CIRCADIAN RHYTHMS

Effects of caffeine on the human circadian clock in vivo and in vitro

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Caffeine's wakefulness-promoting and sleep-disrupting effects are well established, yet whether caffeine affects human circadian timing is unknown. We show that evening caffeine consumption delays the human circadian melatonin rhythm in vivo and that chronic application of caffeine lengthens the circadian period of molecular oscillations in vitro, primarily with an adenosine receptor/cyclic adenosine monophosphate (AMP)–dependent mechanism. In a double-blind, placebo-controlled, ~49-day long, within-subject study, we found that consumption of a caffeine dose equivalent to that in a double espresso 3 hours before habitual bedtime induced a ~40-min phase delay of the circadian melatonin rhythm in humans. This magnitude of delay was nearly half of the magnitude of the phase-delaying response induced by exposure to 3 hours of evening bright light (~3000 lux, ~7 W/m^2) that began at habitual bedtime. Furthermore, using human osteosarcoma U2OS cells expressing clock gene luciferase reporters, we found a dose-dependent lengthening of the circadian period by caffeine. By pharmacological dissection and small interfering RNA knockdown, we established that perturbation of adenosine receptor signaling, but not ryanodine receptor or phosphodiesterase activity, was sufficient to account for caffeine's effects on cellular timekeeping. We also used a cyclic AMP biosensor to show that caffeine increased cyclic AMP levels, indicating that caffeine influenced a core component of the cellular circadian clock. Together, our findings demonstrate that caffeine influences human circadian timing, showing one way that the world's most widely consumed psychoactive drug affects human physiology.

INTRODUCTION

The circadian system is a key regulator of daily sleep-wakefulness timing, as well as of other physiological and behavioral processes. The timing of endogenous circadian clocks can be shifted by environmental factors such as light, feeding, physical activity, and pharmacological agents. For example, bright-light exposure around typical bedtime delays the master circadian clock and sleep timing in humans (*1*–*3*).

Caffeine is a methylxanthine that exerts its actions in part by opposing the effects of the neuromodulator adenosine through competitive binding to serpentine adenosine receptors (*4*, *5*) where it acts as an antagonist with inverse agonist activity (*6*, *7*). Caffeine binding to neuronal adenosine receptors causes release of excitatory neurotransmitters and alters intracellular cyclic adenosine monophosphate (cAMP) signaling and cellular metabolism in a wide range of cells and tissues (*5*, *8*). Caffeine also acts at several intracellular targets to modulate second messengers, including cyclic AMP. Caffeine competitively inhibits phosphodiesterases (PDEs), the enzymes that degrade cAMP. Thus, at the cellular level, caffeine can stimulate cAMP-dependent signaling by more than one mechanism. Caffeine also binds to intracellular calcium channel ryanodine receptors (RyRs) leading to intracellular Ca^{2+} release (*5*). Whether caffeine influences circadian timing in humans is unknown.

Caffeine lengthens the circadian period of conidiation rhythms in the red bread mold *Neurospora crassa* (*9*), the phototactic rhythm of the green alga *Chlamydomonas reinhardii* (*10*), and the activity rhythm of the fruit fly *Drosophila melanogaster* (*11*). Caffeine can also phase-shift the ocular compound action potential rhythm in the sea snail *Bulla goudiana* (*12*). Caffeine can acutely reduce the levels of the pineal hormone melatonin in humans on the day of administration (*13*, *14*) and induce immediate-early gene expression (c-*fos*) in the master circadian clock, the suprachiasmatic nucleus (SCN), in rodent models (*15*). Furthermore, caffeine can advance and delay the phase of the electrical activity rhythm in SCN from isolated rat and hamster brain (*16*, *17*) and lengthen the period of the activity rhythms in mice, as well as the hPer2 (human Period 2) rhythm in human osteosarcoma U2OS cells and the mPer2 (mouse Period 2) rhythm in mouse NIH3T3 fibroblasts (*18*). These findings suggest that caffeine may also influence human circadian timing. We therefore first tested the hypothesis that evening caffeine consumption would phase-delay the endogenous circadian melatonin rhythm. The onset of the melatonin rhythm, tested under constant conditions, is considered the most accurate and precise measure of circadian timing in humans. Melatonin is a hormone with rhythmic concentrations in bodily fluids that are driven by the SCN. Melatonin is also the primary hormonal signal to the body of internal biological night, and its onset initiates a physiological cascade that promotes sleep and associated physiological functions in humans. To understand caffeine's activity at the cellular level, we also explored mechanisms by which caffeine affects circadian timing by examining its influence on the circadian clock in human cells.

RESULTS

Caffeine delays circadian melatonin phase in humans

We investigated how evening caffeine influences the human circadian phase compared with evening exposure to broad-spectrum bright light,

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a potent environmental time cue for the human circadian clock (*1*, *19*). We also tested the combination of caffeine and bright light to determine if, together, they induced a greater phase shift than either alone. We conducted five approximately 49-day-long, circadian phase–shifting trials using a sensitive within-subject design (fig. S1). Circadian phase was examined under constant routine conditions (*2*, *20*) (constant wakefulness, semirecumbent posture, ambient temperature, and dim light, with meals equally distributed across the circadian cycle in hourly snacks) on the day before and after exposure to four randomized, double-blind, placebo-controlled interventions: dim-light placebo (~1.9 lux, ~0.6 W/m^2), dim-light caffeine (2.9 mg/kg body mass, equivalent to 200 mg caffeine in a 69-kg person), bright-light placebo (~3000 lux, equivalent to about one-third of the maximal light exposure provided by light therapy devices; ~7 W/m^2), and bright-light caffeine. Caffeine was administered 3 hours before the participants' habitual bedtime (Fig. 1), and the 3 hours of bright-light exposure began at habitual bedtime.

We found that the dim-light caffeine stimulus induced a significant phase delay that was ~40 min larger than for dim-light placebo—a large effect size (Cohen's d = 0.93). Bright light alone and bright-light caffeine induced phase delays of respectively ~85 and ~105 min more than that with dim-light placebo; these, too, were large effect sizes (d = 2.25 and 3.66) (Fig. 2A). Furthermore, bright-light caffeine significantly delayed the circadian phase more than did caffeine in dim light, also with a large effect size (d = 2.13) (Fig. 2A). No significant differences were observed between the effects of dim-light caffeine and bright-light placebo (P = 0.07) or between the effects of bright-light placebo and bright-light caffeine (P = 0.26), although effect sizes were large and medium, respectively (d = 1.17 and 0.79). Unexpectedly, bright-light caffeine did not induce a greater phase shift than did bright-light placebo. It is possible that the light intensity used was saturating for the phase-shifting response (*21*), and adding caffeine had no additional influence because of a ceiling effect. Thus, it will be important to test lower light intensities to determine whether caffeine potentiates phase shifts by light and also whether caffeine administered at the beginning of the light pulse has an influence on the induced phase shift.

In human circadian research, there have been few studies in which all subjects studied have been exposed to multiple phase-shifting stimuli, and thus, we compared the consistency of the phase-shift response to the stimuli tested. Because the period of the circadian clock is, on average, longer than 24 hours (*22*–*24*), under experimental dim-light placebo conditions, circadian phase in most individuals delays gradually from one day to the next. We observed a significant positive correlation between this gradual phase change under dim-light placebo and the magnitude of phase delay in response to bright-light placebo

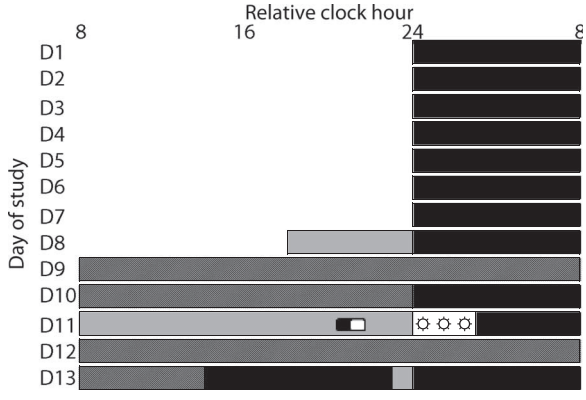

Fig. 1. Protocol for the human experiment. After about 7 days of ambulatory monitoring, participants remained in an environment free of external time cues (days 8 to 13) under dim light during scheduled wakefulness (~1.9 lux, ~0.6 W/m^2) and darkness during scheduled sleep (black bars) in the laboratory. Examples of in-laboratory procedures are as follows: day 8 included an 8-hour sleep opportunity; days 9 and 10 consisted of a 40-hour constant routine (hashed dark gray bars). On day 11, participants received either caffeine or rice powder–filled placebo (pill symbol) 3 hours before habitual bedtime and a 3-hour exposure (☼) to bright light (~3000 lux, ~7 W/m^2) or continued exposure to dim light (light gray bars; ~1.9 lux, ~0.6 W/m^2) beginning at habitual bedtime. Days 12 to 13 consisted of a 30-hour constant routine. Laboratory procedures were repeated four times over ~49 days (D1 to D49; fig. S1). Relative clock hour shown with the 2400 hour assigned to bedtime; actual times were dependent on and relative to the participant's habitual bedtime.

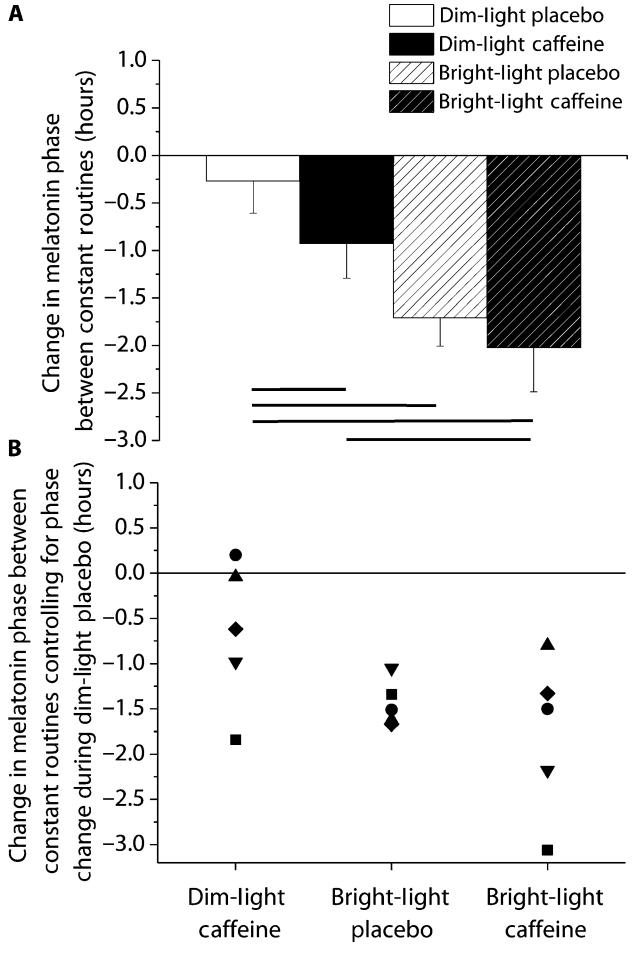

Fig. 2. Phase-shifting response for each condition. (**A**) Average phase shifts. Circadian phase delays are denoted as negative numbers and error bars represent SEM. Lines represent significant differences between conditions at endpoints of the line (Dunnett's test: dim-light placebo versus dim-light caffeine, P = 0.011; dim-light placebo versus bright-light placebo, P = 0.0007; dim-light placebo versus bright-light caffeine, P = 0.0003). Data are mean ± SEM, n = 5. (**B**) Individual differences in the phase-shifting response controlling for phase change during the dim-light placebo control condition. Symbols represent individual subjects.

(Fig. 3B) (r = 0.95, P = 0.015). We also observed a significant positive correlation between the phase shifts induced by dim-light caffeine and bright-light caffeine (Fig. 3A) (r = 0.94, P = 0.016), such that the greater the phase delay in response to dim-light caffeine, the greater the phase delay in response to bright-light caffeine. No correlation was observed between phase shifts induced by bright-light placebo and dim-light caffeine (r = 0.17, P = 0.78) or between bright-light placebo and bright-light caffeine (r = 0.33, P = 0.58).

Furthermore, we quantified whether there was systematic interindividual variability in the phase-shifting response to the stimuli tested using the intraclass correlation coefficient (ICC). The ICC was used to test the consistency of the phase-shift magnitude for the caffeine and light stimuli in individuals. We found ICCs to be in the fair range (ICC = 0.31) for the phase-shift response between the two placebo conditions and in the moderate range (ICC = 0.55) between the two caffeine conditions. The latter shows stable individual differences in response to placebo and to caffeine stimuli because 31 to 55% of the variance in the phase-shift response was explained by stable shifts among individuals. Such findings suggest that there are robust, stable individual differences in the phase-shifting response to caffeine. ICCs between light and caffeine stimuli (for dim-light caffeine and bright-light placebo, slight ICC = 0.12; for bright-light placebo and bright-light caffeine, fair ICC = 0.32; for dim-light placebo and bright-light caffeine, slight ICC = 0.20; for dim-light placebo and dim-light caffeine, fair ICC = 0.39) show that the consistency of individual differences in response to these stimuli is less and suggest that these phase-resetting stimuli may work through different mechanisms on the human circadian clock. Thus, the slight ICCs between phase shifts for dim-light caffeine and bright-light placebo and for dim-light placebo and bright-light caffeine indicate that the proportion of variance in the data explained by systematic interindividual variability is less when comparing light and caffeine stimuli. Because participants were exposed to all interventions, our protocol permitted control for the influence of individual differences in the circadian period (*23*) on the phase-shifting response (*2*, *24*). This procedure increased our sensitivity to detect phase shifts when using a relatively small number of subjects. Four of five subjects in the dim-light caffeine condition and five of five subjects in the bright-light placebo and bright-light caffeine conditions showed delays compared to those in the dim-light placebo control condition. When controlling for responses to the dim-light placebo control condition, we observed individual differences in phase-shifting to caffeine, to bright light, and to their combination (Fig. 2B). Caffeine alone and in combination with bright light showed larger individual differences in the phase-shifting response when compared to bright light only (Pitman-Morgan test: dim-light caffeine, P = 0.06; bright-light caffeine, P = 0.034). It thus appears that caffeine increases individual differences in the phase-shifting response to light, perhaps indicating an influence of caffeine on light-induced phase shifts or an influence of the integration of photic and nonphotic stimuli by the circadian clock. Why larger individual differences in the phase-shifting response to caffeine occur is unclear. They may, however, be related to genetic or epigenetic variations in caffeine pharmacokinetics and sensitivity to caffeine. For example, polymorphism in the adenosine A_{2A} receptor gene (ADORA2A) is linked with individual differences in sleep disruption effects of caffeine and habitual caffeine consumption (*25*–*27*).

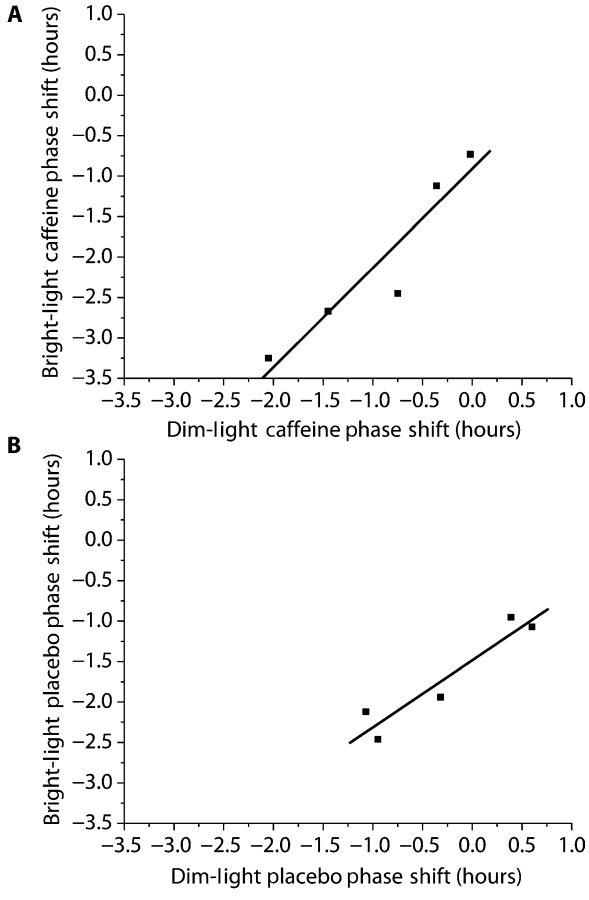

Fig 3. Association between phase shifts induced by different conditions. (**A**) Dim-light caffeine with bright-light caffeine. (**B**) Dim-light placebo and bright-light placebo. Symbols represent individual subjects, and solid line represents the best linear fit to the data.

Caffeine affects circadian timekeeping in human cells through an adenosine receptor–dependent mechanism

The variation in the phase-shifting response in humans could be more readily explained if the mechanism by which caffeine affects the circadian clock were better understood. To directly address this question, we used an in vitro assay based on bioluminescent reporters of circadian transcriptional rhythms in the well-characterized human U2OS cell line. Period lengthening in response to chronic caffeine has been reported both at the behavioral level in rodents and flies (*11*, *18*, *28*) and at the cellular level in isolated rodent SCN ex vivo and human U2OS cells in vitro (*16*, *18*). Human U2OS cells recapitulate the key effects of caffeine investigated here (*18*) and, similar to the SCN, primarily express the higher-affinity, ubiquitous, and abundant A_1 adenosine receptor (A_1R; encoded by the ADORA1 gene) (*8*, *29*) as well as PDEs and RyRs. U2OS cells thus seemed an ideal platform with which to delineate the cellular target of caffeine in the context of its action on circadian rhythms in humans.

As an antagonist of broadly expressed adenosine receptors, caffeine can attenuate the regulation of adenylyl cyclase activity by extracellular adenosine. In the case of the A_1R, caffeine blocks the activation of $G_{i/o}$ by adenosine, effectively increasing cAMP production (*30*, *31*). As noted, caffeine also has activity against intracellular targets: as an inhibitor of PDE, acute caffeine can reduce the rate of cAMP degradation; as an activator of RyR, acute caffeine increases calcium release from intracellular

stores. Dynamic cAMP signaling is required for normal circadian timekeeping, and chronic modulation of cAMP turnover lengthens the period of cellular circadian rhythms (*32*). Furthermore, period lengthening has previously been reported in response to drugs known to be more selective than caffeine (a weak ligand) for either adenosine receptors (CGS-15943, a potent non-xanthine adenosine antagonist/inverse agonist) or PDEs [3-isobutyl-1-methylxanthine (IBMX)] (*32*–*34*). Thus, caffeine, CGS-15943, and IBMX all lengthen the circadian period in cultured human cells; in contrast, we observed that chronic administration of ryanodine had no effect on cellular timekeeping (fig. S2), so we excluded the RyR from further consideration as the caffeine target mediating the effects on circadian rhythms.

To determine whether caffeine's effect on the cellular clockwork is primarily through PDEs or adenosine receptors, we exploited the differing pharmacodynamics and selectivities of CGS-15943 and IBMX during functional assays of cellular timekeeping to compare the relative caffeine-sensitive contributions of the two potential mechanisms. Using the dose-dependent effects of the two drugs on the cellular circadian period detected by bioluminescence, we analyzed their interactions at several concentrations to pharmacologically dissect the mechanism by which caffeine interacts with the cellular clockwork. We also used a noncompetitive inhibitor of adenylyl cyclase [9-(tetra-hydro-2-furanyl)-9H-purin-6-amine (THFA)], which slows the rate of $G_{s\alpha}$-stimulated cAMP synthesis (*32*) and lengthens the circadian period in vitro and in vivo across a wide range of model systems (*32*, *35*, *36*), as a control to confirm that caffeine's effect is cAMP-mediated. We hypothesized that either CGS-15943 or IBMX lengthens the circadian period by acting through the same cellular target as caffeine. When two drugs act at the same site, the target saturates at a lower effective concentration. Therefore, when applied in combination with caffeine, the drug that exhibits less-than-additive period lengthening is acting through the same cellular target as caffeine. If the postulates underlying our hypothesis were correct, we predicted that, because CGS-15943 and IBMX target different mechanisms within the same signaling pathway, the slope of the dose response for the combined action of the two drugs on the circadian period should be equal to the sum of their effects when applied separately. We observed this to be the case [Fig. 4, A and B (upper panels), and figs. S3 and S4]. As anticipated, we found that caffeine lengthened the circadian period reported by bmal1:luc and per2:luc in a dose-dependent manner [figs. S3, A and B, and S4, A and B (left panels)], consistent with previous findings with the Period 2 promoter (*18*). We found that lengthening of the circadian period by caffeine is significantly less than additive with increasing doses of CGS-15943, implying that caffeine and CGS-15943 act on the same target. Moreover,

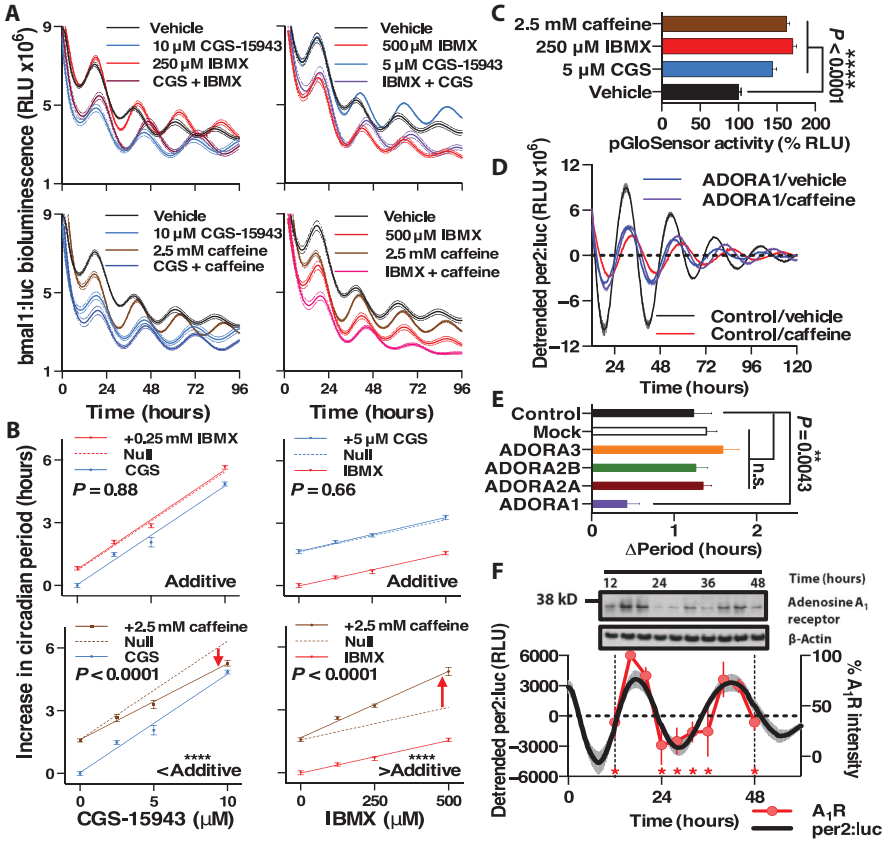

Fig. 4. Caffeine increases the circadian period in cultured human cells in vitro in an adenosine receptor/cAMP–dependent fashion. (**A**) Representative examples of grouped raw bioluminescence data (mean ± SEM, $n = 6$) showing the effect of different concentrations and combinations of IBMX, CGS-15943, and caffeine on human U2OS cells stably expressing bmal1:luc. (**B**) Grouped quantification of circadian period (mean ± SEM, $n = 6$) showing dose-dependent lengthening of the circadian period in response to CGS-15943 (blue, left panels) or IBMX (red, right panels) ± a fixed concentration of another period-lengthening drug [either 0.25 mM IBMX (red), 5 μM CGS-15943 (blue), or 2.5 mM caffeine (brown)]. Solid line depicts the linear regression in each case ($R^2 \geq 0.98$), with broken lines representing the null hypothesis (null, simple additive drug action, that is, no change in the slope). In each subpanel, sum-of-squares *F*-test *P* values are reported, where $P < 0.05$ indicates rejection of the null hypothesis (same slope for both groups). The significance and drug additivity are summarized below. Red arrows indicate that caffeine acts synergistically with IBMX but less than additively with CGS-15943. (**C**) All three drug treatments significantly increase cAMP signaling reported by pGloSensor activity over 6 days, plotted as mean ± SEM ($n = 4$) relative to vehicle control. $P < 0.0001$ by one-way ANOVA and by Bonferroni's multiple comparisons test for each drug versus vehicle. (**D**) ADORA1 knockdown attenuates the period-lengthening effect of 2.5 mM caffeine in U2OS cells; representative detrended group mean ± SEM is shown ($n = 4$). (**E**) Grouped quantification of period lengthening by caffeine after siRNA knockdown of each adenosine receptor isoform ($n = 7$ or 8); $P = 0.0002$, one-way ANOVA. By Bonferroni's multiple comparisons test, $P = 0.0043$ for control versus ADORA1, with no significant difference versus any other group (n.s., $P > 0.67$). (**F**) A_1R is rhythmically expressed in U2OS cells in phase with per2:luc. Upper panel, representative A_1R immunoblot; lower panel, normalized grouped A_1R abundance (mean ± SEM, $n = 3$; $P < 0.0001$ by one-way ANOVA; * indicates $P < 0.0001$ for each time point versus 16 hours by Bonferroni's multiple comparisons test). Per2:luc rhythms, recorded in parallel ($n = 4$), are shown for reference. RLU, relative light units.

caffeine acts more than additively (synergistically) with increasing doses of IBMX, indicating that caffeine and IBMX cannot be competing at the same cellular targets in this context. As anticipated, THFA, an inhibitor of adenylyl cyclase that acts upstream of PDEs but downstream of adenosine receptors in cAMP signal transduction, modulated the effect of caffeine, IBMX, and CGS-15943 (figs. S3 and S4), supporting the role of cAMP as the second messenger system stimulated by caffeine in this context (*32*, *37*). We confirmed this by using a well-characterized posttranslational cAMP intramolecular complementation biosensor (pGloSensor). Caffeine, CGS-15943, and IBMX all increased average cAMP levels over 6 days by ~50%, at concentrations that evoked intermediate increases in period (Fig. 4C).

To further substantiate that the effect of caffeine on the cellular clock is attributable to its action at adenosine receptors, we predicted that incubating human cells with an alternative selective adenosine receptor antagonist would result in a dose-dependent lengthening of the circadian period. We chose the well-characterized xanthine derivative 8-(*p*-sulfophenyl)theophylline (8-SPT) (*31*) because it is membrane-impermeant and therefore cannot act at intracellular sites (PDE and RyRs) and because it is structurally unrelated to CGS-15943. We further predicted that, as with CGS-15943, co-application with 2.5 mM caffeine would reduce the gradient of the dose-dependent period lengthening elicited by 8-SPT. We expected caffeine and 8-SPT to compete for the same target, adenosine receptors, which can be saturated. Both predictions were confirmed to be the case (fig. S5).

We next asked whether a specific adenosine receptor is responsible for the effect of caffeine on cellular rhythms. To address this, we assessed the effect of caffeine on cells treated with small interfering RNA (siRNA) to knock down each of the human adenosine receptors, using cocktails of previously validated target-specific siRNAs at two different siRNA/cell ratios. Only with the knockdown of ADORA1 expression did we observe a significant attenuation of the dose-dependent lengthening of the cellular circadian period (Fig. 4, D and E, and fig. S6). Moreover, by immunoblotting for A_1R in U2OS time courses, sampled at 4-hour intervals, with bioluminescence recorded from parallel cultures, we observed that A_1R protein levels oscillated in phase with per2:luc activity (Fig. 4F). It is thus plausible that the major action of caffeine on the cellular clockwork is to attenuate an endogenous circadian regulation of cAMP-mediated inputs to the transcriptional clockwork, perhaps through functional cAMP/Ca^{2+}-response elements in the Period 1 and Period 2 promoters (*32*). If daily cycles of A_1R expression occur in human cells and tissues in vivo, then they may play a similar role to that proposed for RGS16 and CRYPTOCHROME1/2 in mouse SCN and liver, respectively (*35*, *38*).

Together, our in vitro findings therefore indicate that caffeine influences circadian timing by acting primarily through A_1R, thereby modulating endogenous regulation of cAMP signaling, a core component of the cellular circadian clock (*32*). We cannot exclude the possibility that additional targets for caffeine might be of relevance in vivo.

DISCUSSION

Our findings demonstrate that caffeine affects the phase of the human circadian clock, as measured by the melatonin rhythm driven by the human SCN, and that caffeine primarily affects human cellular circadian clocks by an A_1R/cAMP-dependent mechanism. Administration of evening caffeine induced a phase delay that was nearly half the size of the phase shift induced by evening exposure to bright light. Continuous exposure of human cells in vitro to caffeine also lengthened the period of the cellular circadian clock, in a manner dependent on adenosine receptor/cAMP signaling. Whether caffeine also influences circadian timing in peripheral tissues (*39*) in vivo (for example, liver and extra-SCN neuronal clocks) remains to be determined. Nevertheless, our observation that A_1R levels cell-autonomously oscillate in human U2OS cells is complemented by the observation that ADORA1 transcript levels are also circadian-regulated in mouse in vivo, for example, in the heart, liver, and adipose tissues (*40*).

Although our experiments show that caffeine can delay the human circadian clock in vivo and lengthen the period of the human circadian clock in vitro, further research is needed with similar sensitive within-subject experimental designs and additional circadian phase markers to explore whether caffeine can also phase-advance circadian timing and whether our dose-dependent responses in vitro translate to dose-dependent responses in vivo. These studies were performed in a transformed cell line; therefore, it will be important to confirm whether these observations hold true in nontransformed cells. Future studies should also test the influence of caffeine on circadian timing across a range of cell and tissue types and whether the A_1R remains primarily responsible in each case. Furthermore, we cannot discount a direct interaction between caffeine and photic inputs to the SCN master clock in vivo. For example, adenosine is reported to influence retinal function and transmission of photic information through the retinohypothalamic tract (RHT). Moreover, adenosine administration has been reported to attenuate phase shifts induced by light exposure (*41*), and caffeine has been shown to enhance the period-lengthening effects of light in mice (*28*). Furthermore, adenosine receptor mRNA is expressed in retinal ganglion cells (*42*); whether these include the melanopsin retinal ganglion cells that project to the SCN is unknown. Adenosine may reduce photic input to the SCN by altering RHT transmission and glutamate release onto the SCN (*30*).

The finding that caffeine influences human circadian physiology may have implications for the pathophysiology and treatment of some circadian sleep-wake disorders. For example, in addition to increasing daytime exposure to sunlight and reducing evening exposure to electrical light (*19*), avoiding evening caffeine may help to treat problematic delayed sleep timing through circadian as well as established wakefulness-sleep mechanisms (*5*). Our phase-delay results with caffeine may also confirm the reported association between higher caffeine intake and being an evening chronotype (*43*). Properly timed caffeine use may also be of benefit with respect to shifting circadian timing, potentially assisting with circadian adaptation to large phase delays required when flying across many time zones westward, as well as sustaining wakefulness until bedtime in the new time zone. Trials are needed to test the latter, and it will be important to monitor for caffeine-induced sleep disruption under such conditions, which could worsen jet lag.

MATERIALS AND METHODS

Human participant protocol

Twenty phase-shifting protocols were conducted with five healthy participants [three females; age, 24.0 ± 2.8 years; BMI, 23.9 ± 0.9; weight, 70.6 ± 11.9 kg (mean ± SD)] who maintained regular approximately 8-hour sleep-wakefulness schedules based on habitual sleep and wake times for about 7 days before laboratory visits (range, 4 to 10 days; Fig. 1)

verified by sleep logs, voicemail time-stamped sleep and wake times, and wrist actigraphy (Actiwatch-L, Mini Mitter/Respironics). Participants abstained from over-the-counter medications, supplements, and caffeine for 2 weeks, naps for 1 week, exercise for 3 days, and alcohol for 2 days before and throughout the experiment [exclusion criteria are as follows: medical, psychiatric, or sleep disorders as determined by history, physical, and psychiatric exams; abnormal blood chemistries (comprehensive metabolic panel and complete blood cell counts) or clinical electrocardiogram; drug, nicotine, or medication use; habitual sleep duration of <7 or >9 hours; BMI of <18.5 or >27; pregnancy; shift work or living below the local 1600-m altitude in the previous year; travel of >1 time zone 3 weeks prior]. Urine toxicology and alcohol breath testing were performed each visit. All participants gave written informed consent, and procedures were approved by the University of Colorado Boulder Institutional Review Board and the Colorado Clinical and Translational Sciences Institute Scientific Advisory and Review Committee.

Participants arrived about 6 hours before habitual bedtime each laboratory visit. Double-blind conditions were performed by the Clinical and Translational Research Center pharmacist who provided pills identical in appearance. The allocation sequence was concealed until interventions were assigned, and data were prepared for statistical analysis. The pills were five capsules, containing rice flour placebo or caffeine (total dose, 2.9 mg/kg; Gallipot Inc.). Ceiling-mounted fluorescent lamps (Sylvania OCTRON 32W T8 bulbs) provided broad-spectrum white-light exposure with a color temperature similar to sunlight at midday (6500 K). During the 3-hour light exposure session, subjects were under the direct supervision of research assistants who remained in the suite to ensure that the intended intensity of illumination was achieved. Subjects maintained constant posture while alternating between fixing their gaze on a target for 6 min or free gaze for 6 min. Average light intensities during the fixed gaze were 2985 ± 388 lux (mean ± SD) for bright-light and 1.9 ± 0.4 lux for dim-light interventions. Light exposure was timed to induce a maximal phase delay based on the published phase and illuminance response curves (*3*, *21*). Saliva for melatonin assessment was collected every 30 to 60 min and frozen at −80°C until assayed by enzyme-linked immunosorbent assay (IBL International).

Human phase shift data analysis

Phase shifts were determined as change in timing of dim-light melatonin onset (DLMO) between constant routines. The salivary DLMO was defined as the linearly interpolated time point when melatonin levels exceeded and remained 2 SDs above the stable baseline mean (*20*). Data were analyzed with repeated measures analysis of variance (ANOVA) and using planned comparisons with Bonferroni correction for multiple comparisons. One-tailed Dunnett's test was used for hypothesis-driven comparisons versus dim-light placebo control, and dependent *t* tests were used for caffeine and bright-light comparisons. Associations for phase shifts between conditions were analyzed with Pearson's *r* and intraclass correlations. A two-way mixed-model ANOVA was used to derive ICC with subject as a random factor and phase-shifting stimuli as a fixed factor. The ICC model (*44*) used the following formula:

$$\frac{MS_S - MS_E}{MS_S + (k-1) * MS_E + k/n(MS_C - MS_E)}$$

where MS_S is the mean square subject, MS_E is the mean square error, k is the number of conditions, n is the number of subjects, and MS_C is the mean square condition. The following arbitrary benchmarks from Landis and Koch (*45*) were used to describe the strength of agreement of ICC scores: poor, <0.00; slight, 0.00 to 0.20; fair, 0.21 to 0.40; moderate, 0.41 to 0.60; substantial, 0.61 to 0.80; almost perfect, 0.81 to 1.00. Statistical analyses were performed using Statistica (version 10.0, StatSoft Inc.). Effect sizes (Cohen's *d*) were calculated to determine the size of phase-shifting effects. Standard interpretations of Cohen's *d* effect size were used: small, $d = 0.2$; moderate, $d = 0.5$; large, $d = 0.8$. Pitman-Morgan tests were used for post hoc comparisons of the difference in variance between paired conditions. Circadian DLMO phase relative to bedtime before interventions was similar ($P = 0.41$): dim-light placebo, −1.7 ± 0.9 hours [mean ± SD; 95% confidence interval (CI), −2.8 to −0.6 hours]; dim-light caffeine, −1.5 ± 0.6 hours (95% CI, −2.2 to −0.7 hours); bright-light placebo, −2.3 ± 1.2 hours (95% CI, −3.8 to −0.8 hours); bright-light caffeine, −2.1 ± 0.9 hours (95% CI, −3.2 to −0.9 hours). Phase shift data were normally distributed.

Cell culture protocol

Human U2OS cells stably expressing per2:luc or bmal1:luc were generated as described (*46*). One day before bioluminescence imaging, cells were trypsinized, resuspended in culture medium, seeded into six 96-well white plates (LUMITRAC, Greiner Bio-One) at a density of 10^4 cells per well, and incubated in a humidified incubator for 24 hours (37°C, 5% CO_2). After 24 hours, when the cell monolayer was observed to be 100% confluent in clear duplicate plates, the cell medium was replaced with 100 μl of Air Medium containing various drugs, as described (*47*). Stock solutions of IBMX and CGS-15943 were dissolved in dimethyl sulfoxide (DMSO) at 100 and 20 mM, respectively, before being diluted to working concentrations in Air Medium. THFA, 8-SPT, and caffeine stock solutions (100 mM) were directly mixed in Air Medium. Final DMSO content was controlled in each 96-well plate and never exceeded 0.5%, a concentration observed to have no significant effect on the circadian period of U2OS bioluminescence rhythms under these conditions (figs. S3 and S4). Plates were sealed with adhesive film (Thermo Scientific) and immediately transferred to the recording incubator. Bioluminescence imaging was performed at 37°C over 6 days in the alligator incubator system (Cairn Research) with $n = 6$ replicates for each experimental condition. Drug concentrations that have previously been shown to lie within the quasilinear portion of the dose-response curve [around the median inhibitory concentration (IC_{50}) value], with respect to the effects on cellular circadian period (*18*, *32*, *33*), were used. For comparison of cellular cAMP signaling activity, the U2OS cells stably expressing the pGloSensor bioluminescent cAMP reporter (Promega) were generated, with the recording taking place over 6 days under conditions similar to the circadian bioluminescence assays, except that, to facilitate reporter activity, 1 mM luciferin was included in the recording medium and cells were continuously incubated at 30°C. Total bioluminescence values collected over 6 days were normalized to vehicle controls.

All siRNA reagents were purchased from Santa Cruz Biotechnology. Pools of target-specific siRNAs against each human adenosine receptor (sc-39848, sc-39850, sc-29642, sc-39854) or a control siRNA (sc-37007) were transfected into per2:luc U2OS cells in 96-well plates, using the siRNA Reagent System (sc-45064) following the manufacturer's recommended protocol, with either 1.25 or 3.75 pmol of siRNA per well. Mock, or untransfected, cells were also assayed to control for any nonspecific effect of siRNA transfection on cellular rhythms. Bioluminescence assays, or lysis of parallel cultures for immunoblotting, were begun after 72 hours, when cells were fully confluent.

Western blotting

Cells washed in ice-cold phosphate-buffered saline buffer, then lysed directly in 2×LDS (lithium dodecyl sulfate) sample buffer (Life Technologies) supplemented with 10 mM TCEP [tris(2-carboxyethyl)phosphine], and heated at 70°C for 10 min. Western blotting was performed using the Novex NuPAGE system (Life Technologies) under reducing conditions with 4 to 12% bis-tris gradient gels with MES buffer following the recommended protocol. Protein transfer to nitrocellulose for blotting was performed using the iBlot system (Life Technologies). Blocking and all antibody incubations were performed in 0.25%/0.25% (w/w) bovine serum albumin/nonfat dried milk (Marvel) in tris-buffered saline/0.05% Tween 20 (TBST). After 1 hour in blocking buffer and three brief washes in TBST, primary antibody incubations were performed overnight at 4°C with 1:5000 anti-actin (sc-47778, Santa Cruz Biotechnology), anti-A_1R (55026-1-AP, Proteintech), and antibodies against human adenosine A_{2A}, A_{2B}, and A_3 receptors (ab3461, ab40002, and ab136051, respectively; Abcam). Membranes were washed in TBST three times for 10 min and then incubated with the appropriate 1:10,000 horseradish peroxidase–conjugated secondary antibody (Sigma-Aldrich) for 60 min. After four 10-min TBST washes, chemiluminescence detection was done using Immobilon ECL reagent (Merck Millipore).

Bioluminescence data analysis

Analysis of bioluminescence data was done using a modified version of the CellulaRhythm algorithm (*48*). Raw luminescence data were detrended by fitting a fourth-order polynomial function using nonlinear regression. The detrended traces were then fitted to a damped cosine curve using nonlinear least squares to the following equation:

$$x(t) = \text{baseline} + mt + \text{amplitude}(e^{-kt})\cos(2\pi(t - \text{phase})/\text{period})$$

where m is the slope, k is the damping rate, and t is time. Because of transient luminescence changes after medium changes, the first 20 hours of luminescence data were excluded from the analysis. Graphs were plotted and statistical analyses were performed in GraphPad Prism version 6.0 for Mac (GraphPad Software).

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/7/305/305ra146/DC1

Fig. S1. Forty-nine day protocol for human experiment.

Fig. S2. Ryanodine does not lengthen the circadian period reported by per2:luc in cultured human cells in vitro.

Fig. S3. Caffeine increases the circadian period reported by bmal1:luc in cultured human cells in vitro in an adenosine receptor/cAMP–dependent fashion.

Fig. S4. Caffeine increases the circadian period reported by per2:luc in cultured human cells in vitro in an adenosine receptor/cAMP–dependent fashion.

Fig. S5. Caffeine acts at the same site as 8-SPT to increase the circadian period in cultured human cells in vitro.

Fig. S6. Quantification of adenosine receptor siRNA knockdown efficacy.

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Effects of caffeine on the human circadian clock in vivo and in vitro

Tina M. Burke *et al.*

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Editor's Summary

Your daily drug resets your clock

Your morning cup of coffee may be shifting your circadian clock. Burke *et al*. show that caffeine—widely available, legal, and psychoactive—inserts a delay into the ~24-hour metabolic rhythm that keeps your body running in time with the world.

In a sensitive, within-subject experimental design, five people were kept under highly controlled conditions for 49 days. Before bedtime, they were given various treatments: either a double-espresso caffeine dose, exposure to bright or dim light, or a placebo. The caffeine delayed their internal clock by 40 min, a shift about half as long as bright light, a stimulus known to robustly lengthen the circadian phase.

The authors used cultured cells to determine that the drug acted directly on the adenosine receptor, which increases the intracellular messenger molecule cyclic AMP. The fact that cyclic AMP forms a key cog in the inner workings of the clock links caffeine's biochemical effects to its delay of the circadian rhythm.

Not only do these results reinforce the common advice to avoid caffeine in the evening, but they also raise the intriguing possibility that caffeine may be useful for resetting the circadian clock to treat jet lag induced by international time zone travel.

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