

Adenosine and caffeine modulate circadian rhythms in the Syrian hamster

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Extracellular adenosine accumulates in some brain areas during sleep deprivation. In Syrian hamsters, both sleep deprivation and adenosine A1 agonists can inhibit phase shifts of circadian rhythms to light at night. Sleep deprivation in the day (sleep period) can shift circadian phase. We examined whether the A1 agonist N-CHA mimics this effect. N-CHA (i.p. or i.c.) in the mid-sleep period induced dose-dependent shifts similar to

those induced by 3 h sleep deprivation. The adenosine antagonist caffeine administered systemically at the mid-sleep period induced arousal without shifts, and dose-dependently attenuated shifts to a 3 h sleep deprivation procedure (running in a novel wheel). Adenosine may participate in resetting of the circadian clock by manipulations of behavioral state. *NeuroReport* 12:2901–2905 © 2001 Lippincott Williams & Wilkins.

Key words: Adenosine; Caffeine; Circadian rhythms; N⁶-Cyclohexyladenosine; Sleep; Sleep deprivation

INTRODUCTION

Adenosine, a byproduct of cellular metabolism, functions in the nervous system as a neuromodulator through actions at four major receptor subtypes [1]. Adenosine has been hypothesized to play a role in sleep regulation, based on evidence that adenosine agonists increase slow-wave sleep and decrease arousal, that adenosine antagonists, such as caffeine and theophylline, increase arousal, and that extracellular adenosine accumulates during spontaneous and extended wakefulness and dissipates during subsequent sleep [1–4]. Short-term sleep deprivation (e.g. 3 h in the middle of the usual sleep period), by gentle handling [5] or continuous locomotion (reviewed in [6]), can induce large phase advance shifts of circadian rhythms in the Syrian hamster. Sleep deprivation can also attenuate phase shifts to brief light exposure at night [7]. Adenosine A1 receptor agonists mimic this inhibitory effect [8,9], raising the possibility that adenosine mediates one or more effects of acute sleep deprivation on the circadian clock. Here we report that an adenosine A1 agonist also mimics the phase shifting effects of sleep deprivation, and that the A1 receptor antagonist caffeine induces arousal without phase shifts, and attenuates the phase shifting effect of locomotor activity stimulated during the usual sleep period.

MATERIALS AND METHODS

Animals and housing: Young adult male hamsters (total 81; Charles River, PQ) were housed singly in standard polypropylene cages equipped with 17 cm running wheels monitored continuously by computer. Cages were maintained in ventilated isolation cabinets providing a 14:10 h light:dark cycle.

Surgical procedures: Hamsters ($n = 31$) were prepared with indwelling cannulas (22 gauge, Plastics One, Roanoke, VA) aimed at the suprachiasmatic nucleus (SCN), the site of the light-entrainable circadian pacemaker. Stereotaxic co-ordinates were 0.3 mm lateral from midline and 7.0 mm below the skull surface at bregma, with the incisor bar at 2 mm below the interaural line.

Behavioral procedures: Animals received i.p. or intracerebral injections of the adenosine A1 agonist N⁶-cyclohexyladenosine (N-CHA, RBI), the adenosine antagonist caffeine (RBI) or vehicle (50% DMSO/PBS) 6 h before the time of usual dark onset (zeitgeber time (ZT) 6, where dark onset is designated ZT12, by convention). Injection volumes were set at 1 ml/kg for i.p. injections, and at 0.5 μ l delivered over 30 s for cannula injections. Lights were then turned off for 3–4 days. Phase shifts were quantified by comparing the time of spontaneous activity onset (designated circadian time (CT) 12, by convention) on the second day after drug treatment with the average time of activity onset during the 5 days before drug treatment, as described elsewhere [5,7]. Phase changes were then compared between drug and vehicle conditions.

Immunocytochemistry: To determine whether the adenosine agonist N-CHA mimics the effects of sleep deprivation on basal fos expression in the SCN, hamsters received injections of N-CHA (2 mg/kg, i.p.) at ZT6, and were perfused transcardially with 50 ml PBS, followed by 4% paraformaldehyde at ZT11.5. Brains were postfixed overnight, and then cryoprotected in 20% sucrose for 24 h. Sections (50 μ m) were collected into PBS filled wells and then received the following treatments, separated by PBSx

(0.3% Triton-X-100 in PBS) rinses; 0.3% H₂O₂ in PBSx to inactivate endogenous peroxidase, 10% normal goat serum (NGS, Vector), 48 h at 4°C in the primary *fos* antibody (Oncogene Science, 1:80000 in 1% NGS-PBSx), 1 h in secondary goat-anti-rabbit antibody (Vectastain Elite Kit, Vector), 1 h in avidin-biotin complex (ABC, Vectastain Elite Kit, Vector), diaminobenzidine (DAB) reaction for 5 min (0.04% DAB in Tris buffer + 60 µl 8% NiCl and 10 µl 30% H₂O₂). Tissue was mounted on slides, dehydrated and coverslipped. Digital images were captured and analyzed using MCID (Imaging Research Inc., St. Catherine's, ON, Canada).

Data analysis: The results were evaluated by repeated-measures ANOVA with Student-Newman-Keul's *post hoc* analyses or paired *t*-tests. Pearson correlation coefficients were calculated to quantify relationships between phase shifts and physiological variables. Values are reported as mean $\pm$ s.d.

RESULTS

Pilot experiments established that systemic injections of N-CHA at ZT6 induced phase advance shifts at doses of 2.5 mg/kg (mean shift 113 ± 27 min, $n=2$) and 2.0 mg/kg (50 ± 24 min *vs* 25 ± 22 min in response to vehicle alone; paired *t*-test, $t(11)=2.35$, $p<0.05$), but not at 1.5 mg/kg (27 ± 26 min). Following injections, hamsters were hypoactive for at least 24 h and hypothermic for ~ 4 h. To minimize these acute side effects, N-CHA was microinjected directly into the SCN area. These injections induced significant, dose-dependent phase advance shifts (one-way RM-ANOVA, $F(3,25)=25.9$, $p<0.0001$, Fig. 1a,b,e) at ZT6, and significant phase delay shifts at ZT18 (i.e. 6 h after the usual time of lights off; paired *t*-test, $t(9)=2.868$, $p<0.01$, Fig. 1c-e). All cannula placements were within 400 µm of the SCN (measured by visual inspection of post-mortem, Nissl stained brain sections), and there was no relation between the position of the cannula and the size of phase shifts to N-CHA ($r=0.03$ at ZT6, 0.25 µg dose). Activity was reduced to $8 \pm 12\%$ baseline during the 24 h following N-CHA (0.25 µg) injections. Activity was also strongly suppressed for a day or more after some vehicle injections that were not associated with phase shifts (e.g. Fig. 1d).

Sleep deprivation by gentle handling (ZT6-9; [5]) or wheel running (ZT4.5-7.5; [10]) significantly suppresses basal Fos levels within the SCN. If endogenous adenosine participates in the phase resetting effect of sleep deprivation, then adenosine agonists may also suppress basal SCN Fos expression. As predicted, N-CHA (2 mg/kg, i.p., $n=4$) at ZT6 did significantly suppress basal SCN Fos by comparison with the vehicle control ($n=4$; one-tailed *t*-test, $t(7)=2.28$, $p<0.05$; Fig. 2), although not to the same extent observed following sleep deprivation [5]. Sleep deprivation procedures induce larger shifts on average than those observed here in response to N-CHA, suggesting that phase shift magnitude is related to the degree of Fos suppression.

The circadian clock is significantly reset within 1 h following a 3 h sleep deprivation from ZT6-9, as indicated by a phase advance of the circadian rhythm of light-induced *c-fos* expression in the SCN [5]. To determine if clock resetting by N-CHA is similarly rapid, hamsters

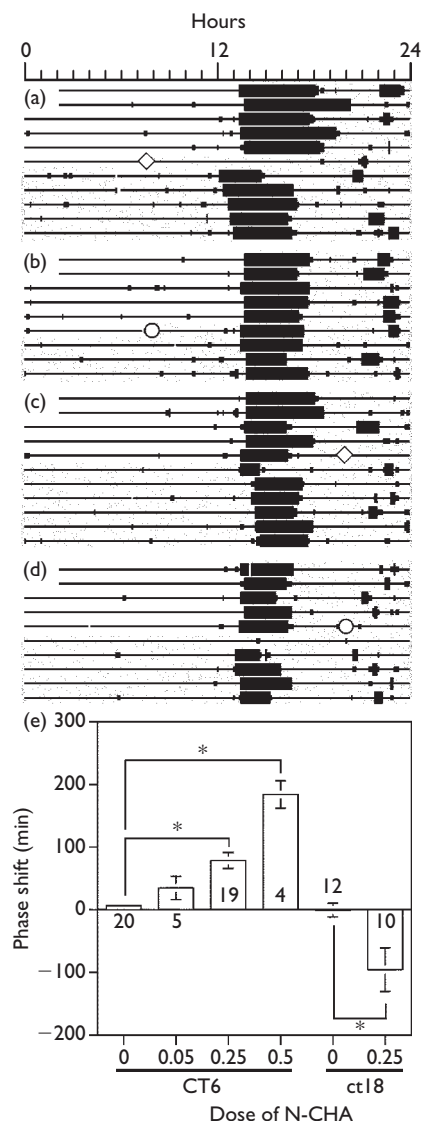


Fig. 1. Actograms from a representative hamster that received either N-CHA (diamonds, 0.25 µg in 0.5 µl 50%DMSO/saline) or vehicle control (circles) at either ZT6 or ZT18. Each horizontal row represents 24 h, with time in 10 min bins plotted from left to right. Vertical defections from the horizontal line represent bins during which wheel revolutions occurred. Shading represents lights-off period. (a) N-CHA at ZT6, (b) Vehicle at ZT6, (c) N-CHA at ZT18, (d) Vehicle at ZT18. (e) Bar graphs of mean ($\pm$ s.e.m.) phase advance shifts to intra-SCN injections of vehicle or N-CHA. *Significant at $p<0.01$.

($n=5$) received N-CHA (0.25 or 0.50 µg in 0.5 µl 50% DMSO/saline, intra-SCN) or vehicle ($n=3$) at ZT6, and a light pulse (15 min, 25 lux) at ZT11 (~ 1 h before the hamster SCN normally expresses Fos in response to light; [11]). If N-CHA phase advances the clock by at least 1 h by this time, then the light pulse should induce Fos in drug but not vehicle-treated animals, and Fos-positive cells should be concentrated ventrolaterally in the SCN, in the spatial pattern characteristic of photically induced Fos early in the active period. Both groups exhibited many Fos-positive cells but these were concentrated (although not

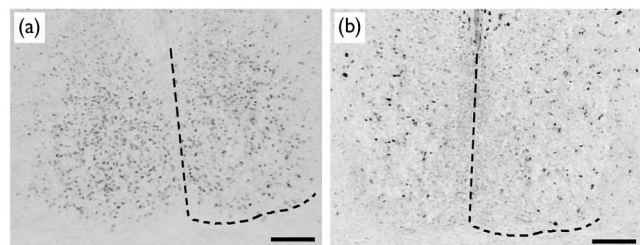


Fig. 2. Coronal photomicrographs of fos-IR at the mid-caudal level of the SCN (medial and ventral borders indicated by dashed lines). (a) Vehicle control injections at ZT6. (b) N-CHA (2mg/kg, i.p.) at ZT6. Animals were perfused at ZT11.5. Scattered Fos-IR cells are evident in N-CHA treated animal, but the number of cells and background Fos are lower than in the control animal. Bar = 100 μ m.

confined) ipsilaterally along the cannula track, and did not show the bilateral spatial distribution characteristic of photic induction. Cell counts taken from representative sections in the mid-caudal ventrolateral SCN, contralateral to the injection site, showed no difference between groups (56 ± 10 vs 42 ± 12 cell counts in vehicle and N-CHA groups, respectively; $t = 1.5$, $p > 0.1$).

The absence of Fos expression in the photic pattern in the N-CHA group could indicate that the clock was not shifted or that N-CHA has an extended inhibitory effect on photic activation of SCN neurons. To test the latter alternative, two additional groups were processed for Fos following N-CHA (0.25μ g, $n = 3$) or vehicle ($n = 3$) at CT8, and a light pulse at CT13, when the Fos response to light is robust. Both groups again exhibited Fos associated with the injection site, but only the vehicle-treated hamsters also expressed a concentration of Fos-positive cells ventrolaterally in the SCN, in the pattern typical of photic stimulation (75 ± 42 vs 17 ± 7 cell counts in vehicle and N-CHA groups, respectively; $t = 2.35$, $p < 0.05$). This result is consistent with recent reports that N-CHA can inhibit photic input to the clock [8,9], and further demonstrates that inhibition can be prolonged (at least 5 h).

Resetting of behavioral rhythms and suppression of basal Fos within the SCN by an adenosine agonist is consistent with a role for adenosine in the induction of phase shifts by acute sleep deprivation. We further tested this hypothesis by determining whether an adenosine receptor antagonist can inhibit phase shifting to sleep deprivation procedures. The adenosine A1 receptor antagonist caffeine (75 mg/kg , i.p., delivered at ZT5.5) induced substantial arousal (assessed at 1 min intervals by trained observer), with behavioral sleep reduced to 6 ± 2 , 14 ± 5 and 31 ± 5 min, respectively, during each of the first 3 h following treatment, compared to hourly averages of 50 ± 2 , 56 ± 1 and 55 ± 1 min sleep following vehicle injections. Despite stimulating arousal, caffeine did not induce phase shifts significantly different from saline (1 ± 19 min vs -2 ± 23 min, paired t -test, $t(10) = 0.390$, $p > 0.10$).

In a second test, hamsters were confined to novel running wheels for 3 h beginning at ZT6, to stimulate arousal by running. This procedure reliably induces large (~ 2 – 3 h) phase advance shifts in hamsters that run continuously [12]. Caffeine dose-dependently attenuated phase shifts to this sleep deprivation procedure (one-way RM-

ANOVA, $F(3,27) = 3.75$, $p < 0.05$, Fig. 3a–c). The high dose and vehicle control tests were repeated in an additional 30 animals, again yielding a significant difference (mean shifts 23 ± 7 min vs 80 ± 13 min, respectively; paired t -test, $t(29) = 5.348$, $p < 0.0001$). There was no effect of caffeine treatment on activity level during wheel confinement (paired t -test for the highest dose vs vehicle, $t(41) = 0.29$, $p > 0.1$).

DISCUSSION

Previous work in Syrian hamsters has demonstrated that phase shifts to light exposure early in the night can be attenuated by the adenosine A1 agonist N-CHA [8,9] or by short-term (6 h) sleep deprivation [7]. Given that sleep deprivation is associated with increased levels of extracellular adenosine in at least some areas of the CNS [2], adenosine may serve as a neurochemical signal by which manipulations of behavioral state alter functional properties of the circadian clock. The results reported herein provide additional support for this hypothesis by demonstrating that N-CHA mimics the clock resetting effect of sleep deprivation, and has a prolonged inhibitory effect on an immediate-early gene response of SCN cells to a photic stimulus. Moreover, the A1 antagonist caffeine dose-dependently inhibited phase shifts to arousal with or without continuous locomotor activity during the usual sleep period.

Whether the effects of N-CHA and caffeine on clock

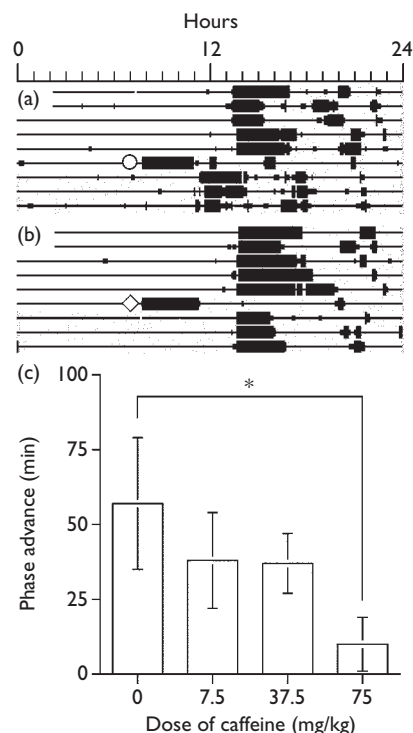


Fig. 3. Actograms from a representative hamster illustrating phase shifts to 3 h wheel confinement procedure at ZT6, following pretreatment at ZT5.5 with (a) PBS (circles) or (b) caffeine (diamonds, 75 mg/kg i.p. in PBS). (c) Bar graph of mean ($\pm$ s.e.m.) phase advance shifts to 3 h of wheel confinement following caffeine or vehicle injections, from eight hamsters providing data at all doses. *Significant at $p < 0.01$.

function are mediated by receptors within the SCN or at sites afferent to the clock is a complex issue to be resolved. Neuronal activity and metabolism within the SCN are maximal during the subjective day, which corresponds to the daily sleep period in the nocturnal Syrian hamster. Neurotransmitters that phase advance the circadian clock when administered to the SCN in the subjective day have a predominantly inhibitory action on SCN neurons (e.g. GABA, benzodiazepines, serotonin, neuropeptide Y) [13,14]. Spontaneous locomotor activity is also associated with suppression of SCN neuronal activity [15,16]. Adenosine and N-CHA, which inhibit neuronal activity via postsynaptic A1 receptors, presumably share with these other stimuli the property of hyperpolarizing SCN neurons, and this common effect on membrane potential may be the means by which phase advance shifts are induced in the subjective day.

Although exogenous adenosine agonists may mimic the phase shifting effect of sleep deprivation by actions at SCN A1 receptors, it is not clear that endogenous extracellular adenosine within the SCN plays this role *in vivo*. Given that neural activity within the hamster SCN is high during the usual sleep period and is attenuated during periods of locomotor activity, extracellular adenosine in the hamster SCN is more likely to accumulate during sleep, and to dissipate when hamsters are aroused. Although direct evidence for this is lacking, this raises the intriguing possibility that the daily rhythm of SCN neural activity, and extracellular accumulation of adenosine, might normally contribute to the relative insensitivity of the circadian clock to light during the day, possibly via presynaptic actions at retinal terminals [17]. These considerations also suggest that we should consider sites outside of the SCN where adenosine accumulation, and exogenous agonists, might exert clock resetting effects similar to those that follow sleep deprivation.

A candidate area is the basal forebrain. N-CHA administered directly to the SCN could diffuse into the basal forebrain directly, or via leakage into the third ventricle. Actions of N-CHA in this region would be expected to induce sleep [18] and alter activity of cholinergic and other neurons afferent to the SCN [19]. The suppression of locomotor activity that followed intra-SCN infusions of N-CHA supports the possibility of actions at a distance from the injection site, although it is also possible that behavioral suppression is a local effect. Wheel running in hamsters is typically decreased by SCN lesions, and N-CHA may simulate a reversible SCN ablation by inhibiting SCN output.

The behavioral suppression induced by peripheral and central adenosine injections had an intriguing similarity to torpor, particularly at the higher systemic doses. Wheel running was greatly suppressed, and the animals were noticeably cool. During most observations, the animals appeared asleep in a prone position that was distinct from the typical sleep posture. Although arousal threshold appeared elevated, this state was rapidly reversed when the animals were disturbed, which enabled us to ensure that the animals received the photic stimulus. Also, the hamsters usually exhibited at least some spontaneous wheel running during the period of maximal behavioral suppression. Whether this was a true state of torpor

triggered by hypothalamic A1 receptor stimulation remains to be examined further. However, clock resetting was likely independent of behavioral and metabolic suppression, for several reasons. First, suppression of locomotor activity at night, by restraint, induces small phase delays of circadian rhythms [20] (Antle and Mistlberger, unpublished observations), rather than the large phase advances evident in the present study. Second, vehicle injections also suppressed activity in some cases, but did not produce large phase shifts. Third, cooling can induce large phase shifts in hamsters, but only if body temperature is reduced below 28° deg for 12h or longer, which is a much more severe hypothermia than that observed in the present study. Moreover, only phase delays have been observed in response to severe cooling [21].

While systemic injections of caffeine presumably access A1 receptors within the SCN, caffeine almost certainly also modulates neuronal activity at sites afferent to the clock. An area of special interest is the raphe, as caffeine is known to promote terminal release of serotonin [22]. Serotonergic cell groups in the midbrain raphe can modulate SCN neuronal activity via a direct projection from the median raphe, and via an indirect projection from the dorsal raphe to the intergeniculate leaflet (IGL) [23], an area of the thalamus thought to convey non-photic input to the clock [6,24]. Conceivably, caffeine-enhanced release of serotonin in the IGL might inhibit activity of IGL neurons that project to the SCN, thereby preventing the full phase resetting effect of wheel running stimulated in the usual sleep period [25]. Such indirect effects may combine with direct effects of caffeine on A1 receptors in the SCN. At high doses (> 30 mg/kg in rats), caffeine inhibits locomotor activity, but we did not see significant suppression of running in hamsters at 75 mg/kg, although suppression was evident at 150 mg/kg (unpublished observations).

CONCLUSION

Additional studies will be necessary to unravel the sites of action of adenosinergic compounds within the circadian system. Nonetheless, the present data provide the first evidence that adenosine, a putative sleep signal within the CNS, may have significance as an endogenous circadian phase resetting stimulus, in addition to its recently hypothesized role as a modulator of photic inputs to the clock. Furthermore, the failure of caffeine to induce phase shifts at pharmacological doses that induce significant behavioral arousal is the first formal demonstration of an important principle that behavioral and pharmacological means of stimulating arousal can have different, even opposing effects on the state of the circadian clock. Given the world-wide use of caffeine, particularly during jet travel and shift-work rotations, when clock resetting is often necessary for adaptation, the potential interaction between this drug and phase resetting stimuli clearly merits further attention.

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