Effects of chronic expression of the HIV-induced protein, transactivator of transcription, on circadian activity rhythms in mice, with or without morphine

Marilyn J. Duncan,1 Annadora J. Bruce-Keller,1 Clayton Conner,1 Pamela E. Knapp,1,3 Ruquiang Xu,3 Avindra Nath,2 and Kurt F. Hauser1,4

1Department of Anatomy and Neurobiology, University of Kentucky Medical Center, Lexington, Kentucky; 2Department of Neurology, Johns Hopkins University Medical School, Baltimore, Maryland; and Departments of 3Anatomy and Neurobiology and 4Pharmacology and Toxicology, and Institute for Drug and Alcohol Studies, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia

Submitted 13 June 2008; accepted in final form 4 September 2008

Duncan MJ, Bruce-Keller AJ, Conner C, Knapp PE, Xu R, Nath A, Hauser KF. Effects of chronic expression of the HIV-induced protein, transactivator of transcription, on circadian activity rhythms in mice, with or without morphine. Am J Physiol Regul Integr Comp Physiol 295: R1680–R1687, 2008. First published September 10, 2008; doi:10.1152/ajpregu.90496.2008.—Patients with human immunodeficiency virus (HIV) infection exhibit changes in sleep patterns, motor disorders, and cognitive dysfunction; these symptoms may be secondary to circadian rhythm abnormalities. Studies in mice have shown that intracerebral injection of an HIV protein, transactivator of transcription (Tat), alters the timing of circadian rhythms in a manner similar to light. Therefore, we tested the hypothesis that chronic Tat expression alters circadian rhythms, especially their entrainment to a light-dark (LD) cycle, by using transgenic mice in which Tat expression in the brain was induced via a doxycycline (DOX)-sensitive, glial fibrillary-associated, protein-restricted promoter. Because opiate substance abuse, which shares comorbidity with HIV infection, also disrupts sleep, a final experiment assessed the effects of morphine exposure on circadian rhythms in wild-type and Tat transgenic mice. Mice housed in cages equipped with running wheels were fed chow with or without DOX. Experiment 1 revealed a small but significant (P < 0.05) difference between groups in the phase angle of entrainment and a 15% decrease in the wheel running in the DOX group (P < 0.005). During exposure to constant darkness, DOX did not alter the endogenous period length of the circadian rhythm. Experiment 2 investigated the effect of DOX on circadian rhythms in wild-type and Tat (+) mice during exposure to a normal or phase-shifted LD cycle, or morphine treatment without any change in the LD cycle. Tat induction significantly decreased wheel running but did not affect entrainment to the normal or shifted LD cycle. Morphine decreased wheel running without altering the phase angle of entrainment, and the drug’s effects were independent of Tat induction. In conclusion, these findings suggest that chronic brain expression of Tat decreases locomotor activity and the amplitude of circadian rhythms, but does not affect photic entrainment or reentrainment of the murine circadian pacemaker.

TRANSLATIONAL PHYSIOLOGY

INFECTION WITH HUMAN IMMUNODEFICIENCY virus (HIV) induces expression of many viral and/or cellular factors that are not intrinsically infectious but may be involved in mediating some of the neural and psychological symptoms associated with HIV-acquired immunodeficiency syndrome (AIDS), including alterations in sleep architecture and quality, fatigue, movement, and cognition. It has been postulated that these symptoms may be secondary to abnormalities in the circadian timing system (8), which is a major regulator of the timing of sleep (see Ref. 27 for review) and is also a factor that influences cognition (7, 9, 12). Abnormalities in circadian rhythms of activity, body temperature, hormone secretion, and circulating immune cells have been reported in HIV-AIDS patients, as well as in nonhuman primates infected with simian immunodeficiency virus (SIV) (17, 18, 36).

Recently, one HIV-induced protein, transactivator of transcription (Tat), has been shown to directly affect the timing, i.e., phase, of the mammalian master circadian pacemaker located in the hypothalamic suprachiasmatic nucleus (SCN) (8). Tat is released extracellularly by HIV-infected glial cells and macrophages in the brain. It may then directly interact with neurons or be transported anterogradely or retrogradely along neuronal pathways (4). It may also activate uninfected glial cells to release a variety of factors that may, in turn, alter neuronal function (38). Administration of Tat protein to the murine SCN region in vivo or in vitro mimics the effects of acute light exposure on the circadian timing system (8). For example, similar to light pulses, SCN administration of Tat in the early subjective night induces phase delays in locomotor activity rhythms, while administration in the mid- to late subjective night induces phase advances (8). These Tat-induced circadian phase shifts are mediated by activation of N-methyl-D-aspartate receptors (8), as are photic phase shifts (10, 11, 26). Thus, by stimulating glutamatergic neurotransmission, administration of Tat can acutely regulate the circadian timing system in a rodent. This finding suggested that chronic exposure to Tat as a result of HIV infection may alter the expression of circadian rhythms in humans, especially entrainment of circadian rhythms to the light-dark (LD) cycle (8). Furthermore, because many findings in a variety of species show that disrupted or dysynchronous circadian rhythms are...
associated with increased incidence of cardiovascular disease, increased tumor growth, decreased responsiveness to chemotherapy, memory deficits, and decreased longevity (7, 13, 14, 19–21, 29, 32, 45), it is possible that chronic Tat expression in the brain might contribute to HIV morbidity or mortality.

Because Tat induces activation of glutamate receptors, which mediates the phase-shifting effects of light on circadian rhythms, chronic Tat exposure may interfere with entrainment to a lighting cycle or even mimic the effect of exposure to constant light. During exposure to constant light, circadian rhythms typically “free-run,” such that they gradually lose synchrony with external clock time (33, 34). Also, constant light exposure lengthens the endogenous circadian period in nocturnal rodents (33, 34). To investigate the effects of chronic Tat expression on circadian rhythms, we used transgenic mice in which the Tat promoter has been linked to the doxycycline (DOX)-inducible tet promoter. We tested the following hypotheses: 1) chronic Tat expression interferes with entrainment to a LD cycle; 2) chronic Tat expression increases the endogenous period length during exposure to constant darkness; and 3) chronic Tat expression alters the rate of entrainment to a new lighting cycle. Furthermore, because opiate addiction can be associated with HIV infection, and because opiates can aggravate the pathophysiological effects of HIV in the central nervous system (CNS) clinically (1, 2) and in experimental models of HIV encephalitis (16, 30), we also tested the effect of the chronic exposure to the opioid drug, morphine, on circadian rhythms in mice expressing Tat.

MATERIALS AND METHODS

Experimental animals and housing conditions. Male mice, C3-C57Bl/6, 5–9 mo old, were used for these studies. Transgenic mice possessed an HIV-1 tat gene whose expression was driven by a tetracycline (tet)-inducible promoter. These transgenic mice were generated as follows: a tet “on” system was used for generation of inducible constructs. Tat-1 (HIV-1 IIIb) was cloned downstream of a tet responsive element (TRE) in the pTREX vector (Clontech, Mountain View, CA). Reverse tet transactivator (RTTA) was first cloned in pEGFP vector (Clontech) at the BamHI EcorI I site and further subcloned in giall fibrillary-associated protein (GFAP) promoter-driven vector pGFaLac-1 at the BamHI BgII II site. The construction of these plasmids and their validation using in vitro systems is described in a previous publication from the founder’s laboratory (6). For creation of transgenic lines, plasmid vectors were selectively digested with restriction enzymes, and intact regulatory regions with respective genes and poly A sites were gel extracted. The purified fragments were used for inoculation of eggs. The founder animals (C3H x C57Bl/6) for each construct were checked for respective genes by PCR and southern blotting. Each founder line was then inbred to produce mice homozygous for the selected genes. Nervous system-restricted, inducible transgenic mice were created by crossing mice expressing the Tat gene under the TRE with mice engineered to express a GFAP promoter-driven RTTA. Pure transgenic lines were derived by crossing homologous lines containing both the GFAP-RTTA and TRE-Tat genes. Thus the inducible Tat transgenic mice [Tat(+) mice] described in this paper expressed both GFAP-RTTA and TRE-Tat genes, while control Tat(−) mice expressed GFAP-RTTA but not the TRE-Tat gene. It should be noted that GFAP-immunoreactive cells are abundant in the SCN (28).

Before experimentation, the mice were group housed and exposed to an alternating 14-h light (L)/10-h dark (D) cycle with lights on at 7 AM. During experiments, the mice were individually housed in cages (32 by 14 by 12 cm) equipped with running wheels (11 cm diameter) electronically interfaced with hardware and software for recording and analyzing circadian locomotor activity data (ClockLab, Actimetrics, Willmette, IL). The cages were enclosed in ventilated, light-tight compartments, with 12-h L/12-h D photoperiod. The light was provided by green light source, because previous studies have shown that circadian timing system is most sensitive to green light (~500 nm) (40). Water and food, either normal mouse chow (NC) (Harlan) or chow formulated to contain 6 mg of DOX per gram (DOX) were available ad libitum. Cages, food, and water were changed weekly in both studies. All animal procedures, which were consistent with American Association for Accreditation of Laboratory Animal Care guidelines, were reviewed and approved by the University of Kentucky Institutional Animal Care and Use Committee.

Experiment 1. Effect of DOX ingestion in Tat transgenic mice on expression of circadian locomotor activity rhythms during exposure to a LD cycle or constant darkness. The mice were exposed initially to an alternating LD cycle (12-h L/12-h D) with lights on at 7 AM. The mice were fed either NC or DOX chow (as described above, N = 6 per group). The phase angle of entrainment (time of nocturnal activity onset relative to time of lights off) and the total amount of activity per week, were recorded for each animal. After 4 wk, the mice were transferred to constant darkness by leaving the lights off after the onset of the dark phase. The mice remained in constant darkness for 7 wk. During this time, dim red light was used for monitoring the animals and changing cages, food, and water. The amount of total wheel running activity per week was determined. Also, the endogenous period length was calculated by periodogram analysis using ClockLab software. Body weight was monitored weekly as a possible indicator of nonspecific deleterious effects of the DOX-containing chow. At the end of the study, the mice were anesthetized and perfused transcardially with 4% paraformaldehyde in neutral phosphate buffer. Brains were dissected, washed in PBS, and flash frozen in optimum cutting temperature compound.

To confirm that Tat was expressed within the SCN, frozen, 20-μm thick sections were cut serially through the SCN in the coronal plane. With the use of a dissecting microscope at ×25–40 magnifications, the region of the SCN was microdissected from four adjacent frozen sections per mouse. The tissue was collected using sterile, RNase-free micropipette tips and transferred immediately to RNA extraction buffer. RNA was isolated from tissue sections using the Absolutely RNA NanoPrep kit (Stratagene), following manufacturer’s instructions, and the RNA samples were treated with RNase-free DNase to remove genomic DNA contaminant. Total RNA was then reverse transcribed into cDNA using random hexamer primers by the high-capacity cDNA reverse transcription kit (Applied Biosystems) at 25°C for 10 min and 37°C for 2 h in 20-μl total volume. cDNA was used as a template in PCR amplification to measure Tat expression using the following primers: forward, 5'-gcc cgg gca tcc agg aag tc; reverse, 5'-cgt cgc tgg tct ctc ctc tct ct. PCR was performed using a Touchdown cycle program. The amplified products were analyzed on 2% agarose gels with ethidium bromide and photographed using a Kodak Image Station 440 system.

Experiment 2. Effect of DOX ingestion in wild-type or Tat transgenic mice on expression of circadian locomotor activity rhythms during exposure to a normal or shifted LD cycle and after exposure to morphine. This experiment investigated whether chronic expression of Tat affects the rate of entrainment to a phase-shifted LD cycle, and whether chronic morphine treatment influences circadian rhythms. The second issue was considered because many HIV-infected patients are addicted to opiates. Morphine exposure has been demonstrated to reset the phase of circadian rhythms in mice (25), and opiate addicts show altered sleep-wake patterns (31). Importantly, opiates in combination with HIV can exacerbate some of the CNS effects seen with HIV alone. Therefore, we anticipated that morphine and Tat induction might selectively interact to affect circadian activity. Last, this study included wild-type mice because information from other on-going studies and the results of experiment 1 indicated that the Tat transgenic mice exhibit low levels of Tat expression even in the absence of ingestion of DOX. Therefore, to evaluate any nonspecific effects of
DOX ingestion on circadian rhythms, both Tat(+) and Tat(−) mice were fed the DOX chow in this experiment.

Male mice were weighed at the beginning of the study and randomly assigned to one of four groups (N = 6 each): 1) wild-type mice fed DOX chow, 2) wild-type mice fed NC, 3) Tat(+) mice fed DOX chow, or 4) Tat(+) mice fed NC. The average initial body weight was not significantly different among the groups. The mice were individually housed in cages equipped with running wheels, and running activity was recorded continuously. Food and water were provided ad libitum. At weekly intervals, the mice were weighed, and fresh cages and water bottles were provided.

During phase 1, the mice were exposed to a LD cycle (12-h L/12-h D, lights on from 7 AM to 7 PM) for 3 wk. The phase angle of entrainment (time of nocturnal activity onset relative to time of lights off) and the amount of running activity (wheel revolutions per day) were recorded and analyzed.

During phase 2, the timing of the LD cycle was shifted by 6 h, such that lights were on from 1 AM to 1 PM. (Thus, on the day of the LD cycle change, the animals experienced only 6 h of darkness, instead of 12). This method of advancing the LD cycle has previously been used in studies of phase shifting in mice (35). The number of days required for reentrainment to the shifted LD cycle and the phase angle of entrainment were determined, as was the amount of wheel-running activity.

Phase 3 of the study involved administration to morphine to all of the mice. Under isofluorane anesthesia, sterile surgery was conducted to implant subcutaneously a timed-release, morphine-containing pellet, obtained from the National Institute on Drug Abuse (Rockville, MD). The pellets, which released 5 mg of morphine per day, were replaced with fresh pellets after 5 days. The phase angle of entrainment and the amount of running were determined.

Statistical analysis. Differences between groups in phase angle, total wheel-running activity, endogenous period length, or body weight were assessed with repeated-measures two-way analysis of variance examining the main effects of treatment and time, and their

![Fig. 1. Inducible transactivator of transcription (Tat) expression in the suprachiasmatic nucleus (SCN) region. RNA was extracted from microdissected SCN tissue and analyzed by RT-PCR, as described in MATERIALS AND METHODS. Images depict the presence or absence of an amplification product with the predicted size of 141 bp in samples derived from the SCN of two Tat(−) mice and four Tat(+) mice, as well as positive and negative (nontemplate) control samples. The four Tat(+) mice included two that were fed doxycycline (DOX) chow (lanes 4 and 6), and two that received normal chow (NC) (lanes 3 and 5).](image1)

![Fig. 2. Representative activity records for Tat(+) mice. The activity records are double plotted, such that the horizontal axis represents 48 consecutive h, with clock times indicated on the top. The vertical axis represents successive 48-h periods, from top to bottom. The top line represents days 1 and 2, the next line represents days 2 and 3, the third line shows days 3 and 4, etc. Wheel-running activity is indicated by the dark marks. The animals were exposed to a light-dark cycle (represented by the horizontal black and white bars shown at the top) for the first 4 wk and then were exposed to constant darkness (indicated in gray). Triangles represent times that cages were changed.](image2)
RESULTS

Experiment 1. Effect of DOX ingestion in Tat transgenic mice on expression of circadian locomotor activity rhythms during exposure to a LD cycle or constant darkness. Previous studies detected robust expression of HIV-1 Tat mRNA in the cortex, striatum, and hippocampus of inducible Tat(+) transgenic mice, while Tat transcripts were not detected in wild-type Tat(−) mice or in tissues lacking a GFAP promoter (5), including the spleen and liver (Nath A. and Chauhan A., unpublished results). Nevertheless, to confirm that Tat was expressed in the region of the SCN following chronic DOX administration, SCN tissue was microdissected from frozen tissue sections, RNA extracted, and Tat mRNA expression assessed by RT-PCR analysis, as described in MATERIALS AND METHODS. Evaluation of PCR band intensities confirmed the presence of Tat mRNA in the region of the SCN following chronic DOX exposure in Tat(+) mice, while no signal representing Tat mRNA was observed in the Tat(−) mice (Fig. 1). Interestingly, a modest but detectable level of expression of Tat mRNA in the SCN was observed in Tat(+) mice that were not administered DOX, although Tat expression in these mice was much lower than in DOX-induced Tat(+) mice (Fig. 1). These data are in agreement with a previous study, in which we found that, for some outcome measures, vehicle-treated Tat(+) mice without DOX showed intermediate levels of change (e.g., reactive astrogliosis) compared with Tat(−) mice or Tat(+) mice treated with DOX, suggesting the Tat transgene was inherently “leaky” (5).

During 4 wk of exposure to a LD cycle, all of the mice in both the DOX-containing chow and NC groups exhibited normal entrainment to the LD cycle with nearly all of the wheel-running activity confined to the dark phase (Fig. 2). There was a small but statistically significant (P < 0.05) difference between the groups in the phase angle of entrainment, i.e., the relationship of the onset of nocturnal activity to the time of lights off. Bars represent means ± SE. Two-way ANOVA revealed a significant effect of treatment (P < 0.02) but not time (P = 0.83) and a significant interaction effect (P < 0.05). *P < 0.01, compared with NC-fed mice at the same week, based on Bonferroni’s test.

The average body weight for each experimental group was stable across the study but was slightly but significantly greater (P < 0.05) in the DOX chow group compared with the NC group mice (means ± SE, DOX: 27.9 ± 0.2 g vs. NC: 26.7 ± 0.2 g).

Experiment 2. Effect of DOX ingestion in wild-type or Tat transgenic mice on expression of circadian locomotor activity rhythms during exposure to a normal or shifted LD cycle and after exposure to morphine. All mice were entrained to the normal LD cycle (phase 1) and successfully reentrained to the 6-h phase-advanced LD cycle (phase 2) (Fig. 5). During exposure to either lighting cycle, the phase angle of entrainment was not significantly affected by either genotype or food or an interaction of these conditions (data not shown). Further-
more, the number of days (mean of 8 for all groups) required for stable reentrainment after the phase advance in the lighting cycle was not significantly affected by genotype ($P = 0.89$) or food ($P = 0.96$), nor was a significant interaction of these effects observed ($P = 0.89$).

In contrast to the absence of effects on entrainment, there were significant main effects associated with the Tat transgene and/or its induction (DOX treatment) on running wheel activity ($P \leq 0.0021$) when assessed using a repeated-measures (within-subjects design) ANOVA and post hoc Duncan’s multiple-comparisons test (Statistica version 6; Statsoft, Tulsa, OK) (Fig. 6). Tat induction significantly decreased activity compared with wild-type mice receiving control ($P < 0.001$) or DOX-treated feed ($P < 0.05$), or Tat(+) mice receiving control feed ($P \leq 0.005$) (Fig. 6). Tat induction caused marked reductions in activity compared with other groups throughout the experiment, irrespective of whether a 12-h L/12-h D cycle was advanced (Fig. 6, “Light:Dark Advance”) or whether chronic morphine was administered (Fig. 6, “Morphine”).

Advancing the 12-h L/12-h D cycle significantly increased running activity in individual mice (Fig. 6). There was a significant main effect of increase in running activity in mice when the time of lights on was advanced from 0700 (EST) to 0100 ($P < 0.001$). When individual mice were compared before and after the 6-h advance of the light phase, however, only wild-type mice receiving control feed showed marked increases in activity (*$P \leq 0.02$ vs. “Light:Dark Regular” activity before light cycle advance). Mice in all groups showed increases (28.1–53.6%), albeit not significant, in running activity after the “lights-on” period was advanced, which likely contributed to the highly significant main effect. Tat induction caused marked reductions in running activity compared with wild-type mice receiving control feed after advancing the lights-on period ($§P < 0.05$).

Unlike advancing the LD cycle, which increased activity, continuous morphine exposure (5 mg/day, subcutaneous) for 3 wk significantly reduced wheel running across all groups ($P < 0.005$) (Fig. 6, “Morphine”). Marked reductions in all groups were noted when activity during the “Light:Dark Advance” period before morphine treatment was compared with activity after morphine exposure. Moreover, since lighting conditions remained identical before and after mor-
In contrast to entrainment, the amount of wheel-running activity was significantly affected by Tat expression. In both studies, the total amount of running activity was decreased in the Tat(+) mice fed DOX chow. Furthermore, because the wheel-running activity occurred almost exclusively at night, the amplitude of the circadian rhythm of wheel running was lower in this group, reminiscent of previous findings concerning circadian rhythms after HIV infection. Interestingly, in the absence of DOX, Tat(+) mice displayed an intermediate level of wheel running that was in between that observed for wild-type mice or Tat(−) mice receiving DOX. Although not significantly different from wild-type mice receiving DOX, the trend suggested that there is limited expression of the Tat transgene in the absence of DOX. Ongoing studies in Tat transgenic mice suggest that this assumption is correct and that “leakiness” of the Tat transgene may be revealed in some outcome measures. For example, intermediate levels of reactive astrogliosis have been observed in Tat(+) mice in the absence of DOX compared with both Tat(−) mice or Tat(+) mice receiving DOX (5).

In HIV patients, circadian rhythms in circulating hormones often exhibit reduced amplitude, and, in some cases, the daily variation no longer constitutes a statistically significant rhythm (3, 36, 37). Reduction in the amplitude of the circadian rhythms of body temperature rhythm and activity has also been observed in Rhesus monkeys infected with SIV (17, 18). Furthermore, SIV-infected monkeys exhibited lower levels of motor activity (17, 18). Thus, if DOX induced Tat expression in the mice in the present study (as expected), then the current findings suggest that Tat expression may be involved in decreasing activity and circadian rhythm amplitude during infection with SIV or HIV.

During exposure to constant darkness, the DOX chow-fed mice, as well as the NC-fed mice, continued to exhibit circadian rhythms in locomotor activity. This finding indicates that DOX exposure, and Tat expression, do not interfere with the generation and expression of circadian (i.e., endogenously generated) rhythms. Furthermore, the endogenous circadian period of the locomotor activity rhythm was ~24 h and was not different between the DOX chow and NC groups. This finding shows that DOX did not affect this basic parameter of the circadian timing system and also suggests that the DOX-fed mice were not responding to constant darkness as if it were constant light, which typically increases the endogenous period length of circadian rhythms in nocturnal rodents (33).

Similar to Tat expression, chronic morphine treatment did not disrupt circadian wheel-running rhythms or significantly alter their entrainment to the LD cycle. In contrast, previous studies show that chronic morphine exposure can alter circa-
EFFECTS OF CHRONIC TAT EXPRESSION ON CIRCADIAN RHYTHMS

Perspective and significance. Infection with the HIV or SIV virus is associated with decreased locomotor activity and/or alterations in circadian rhythms, especially reduction in rhythm amplitude (3, 17, 18, 36, 37, 41, 44). Indeed, one longitudinal study of SIV infection demonstrated progressive decreases in circadian rhythm amplitude preceding ultimate loss of circadian rhythms (17, 18, 36). The present findings suggest that lower activity levels and reduced circadian rhythm amplitude in HIV-infected patients may be caused, at least in part, by chronic expression of the HIV-induced protein, Tat, in the brain. Concurrent exposure to chronic morphine further inhibits the amplitude of the activity rhythm, suggesting some potential common mechanisms with HIV Tat. These findings add to a growing body of evidence that exposure to HIV proteins within discrete regions of the CNS mediates specific physiological and behavioral symptoms that are characteristic of HIV-AIDS patients.

Acknowledgments

We thank Dr. Bruce O’Hara for generously providing the ClockLab equipment for recording circadian wheel-running rhythms in mice. We also appreciate the technical assistance provided by Mairi Clements and Verda Davis, as well as comments on earlier versions of the manuscript from Dr. Kathleen M. Franklin.

Present address of A. J. Bruce-Keller, Division of Basic Research, Pennington Biomedical Research Center, Louisiana State University, 6400 Perkins Rd., Baton Rouge, LA 70808.

Grants

This work was supported by National Institutes of Health Grants P01 DA19398 (A. Bruce-Keller, P. E. Knapp, K. F. Hauser), P20 RR15592 (A. Bruce-Keller), RO1 NS039253 (A. Nath), and R01 AG13418 (M. J. Duncan).

References

9. Devan BD, Goad EH, Petri HL, Antoniadis EA, Hong NS, Ko CH, Leblanc L, Lebovic SS, Lo Q, Ralph MR, McDonald RJ. Circadian phase-shifted rats show normal acquisition but impaired long-term reten-


