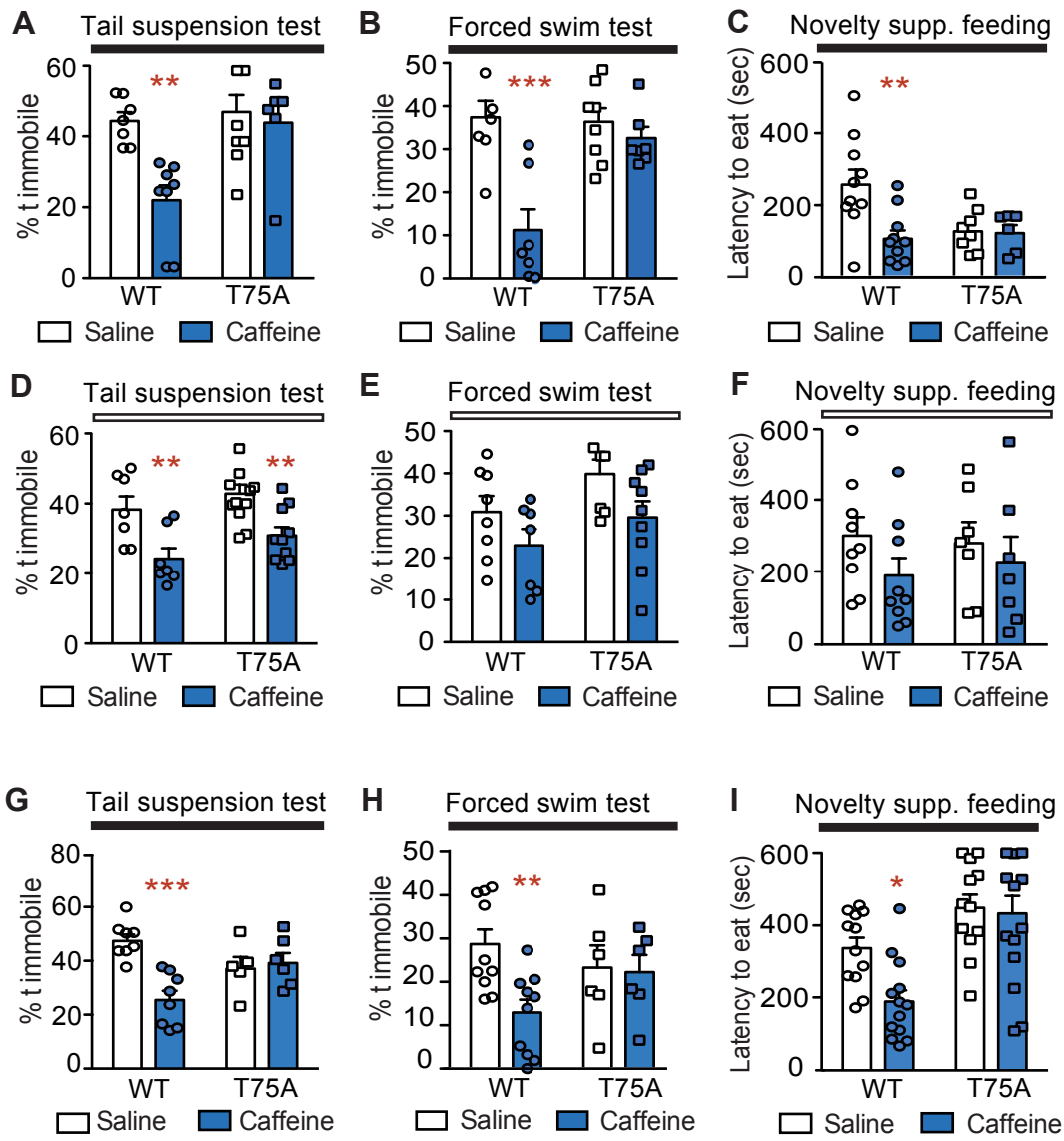


Fig. 3