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Diurnal rhythms in cocaine sensitization and in Period1 levels are common across rodent species

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Abstract

Circadian and seasonal rhythms in psychostimulant-induced behaviors have been reported in different species including humans. Using inbred mice, we recently reported that both cocaine sensitization and striatal "clock" gene *Period1* (PER1 for protein) levels demonstrate a diurnal pattern that is maintained by the rhythm of pineal products *N*-acetylserotonin (NAS) and melatonin. It is well known that genetic background differences in inbred mice affect their behavioral traits. Therefore, to test whether our initial observations were limited to these mouse strains or whether these traits are common across rodent species we have tested additional strains of mice (CBA/J and AKR/J) and rats (Sprague–Dawley). We found that regardless of the species/strains, subjects with regular NAS and melatonin rhythms present diurnal cocaine sensitization and striatal PER1 rhythm. Since there is a growing interest in clock gene–mediated circadian mechanisms, these results may be important in designing experiments (e.g., time of day and subject strain) to study the role of these genes in psychiatric disorders such as addiction and depression.

Keywords: Cocaine; Addiction; Circadian rhythms; Dosing time; Mice; Rats; Clock genes; Striatum

1. Introduction

The circadian properties of cocaine-induced behaviors such as locomotor sensitization and reward have recently been reported in rodents (Iijima et al., 1995; Abarca et al., 2002; Uz et al., 2002; Uz et al., 2003). Circadian changes were also observed in cocaine self-administration in rats (Baird and Gauvin, 2000). In humans, it has been reported that chronic cocaine users show seasonal rhythmicity in drug abuse and craving (Satel and Gawin, 1989; Sandyk and Kanofsky, 1992).

Since the pharmacokinetic profile of cocaine does not show circadian rhythms (Baird and Gauvin, 2000), circadian-rhythm-driven cellular mechanisms are being questioned as possible mediators of cocaine-induced behaviors. Recently, the transcription factor "clock" genes (i.e., *Per1*) have been proposed as such mediators of cocaine-induced behaviors (Abarca et al., 2002; Uz et al., 2003). Our previous studies using inbred mice (i.e., C3H/HeJ and C57BL/6J) with opposite *N*-acetylserotonin (NAS) and melatonin profile and pinealectomy as a NAS/melatonin deficiency model revealed that both cocaine-induced behavioral sensitization and *Per1* rhythms are controlled by pineal NAS/melatonin system (Uz et al., 2002, 2003). Addictive behaviors such as behavioral sensitization are complex and controlled by a number of genes (Phillips, 1997). It is known that inbred mice may present different behavioral phenotypes due to the genetic background differences (Crawley et al., 1997). Therefore, to test whether pineal-gland-driven circadian genetic mechanisms are common across the various rodent species, we used additional inbred mice strains with different NAS/melatonin profiles (NAS/melatonin-deficient AKR/J and NAS/melatonin-proficient CBA/J) and rats (Sprague–Dawley) and studied the correlation between cocaine-induced diurnal locomotor sensitization and striatal *Per1* expression rhythms.

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2. Materials and methods

2.1. Animals and drug treatment

Male CBA/J (n=28) and AKR/J (n=28) mice, 2 months old and weighing 25–30 g, were purchased from Jackson Laboratories (Bar Harbor, ME). Two-month-old male Sprague–Dawley rats (n=40) weighing 250–275 g, were purchased from Harlan (IN). Animals were housed in groups of three and had free access to laboratory chow and water except during behavioral experiments. They were kept in a temperature-controlled room under conditions of 14 h light:10 h dark cycle (lights on at 5 a.m.; zeitgeber time 00 [ZT00]). The experimental protocol was approved by the Institutional Animal Care Committee. Cocaine hydrochloride (Sigma Chemical, St. Louis, MO) was dissolved in sterile physiological saline (0.9% NaCl) and administered intraperitoneally in an injection volume of 0.05 ml/25 g body weight for mice and 0.25 ml/250 g body weight for rats. The time for both injection and sample collection was chosen based on published reports on diurnal NAS/melatonin rhythms for mice (Goto et al., 1989) and rats (Pang and Brown, 1983).

2.2. Quantitative PER1 Western immunoblotting

To quantitate the levels of PER1 protein, striatal samples were taken from both mouse strains (i.e., CBA/J and AKR/J) at ZT05 (for daytime; n=5) and ZT21 (for nighttime; n=5) and from rats over a 24-h period every 4 h starting at ZT01 (n=3–4 for each time point). During the dark periods, samples were collected under dim red light. The subject size was five per time point for each strain. Samples were analyzed as described earlier (Uz et al., 2003). Briefly, tissue samples were homogenized in a buffer containing 20 mM Tris–HCl, 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 5 mM ethylenediaminetetraacetic acid, 1.5 mM pepstatin, 2 mM leupeptin, 0.5 mM phenylmethylsulfonylfluoride, 0.2 U/ml aprotinin, and 2 mM dithiothreitol. After reading the protein content of the tissue homogenate, 40- to 80-μg samples and gel loading solution (50 mM Tris–HCl [pH 6.8], 4% β-mercaptoethanol, 1% sodium dodecylsulfate [SDS], 40% glycerol, and a trace amount of bromphenol blue) were mixed and boiled for 3 min. They were loaded onto a 7.5% (wt/vol) acrylamide gel using the Mini Protean II gel apparatus (Bio-Rad, Hercules, CA). The gels were run using 25 mM Tris-base, 192 mM glycine, and 0.1% (wt/vol) SDS at 150 V. The proteins were subsequently transferred electrophoretically to an ECL nitrocellulose membrane (Amersham, Piscataway, NJ) using the Mini TransBlot transfer unit (Bio-Rad) at 150 mA constant current overnight. Transfer buffer contained 25 mM Tris-base, 192 mM glycine, and 20% methanol. Membranes were washed with TBST buffer (10 mM Tris-base, 0.15 M NaCl, and 0.05% Tween 20) for 10 min. The blots were incubated with anti-mouse PER1 antibody (4 μg/ml; Alpha Diagnostic International, San Antonio, TX), which is specific for both mice and rats, in 5% (wt/vol) powdered nonfat milk in TBST, 2 ml NP-40, and 0.02% (wt/vol) SDS (pH 8.0) overnight. The blots were then washed with TBST and incubated with the horseradish-peroxidase-linked secondary antibody (anti-rabbit IgG; 1:1000; Amersham) for 4 h at room temperature and processed with an ECL kit; blots were then washed with TBST and exposed to Hyperfilm ECL (Amersham). To normalize the signal for PER1 protein, the presence of noninducible β-actin protein was measured on the same blot using a mouse monoclonal antibody against the β-actin (1:5000; Sigma). The optical densities of the PER1 bands were corrected by the optical density of the corresponding β-actin bands on the film using the Loats image analysis system (Loats Associates, Westminster, MD).

2.3. Locomotor activity measurements, behavioral sensitization

Mice (CBA/J, n=18 and AKR/J, n=18) and rats (n=9 for day and n=10 for night) that had not been previously exposed to the testing monitors were placed individually in either mouse or rat activity cages for a 30-min adaptation period. Their locomotor activity was measured with the Cage Rack Photobeam Activity Measurement System (San Diego Instruments, San Diego, CA) equipped with computer-monitored photobeam frames. Immediately after the adaptation period, animals were injected with cocaine HCl (20 mg/kg ip for CBA/J, 10 mg/kg ip for AKR/J, and 15 mg/kg ip for Sprague–Dawley) and returned to their activity cages; the recording procedure was continued for an additional 30-min period. The drug dosage was chosen based on published results where dose–response studies were performed on inbred mice (Uz et al., 2002) and rats (Kosten et al., 1994). Times of injections were as follows: daytime, ZT05–06, and nighttime, ZT20–21. The movement of each animal was recorded as the number of beam interruptions and reported as locomotor activity (ambulation). The experiment room was illuminated by normal fluorescent light (about 150 lx at the bench level) operated with a light timer; the nighttime drug administrations were done under dim red light (0.1 lx at the bench level). After each experimental session, animals were returned to their home cages. To measure locomotor sensitization, the same procedure was repeated for three consecutive days and nights for mice and five consecutive days and nights for rats.

2.4. Statistical analysis

Optical density values of protein samples (day vs. nighttime) calculated as ratios (mean±S.E.M.) were evaluated by t test. Locomotor activity scores (mean±S.E.M.)

were analyzed by one-way ANOVA followed by the Dunnett's multiple comparison test. Significance was accepted as $P<0.05$.

3. Results

3.1. Circadian changes in striatal PER1 protein levels

Previously, we established the circadian rhythm and the time points when PER1 protein levels in NAS/melatonin-normal mice show a significant difference (Uz et al., 2003). When these time points were compared (ZT05 of the day vs. ZT21 of the night), we found that NAS/melatonin-deficient AKR/J did not demonstrate any differences, whereas NAS/melatonin-normal CBA/J presented high daytime levels for PER1 protein (Fig. 1).

To investigate the rhythm of PER1 in the rat striatum, we measured the 24-h-cycle content of PER1 protein. We found circadian PER1 rhythms with elevated daytime levels (Fig. 3A). Circadian cosinor analysis (Nelson et al., 1979) of PER1 protein levels revealed peak levels around ZT08 (amplitude=0.1, phase=199°). There was a statistically significant decrease at nighttime (ZT21) compared to daytime (ZT05) values (ZT05: 0.48±0.09 and ZT21: 0.14±0.01; $P=0.03$; t test).

3.2. Diurnal behavioral cocaine sensitization is present only in NAS/melatonin-proficient subjects

Neither NAS/melatonin-normal CBA/J mice nor NAS/melatonin-normal Sprague–Dawley rats developed sensitization to repeated administration of cocaine when given at night, ZT20 (Figs. 2 and 3B). When cocaine was given during the day (ZT05), both CBA/J mice and rats developed behavioral sensitization to cocaine. On the other hand, NAS/melatonin-deficient AKR/J mice developed cocaine sensitization to both day and nighttime injections (Fig. 2).

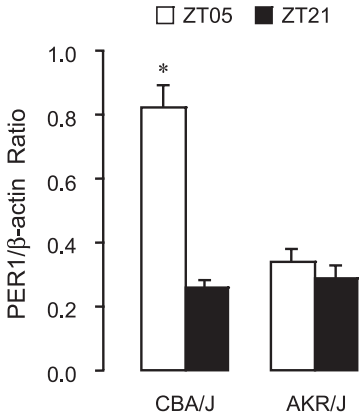

Fig. 1. Strain differences for day vs. nighttime comparisons in striatal PER1 protein levels. PER1 protein levels were measured for the day (ZT05) and nighttime (ZT21) levels with Western immunoblotting in striatal samples ($n=5$) taken from two different inbred strains of mice. NAS/melatonin-deficient AKR/J did not show any difference in protein levels, whereas NAS/melatonin-normal CBA/J demonstrated an increase during the day. Optical density values of protein samples calculated as ratio (mean±S.E.M.) were evaluated by t test. Significance was accepted as $P<0.05$. The zeitgeber time 00 (ZT00) is defined as lights on.

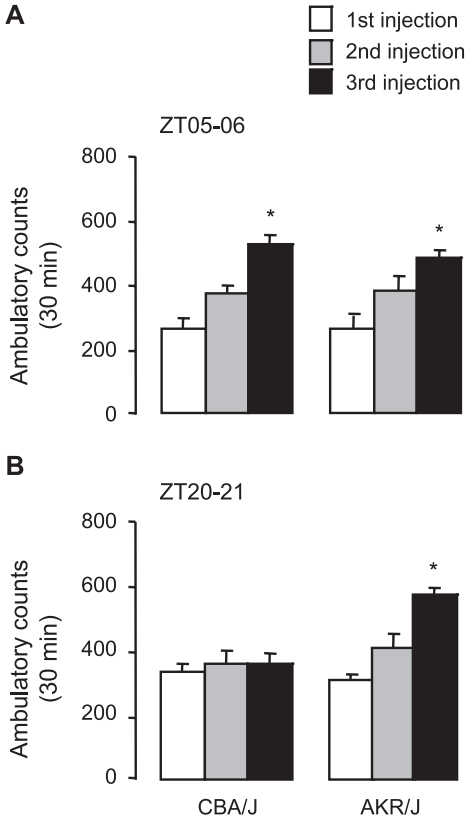

Fig. 2. Pineal products NAS and melatonin are important in the development of diurnal cocaine sensitization. Cocaine sensitization was studied in two different strains, AKR/J and CBA/J. Cocaine was administered for three consecutive days either during the day (ZT05–06) (panel A) or at night (ZT20–21) (panel B). Results (mean±S.E.M.; $n=6$–9 per group per time point) are expressed as locomotor activity; i.e., the number of beam breaks during a 30-min test session. Note the development of sensitization to cocaine (increased locomotor activity after the third injection) in all groups injected at ZT05–06 (daytime) and the absence of sensitization only in NAS/melatonin-normal CBA/J mice at ZT20 (nighttime). *$P<0.05$ in comparison with the corresponding first injection values (Dunnett's test).

4. Discussion

Following up our previous correlation studies between the rhythms of striatal PER1 protein levels and cocaine sensitization (Uz et al., 2003), using additional inbred mouse strains and rats in the present report, we further demonstrate that both development of cocaine-induced behaviors and striatal PER1 rhythms are controlled by the presence/rhythm of pineal NAS and melatonin. We found that diurnal cocaine sensitization is a common phenomenon across species and strains that have regular NAS and melatonin rhythms.

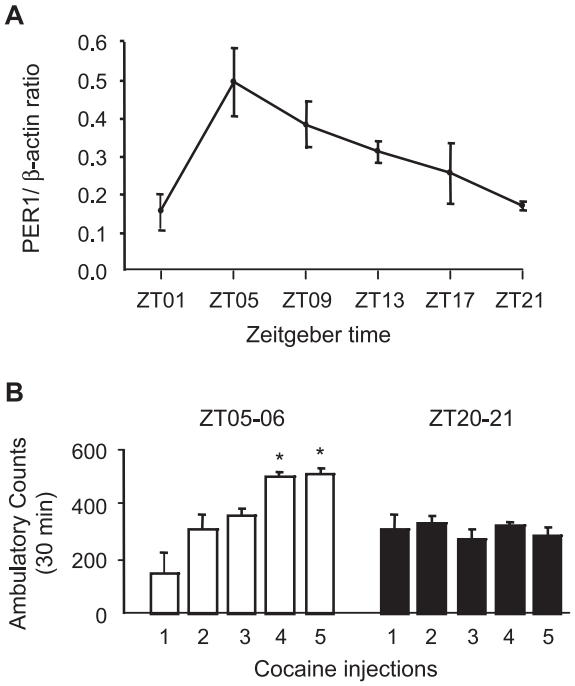

Fig. 3. Diurnal striatal PER1 protein rhythm and cocaine sensitization in the rat. Panel A shows striatal PER1 levels (mean±S.E.M., n=3–4 for each time point). Cocaine was administered for five consecutive days either during the day (ZT05–06, high PER1 levels) or at night (ZT20–21, low PER1 levels). Results (mean±S.E.M., n=9–10) are expressed as ambulatory counts (panel B). *P<0.05 in comparison with the corresponding first day values (Dunnett's test). Zeitgeber time 00 (ZT00) is defined as lights on. Sensitization was observed only when injections were administered at ZT05–06.

Studies on clock gene–mediated circadian mechanisms are important in the field of psychiatry since disorders such as addiction and depression present circadian and seasonal rhythms in their course. Clock genes are widely expressed, not only in the suprachiasmatic nucleus (SCN), where they have been studied extensively, but also outside the SCN in the brain (Shieh, 2003; Uz et al., 2003). It is believed that this SCN-independent clock gene expression plays an important role in regulating local/regional rhythms related to physiology and behavior, such as cocaine-induced behaviors (i.e., nonclock function) (Reppert and Weaver, 2002).

Since the expression rhythms of clock genes outside the SCN are controlled by the pineal gland and its products NAS and melatonin (Messager et al., 2001; von Gall et al., 2002; Uz et al., 2003), it is important to consider the presence and/or secretion rhythms of these products in the subjects (i.e., mice and rats) used for studies on clock gene–mediated behaviors and in the creation of knockout mice for these genes. Many inbred mice strains, including commonly used C57BL/6J and AKR/J, are deficient for NAS and melatonin due to natural mutations on the enzymatic pathway for their synthesis (Goto et al., 1989; Roseboom et al., 1998; Uz and Manev, 2001). Only a few mice strains, such as CBA/J and C3H/HeJ, are proficient for NAS and melatonin. We have reported that C3H/HeJ mice with normal NAS/melatonin rhythms demonstrate circadian PER1 protein levels in the striatum, whereas NAS/melatonin-deficient C57BL/6J mice do not present this circadian profile (Uz et al., 2003). Similar to our findings, Sun et al. (1997) reported that NAS/melatonin-deficient C57BL/6J mice do not demonstrate *Per1* rhythm in the hypophyseal pars tuberalis (PT). When these inbred mice were compared for the development of behavioral sensitization induced by cocaine, we found that NAS/melatonin-normal CBA/J mice demonstrated diurnal cocaine sensitization correlated with diurnal striatal PER1 levels (Figs. 1 and 2). On the other hand, AKR/J mice deficient for NAS and melatonin develop sensitization to cocaine both during the day and at night and show steady levels of striatal PER1 (Figs. 1 and 2).

Circadian rhythms of psychostimulant-induced behavioral sensitization in rats have been studied using amphetamine (Gaytan et al., 1999) and methylphenidate (Gaytan et al., 2000). In the home-cage-testing environment, these authors reported variable sensitization rhythms depending on the dose and the drug used. Moreover, circadian changes were observed in cocaine self-administration in rats (Baird and Gauvin, 2000). Our results demonstrate for the first time the development of circadian behavioral sensitization to cocaine in rats. The Sprague–Dawley rats we used in our studies are outbred and demonstrate circadian blood and brain melatonin levels with peak values around ZT19–21 (Pang and Brown, 1983). Therefore, high nighttime levels of circadian NAS and melatonin could be responsible for blocking the development of cocaine sensitization at night. In the same vein, Sircar (2000) reported that repeated daily melatonin injections prior to the administration of cocaine for 5 days prevented the development of cocaine-induced behavioral sensitization in rats.

mRNA localization studies showed that clock genes such as *Per1* are present in various brain areas such as the caudate–putamen and the nucleus accumbens of the striatum relevant to psychostimulant-induced behaviors in the rat (Masubuchi et al., 2000; Shieh, 2003). Our results demonstrate that PER1 protein levels show diurnal rhythms in the rat striatum that is correlated with diurnal cocaine-sensitization differences (Fig. 3). In the same vein, we recently reported that cocaine-induced drug seeking (reward) presents diurnal rhythms in mice with intact pineal gland (i.e., sham operated), whereas pinealectomized mice showed similar cocaine reward both during the day and at night (Kurtuncu et al., 2004). We found less reward to cocaine at night when striatal PER1 levels are the lowest. Using *Per1* knockout mice, Abarca et al. (2002) reported abolished cocaine reward in these mice compared to their wild-type controls.

It has been proposed that the presence of an E-box motif in a gene promoter is crucial for gene cycling and the genes with E-box have circadian expression rhythm potential (Kyriacou and Rosato, 2000). High and low PER1 protein levels during the cycle may interact differently with other

clock proteins and in concert may modify the expression of a second group of genes (i.e., clock-controlled genes). The genes related to the dopamine system (i.e., a known target for cocaine action), such as the dopamine transporter (Kawarai et al., 1997), the cocaine–amphetamine-regulated transcript (Yamada et al., 2002), and dopamine 1 (Zhou et al., 1992), 2 (Valdenaire et al., 1994), and 3 (D'Souza et al., 2001) receptors all contain putative E-box sequences in their promoter regions. It is possible that in the absence of a pineal-driven *Per1* rhythm, the expression or the cycling patterns of these genes in the striatum may change, thereby altering the susceptibility of the system to cocaine sensitization (i.e., circadian sensitization is absent in melatonin-deficient mice). It has been shown that for cocaine sensitization to occur, a synergism between the activities of dopamine 1 and 2 receptors is essential (Capper-Loup et al., 2002) and we postulate that circadian (e.g., Per-dependent) mechanisms could influence one or both of these receptor subtypes and thereby modify their synergistic activity. For example, such a circadian variation in dopamine receptor-like responsiveness to quinpirole has recently been observed in fruit flies (Andretic and Hirsh, 2000).

Collectively, the results of this study are consistent with our proposal that the pineal products NAS and melatonin allow for a rhythmic expression of striatal *Per1* and are critical for the nighttime suppression of the development of locomotor cocaine sensitization. We expect this line of research to help us understand the underlying circadian mechanisms of addictive behavior and to bring attention to the selection of the subjects (i.e., strain or species), time of treatment, and time of behavioral experiments in studying clock genes and behaviors mediated by these genes. Further research is needed to discern how NAS and melatonin and their receptors (i.e., melatonin receptors) participate in regulating *Per1* expression and, in turn, clock-controlled genes.

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