

# The Pineal Gland is Critical for Circadian *Period1* Expression in the Striatum and for Circadian Cocaine Sensitization in Mice

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Sensitization to psychostimulants can be influenced by circadian rhythms. The pineal gland, the main source of circadian melatonin synthesis, may influence behavioral sensitization to cocaine; mice with normal melatonin rhythms do not get sensitized at night. Clock genes such as *Period1* (*Per1*) show rhythmic region- and strain-dependent expression in the mouse brain, and mice mutant for the *Per1* gene lack cocaine sensitization. Here, for the first time we show circadian changes of PER1 protein levels in the mouse striatum, a brain region crucial for the development of locomotor sensitization to cocaine. In male C3H/HeJ mice, we found peak striatal PER1 protein levels during the day; this was preceded by a *Per1* mRNA peak 16 h earlier. Pinealectomized mice did not show this circadian pattern. We analyzed circadian cocaine sensitization at times when striatal PER1 protein levels in control mice (naive and sham-pinealectomized) were high and low, respectively. Only mice with circadian changes in striatal *Per1* expression showed the night-time absence of cocaine sensitization, whereas pinealectomized mice were without circadian changes in striatal *Per1* and were sensitized to cocaine regardless of diurnal rhythm. Our results indicate that both the striatal circadian *Per1* expression and diurnal locomotor cocaine sensitization are strongly influenced by pineal products. Since we found evidence for the expression of melatonin receptor mRNA in the striatum, we suggest that further studies on pineal-driven mechanisms will help us better understand the mechanisms of drug abuse and identify novel targets for the prevention and/or treatment of addictions.

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## INTRODUCTION

Psychostimulants, such as cocaine and amphetamine, show circadian rhythmicity in their behavioral effects in rodents (Gaytan *et al*, 1999, 2000; Uz *et al*, 2002a), moreover chronic cocaine users show seasonal rhythmicity in their cravings and in drug abuse (Satel and Gawin, 1989; Sandyk and Kanofsky, 1992).

Behavioral sensitization is a process in which repeated exposure to a drug results in increased behavioral responses and that is common across the species; that is, from fruit flies (*Drosophila*) to humans (Robinson and Berridge, 1993; Prinssen *et al*, 1996; Andretic *et al*, 1999; Sax and Strakowski, 2001). Recent work using *Drosophila* has identified a family of circadian 'clock genes' as an important contributor to the development of cocaine sensitization (for a review, see Wolf, 1999; Hirsh, 2001). Thus, flies mutant for

these genes, including *Period* (*Per*), do not sensitize to cocaine (Andretic *et al*, 1999).

It is believed that the mammalian clock system is hierarchical, with a master clock located within the suprachiasmatic nucleus (SCN). In the mouse circadian clock, Clock and Bmal1 are two transcription factors critical for the regulation of expression of three period genes (*Per1*, *Per2*, and *Per3*) and two cryptochrome genes (*Cry1* and *Cry2*) (Etchegaray *et al*, 2003; Morse and Sassone-Corsi, 2002). In a follow-up on *Drosophila* work (Andretic *et al*, 1999), it was found that mice deficient (ie 'knockout') for *Per1*, but not *Per2*, do not develop behavioral sensitization to cocaine (Abarca *et al*, 2002).

Recently, we provided first evidence that the development of behavioral sensitization to cocaine in mice depends on circadian rhythms (Uz *et al*, 2002a). Thus, mice with regular pineal melatonin rhythms sensitize to cocaine during the day, but do not show cocaine sensitization at night when melatonin levels are the highest.

The pineal gland is a key organ that influences mammalian circadian and seasonal rhythms. In this gland, melatonin is synthesized from serotonin via arylalkylamine *N*-acetyltransferase (AANAT), which produces rate-limiting intermediate *N*-acetylserotonin (NAS). In rodents, AANAT mRNA content (Roseboom *et al*, 1996; Uz *et al*, 2002b) and

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NAS and melatonin levels (Goto *et al*, 1989; von Gall *et al*, 2000) are marked by a circadian rhythm with high levels at night and low or even undetectable levels during the day. Recent findings indicate that the pineal gland also contributes to circadian cocaine sensitization; mice mutant for AANAT, whose pineal glands are incapable of synthesizing NAS and melatonin, do not show diurnal cocaine sensitization (Uz *et al*, 2002a).

It is possible that pineal products might influence not only circadian behavioral sensitization but also *Per* rhythm, particularly in the striatum, a brain region directly involved in behavioral cocaine sensitization. Although the rhythmic expression of *Per1* in the SCN is an autonomous process independent of the pineal gland, there are regions outside the SCN where the rhythm of *Per1* expression depends on this gland. For example, using pinealectomy (removal of the pineal gland) in hamsters, Messager *et al* (2001) demonstrated that pineal products are essential for rhythmic *Per1* expression in the hypophyseal pars tuberalis (PT), but not in the SCN. Similar results were obtained in experiments with mice (von Gall *et al*, 2002).

Here, we hypothesize that the pineal gland may serve as an independent and critical oscillator for the striatal *Per1* rhythm via its products, NAS and/or melatonin, and that this pineal gland-driven *Per1* rhythm is essential for the development of diurnal behavioral sensitization to cocaine. Thus, using diurnal rhythms as a model in normal and pinealectomized mice, we characterized the striatal expression of *Per1* mRNA, PER1 protein, and the development of behavioral cocaine sensitization.

## MATERIALS AND METHODS

### Animals and Drug Treatment

Male C3H/HeJ mice, 8 weeks old and weighing 25–30 g, were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Pinealectomized and sham-operated C3H mice of the same age and weight were purchased from Taconic (Germantown, NY, USA). Animals (36 naive, 60 sham-pinealectomized, and 60 pinealectomized) 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 am; Zeitgeber time 00 [ZT00]). Cocaine hydrochloride (Sigma Chemical Co., St Louis, MO, USA) was dissolved in sterile physiological saline (0.9% NaCl) and administered intraperitoneally (20 mg/kg; i.p.) in an injection volume of 0.05 ml/25 g body weight. This dose of cocaine was selected based on our previous dose-response studies (Uz *et al*, 2002a). The experimental protocol was approved by the Institutional Animal Care Committee.

### Sample Preparation

Brain samples were taken every 4 h over a 24-h period, starting at ZT01 (during the dark periods, samples were collected under dim red light) or at ZT05 and ZT20 only. Since the caudate-putamen (CPu) and nucleus accumbens (NAc) of the striatum play an important role in the initiation and maintenance of behavioral sensitization

(Robinson and Berridge, 1993), we analyzed these areas in samples obtained from individual animals, and quantified their *Per1* mRNA and protein levels. For this purpose, the CPu and NAc were excised from 1-mm-thick coronal brain slices (Bregma 1.54–0.74 mm; Paxinos and Franklin, 2001) using a 12-gauge syringe needle as described elsewhere (Terwilliger *et al*, 1991). In these experiments, due to the small sample sizes, different sets of animals were used to quantify mRNA and protein amounts, respectively. Since we observed similar mRNA and protein expression patterns in these specific areas, for all other experiments we used the entire striatum. For this purpose, the striatum was isolated by gross dissection and processed individually; each sample was split in half for mRNA and protein assay, respectively. Punched and gross-dissected tissues were frozen immediately on dry ice and stored at –80°C until assay.

### RNA Extraction and Quantitation

Due to the small size of mouse brain samples, the total RNA was extracted applying the chemical isolation method with TRIzol<sup>®</sup> reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. To eliminate possible DNA contamination, RNA samples were treated with a DNase reagent, DNA-free<sup>™</sup> (Ambion, Inc., Austin, TX, USA). The yield of total RNA was determined by measuring the absorbance of an aliquot of the precipitated stock at a wavelength of 260/280 nm. Furthermore, to test possible DNA contamination, each sample was run by RT-PCR without adding the reverse transcriptase enzyme (see below).

Amplification primers were ordered from Integrated DNA Technologies, Inc. (Coralville, IA, USA). The primer pairs were designed to allow amplification of 149–536 bp for the mouse *Per1* gene (*mPer1*; Sun *et al*, 1997) (F:5'-ATGGCT-CAAGTGGCAATGAGTCCA-3'; R:5'-CTGGTTAGCCT-GAACCTGCTTGAC-3'), of 176–473 bp for the *cyclophilin* (*cyc*; Hasel and Sutcliffe, 1990) (F:5'-ATTTGGCTA-TAAGGTTTCCTC-3'; R:5'-ACGCTCCATGGCTTCCA-CAAT-3'), of 367–874 bp for the melatonin 1 receptor (MT1; Roca *et al*, 1996) (5'-GGATCGCTATGAACCGT-TACTGCT; 3'-AGCTGTTGAAGTACGCCAGGTAGT), and of 10–343 bp for the melatonin 2 receptor (MT2; Weaver *et al*, 1996) (5'-TCTGTCATAGTACCACCTACCACC; 3'-TGGTTAGGAACTGCGCAAATCAC). Each primer contained comparable G+C content to minimize variability in hybridization efficiency at the annealing temperature.

To allow coamplification of *mPer1* with the more abundant *cyc* mRNA, pilot studies were conducted to determine optimal relative primer concentration and cycle number, whereby the PCR would still be within the exponential phase of amplification for all transcripts. The assay is described elsewhere (Uz and Manev, 1998). Briefly, striatal total RNA from each sample (0.5 µg of starting amount) was denatured at 80°C for 6 min and then reverse transcribed with cloned Moloney murine leukemia virus (M-MLV) reverse transcriptase (200 U; Invitrogen, Carlsbad, CA, USA) in reverse transcription (RT) buffer containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, and 1 mM deoxynucleotide triphosphate (dNTPs; Invitrogen, Carlsbad, CA, USA) using random hexamers (5 mM; Pharmacia Biotech, Piscataway, NJ, USA) and

ribonuclease inhibitors (HPRI, 28 U; Amersham, Piscataway, NJ, USA) in a volume of 20  $\mu$ l. The RT mixture was incubated at 37°C for 60 min to promote cDNA synthesis. The reaction was terminated by heating the samples at 98°C for 5 min and then rapidly chilling on ice. The cDNA aliquots containing reverse-transcribed material and specific primer pairs (*mPer1/cyc*, 0.5  $\mu$ M/0.1  $\mu$ M) were amplified with Hot *Tub* DNA polymerase (Amersham, Piscataway, NJ, USA) in a thermal cycler (9600, Perkin-Elmer, Wellesley, MA, USA). The PCR mixture was amplified for 24 cycles with denaturation (94°C, 15 s), annealing (60°C, 30 s), and elongation (72°C, 30 s) amplification steps. The reaction was terminated with a 5-min final elongation step. Trace amounts of [<sup>32</sup>P]dCTP (0.5  $\mu$ Ci/sample; Amersham, Piscataway, NJ, USA) were included during the PCR step for subsequent quantification. To quantify the amount of the product corresponding to the amplified mRNA, the ethidium bromide-stained bands were excised from the electrophoresis gels and counted (Uz and Manev, 1998).

The MT1 and MT2 receptor-specific primers were used only to demonstrate the expression of their respective mRNAs in the CPu and NAc areas of the striatum. RT-PCR conditions for these primers were the same as for *mPer1*, except the number of cycles, 30.

### Quantitative mPER1 Western Immunoblotting

To quantitate the levels of mouse PER1 protein (mPER1) in the striatum, striatal tissue was homogenized in a buffer containing 20 mM Tris-HCl, 2 mM ethylene glycol-bis ( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 5 mM ethylenediamine tetraacetic 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, 40–80  $\mu$ g samples and gel loading solution (50 mM Tris-HCl [pH 6.8], 4%  $\beta$ -mercaptoethanol, 1% sodium dodecylsulfate (SDS), 40% glycerol, and a trace amount of bromophenol blue) were mixed and boiled for 3 min. They were loaded onto a 7.5% (w/v) acrylamide gel using the Mini Protean II gel apparatus (Bio-Rad, Hercules, CA, USA). The gels were run using 25 mM Tris-base, 192 mM glycine, and 0.1% (w/v) SDS at 150 V. The proteins were subsequently transferred electrophoretically to an ECL nitrocellulose membrane (Amersham, Piscataway, NJ, USA) using the Mini TransBlot transfer unit (Bio-Rad, Hercules, CA, USA) at 150 mA constant current overnight. The 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  $\mu$ g/ml; Alpha Diagnostic International, Inc., San Antonio, TX, USA) in 5% (w/v) powdered nonfat milk in TBST, 2 ml NP-40, and 0.02% (w/v) 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, Piscataway, NJ, USA) for 4 h at room temperature and processed with an ECL kit; blots were then washed with TBST and exposed to Hyperfilm ECL (Amersham, Piscataway, NJ, USA). To normalize the signal for mPER1 protein, the presence of noninducible  $\beta$ -actin

protein was measured on the same blot using a mouse monoclonal antibody against the  $\beta$ -actin (1:5000; Sigma, St Louis, MO, USA). The mPER1 protein produced the expected size band at 140 kD as described by others (von Gall et al, 2001), while the  $\beta$ -actin band appeared at 42 kD (Figure 1a). The optical densities of the mPER1 bands were corrected by the optical density of the corresponding  $\beta$ -actin bands on the film using the Loats Image Analysis System (Loats Associates, Inc., Westminster, MD, USA).

### Locomotor Activity Measurements, Behavioral Sensitization

Mice who had not been previously exposed to the testing monitors were weighed and placed individually in mouse activity cages for a 30-min adaptation period. After this period, locomotor activity was measured for 30 min using the Cage Rack Photobeam Activity Measurement System (San Diego Instruments, Inc., San Diego, CA, USA) equipped with computer-monitored photobeam frames. Immediately after the second 30-min interval, animals were injected with cocaine and returned to their activity cages; the recording procedure continued for an additional 30-min period. Times of injections were daytime: ZT05–10 am, and night-time: ZT20–1 am. The movement of each animal was recorded as the number of beam interruptions and reported as locomotor activity (ambulation). The experimental room was illuminated by normal fluorescent light (about 150 lux at the bench level) operated with a light timer; the night-time drug administrations were carried out under dim red light (0.1 lux at the bench level). After each experimental session, animals were returned to their home cages. To measure the development of locomotor sensitization, the same procedure was repeated for 3 consecutive days and nights (Uz et al, 2002a).

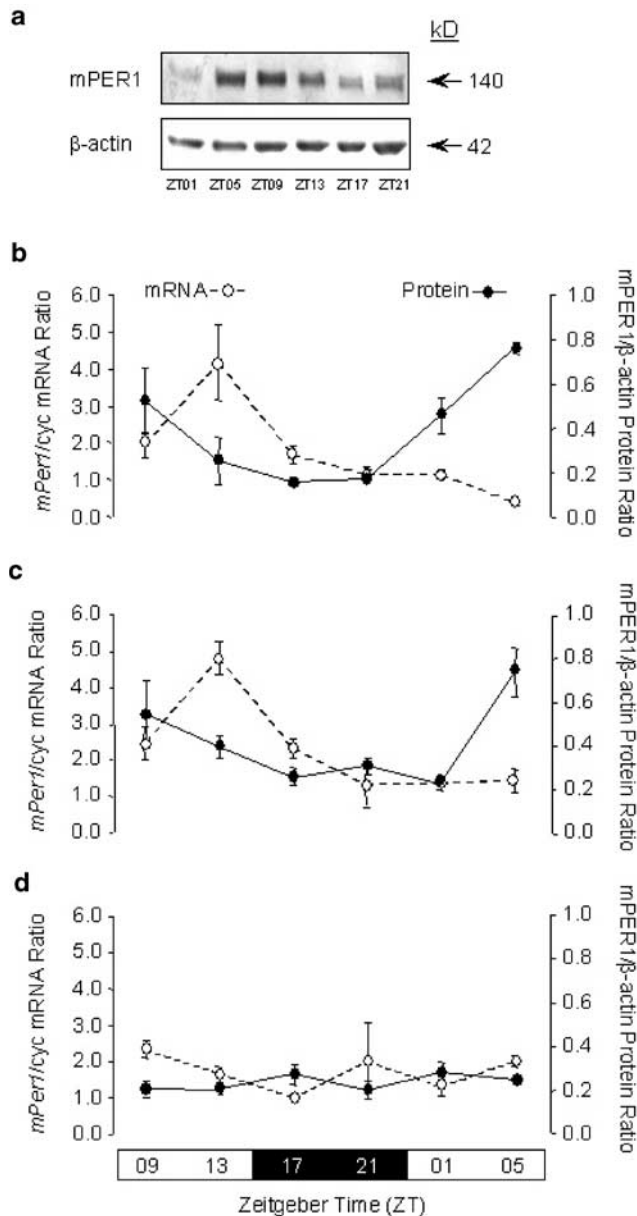
### Statistical Analysis

Optical density values and radioactivity counts (both calculated as ratio, mean  $\pm$  SEM) were evaluated by analysis of variance (ANOVA) followed by *t*-test. Locomotor activity scores (mean  $\pm$  SEM) were analyzed by two-way ANOVA with the factors of the strains and the time of the day separately, followed by the Dunnett's multiple comparison test. Significance was accepted as  $p < 0.05$ .

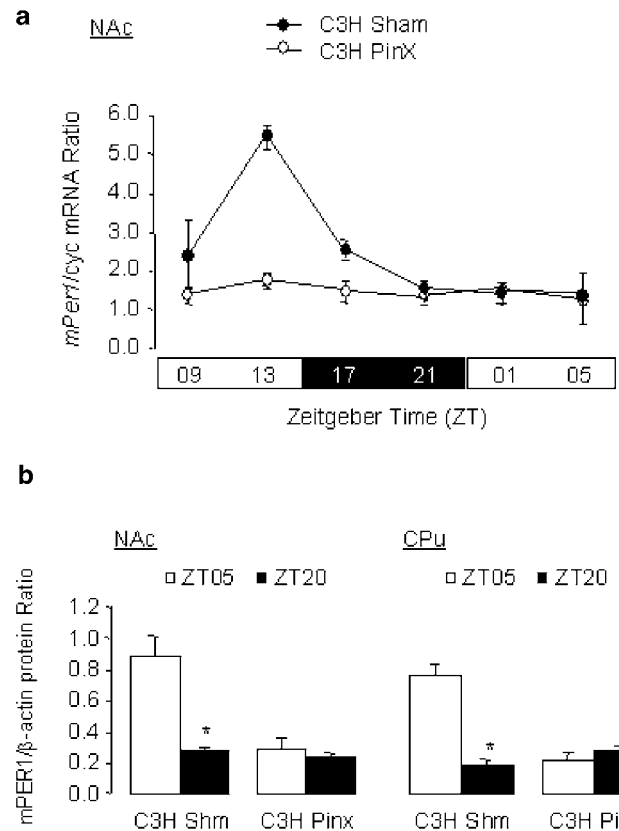
## RESULTS

### Circadian Striatal *mPer1* mRNA and Protein Expression

To investigate the rhythm of *mPer1* gene expression in the striatum, we used NAS/melatonin-proficient naive C3H inbred mice and measured the 24-h-cycle content of *mPer1* mRNA applying RT-PCR. We also measured mPER1 protein levels employing Western immunoblotting with a specific mPER1 antibody. We found that diurnal *mPer1* mRNA expression in the striatum peaked at ZT13. Following this mRNA peak, within 8 h mPER1 protein levels started to elevate and peaked at ZT05 (daytime; 16 h following the mRNA peak). The PER1 levels in the striatum were at their lowest between ZT17 and ZT21 (nighttime) (Figure 1 a and b).



**Figure 1** Removal of the pineal gland abolishes the *mPer1* mRNA and mPER1 protein rhythm in the striatum of C3H mice. Mice with an intact pineal gland, either naive (a, b) or sham-operated (c), demonstrate similar rhythmic *mPer1* gene expression in the striatum, whereas pinealectomized mice show no rhythm over a 24 h period (d). Panel (a) shows an example of the mPER1 Western immunoblot from a striatal sample taken from naive C3H mice. The mPER1-specific antibody detected an expected size (around 140 kD) band. For the quantitative assay, the period protein and mRNA signals were normalized by the corresponding  $\beta$ -actin or cyclophilin (cyc) signals, respectively. The results of the quantitative assay (panels b–d) are the mean  $\pm$  SEM, obtained from three animals from each time point ( $n = 18$  for each group, that is, naive, sham-operated, and pinealectomized). The results are shown as ratios of *mPer1*/cyclophilin (cyc) for mRNA (broken lines with open circles) and mPER1/ $\beta$ -actin for protein (solid lines with closed circles). Note that in both control groups, the peak levels of mRNA (ZT13) precede the peak levels of protein (ZT05) and that no rhythm is observed in pinealectomized mice. The zeitgeber time 00 (ZT00) is defined as lights on.



**Figure 2** Pinealectomy affects the rhythm of the *mPer1* gene in the NAc and the CPU of the striatum. The *mPer1* mRNA levels were assayed in the NAc of control (closed circles) and pinealectomized (open circles) mice (18 per group; three per each time point) over a 24 h period (a). Note the absence of a ZT13 peak in the pinealectomized mice. The mPER1 protein content was assayed in both the NAc and CPU at the time points selected for cocaine injections (ie daytime, ZT05 vs night-time, ZT20; three mice per group) (b). The nighttime values are significantly lower compared with the corresponding daytime values (\* $p < 0.05$ ; t-test). Note that pinealectomized and control mice show similar (ie low) daytime and night-time mPER1 values (b).

Pinealectomy had a major effect on the circadian expression of striatal *mPer1*. Hence, the rhythm of both *mPer1* mRNA and mPER1 protein was intact in sham-pinealectomized mice (Figure 1c) and absent in pinealectomized C3H mice (Figure 1d). Moreover, in pinealectomized mice the striatal content of *mPer1* mRNA and mPER1 protein was relatively low; that is, in the range of the low diurnal levels observed in naive or sham-pinealectomized mice.

Similar results were obtained in two subregions of the striatum, the NAc and CPU. Thus, the rhythm of *mPer1* mRNA in the NAc of control mice was similar to the rhythm in the entire striatum, whereas in the NAc of pinealectomized mice this rhythm was absent (Figure 2a). In both the NAc and CPU, mPER1 protein levels were decreased by pinealectomy, measured at ZT05. At ZT20, mPER1 protein levels were low in both control and pinealectomized mice (Figure 2b).

### Diurnal Behavioral Cocaine Sensitization

We previously reported that NAS/melatonin-proficient mice (ie C3H) show circadian sensitization to cocaine; they do

not develop sensitization when the drug is given repeatedly at night, but sensitization is developed after repeated daytime drug administrations. On the other hand, AA-NAT-mutant C57BL (ie NAS/melatonin-deficient) mice lack this diurnal pattern and sensitize to cocaine both during the day and night (Uz et al, 2002a). Here, we used the naive C3H mice to replicate our original findings (a positive control), and we added additional groups; pinealectomized C3H mice and their sham-operated controls. We confirmed the pineal NAS status of control mice at two time points selected for cocaine administration, that is, the daytime—ZT05, and the nighttime—ZT20. Hence, the pineal NAS was detectable at ZT20 and absent at ZT05 (data not shown).

We selected ZT05 and ZT20 as the times for cocaine administration based on our findings that in normal C3H mice, these are the time points when striatal *mPER1* protein contents are high and low, respectively. Figure 3 shows that all the above-noted experimental groups developed cocaine sensitization after the third daytime injection, whereas the nighttime injections failed to produce sensitization both in naive and sham-pinealectomized control mice. On the other hand, nighttime cocaine injections did produce sensitization in the pinealectomized mice (Figure 3).

### Melatonin Receptor mRNA in the Striatum

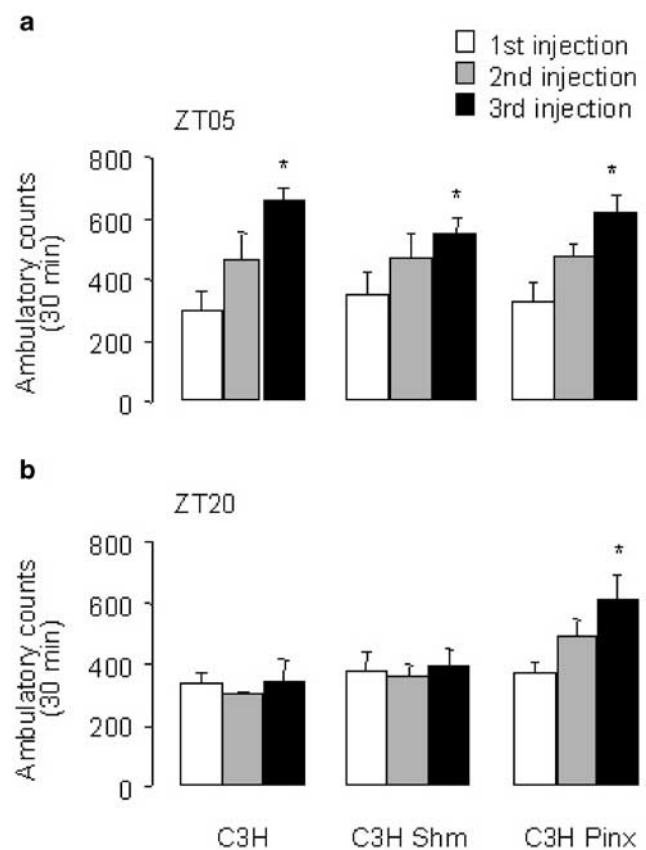
We investigated whether the mRNAs for two melatonin receptor subtypes, MT1 and MT2, are expressed in the CPU and NAc regions of the striatum. Figure 4 shows that both mRNAs are present in the mouse striatum.

### DISCUSSION

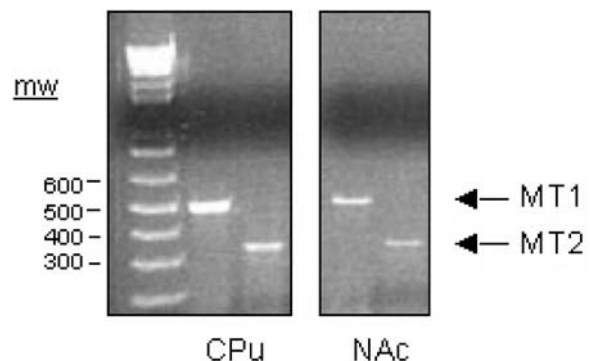
Behavioral sensitization, also termed reverse tolerance, is characterized by progressive and long-lasting behavioral changes that develop after repeated administration of stimulant drugs (Robinson and Berridge, 1993) and may involve lasting alterations in gene expression. Cocaine-induced behavioral sensitization has been commonly used as an experimental model across the species (Prinssen et al, 1996; Andretic et al, 1999; Sircar, 2000), since it replicates repetitive drug taking in humans and also promotes the drug cravings and further addictive behaviors seen in drug dependence (Robinson and Berridge, 1993; Sax and Strakowski, 2001).

The main finding of our work is that the pineal gland has a prominent permissive role both for the circadian expression of striatal *mPer1* and for the development of diurnal behavioral sensitization to cocaine. Thus, we observed a strong positive correlation between the rhythmic *mPER1* protein level changes in the striatum and the development of diurnal locomotor cocaine sensitization. Consequently, when striatal *mPER1* rhythm was abolished, for example, by pinealectomy, diurnal cocaine sensitization was abolished as well and the mice became cocaine-sensitized despite their diurnal cycle.

A number of studies have established that alterations in the functioning of basal ganglia are linked to the appearance of sensitization phenotypes and that the striatum is a region crucial for the development and maintenance of behavioral



**Figure 3** The critical role of the pineal gland in the development of a diurnal pattern of cocaine sensitization. Cocaine sensitization was studied in naive (C3H), sham-pinealectomized (C3H Shm) mice, and pinealectomized (C3H Pinx) mice. Cocaine was administered for 3 consecutive days either during the day (ZT05) (panel a) or at night (ZT20) (panel b). Results (mean  $\pm$  SEM;  $n = 9$ /group/time point) are expressed as locomotor activity; that is, 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, the absence of sensitization in control mice at ZT20, and the reappearance of night-time sensitization in pinealectomized mice. \* $p < 0.05$  in comparison with the corresponding first injection values (Dunnett's test).



**Figure 4** Melatonin receptor MT1 and MT2 mRNAs are expressed in the CPU (left panel) and the NAc (right panel) of the mouse striatum. The presence of MT1 and MT2 mRNAs was detected using the corresponding primer pairs and the RT-PCR assay (see Materials and methods for details).

sensitization to cocaine (Robinson and Berridge, 1993). Work with methods that disrupt gene expression has demonstrated that several genes may be required for the

development of cocaine sensitization in mice (Laakso *et al*, 2002). Abarca *et al* (2002) used knockout mice that do not express *mPer1* or *mPer2* and found that the *mPer1*, but not the *mPer2* gene, is required for the development of cocaine-induced locomotor sensitization. The importance of the *Per* pathway is suggested by the finding that for cocaine sensitization to occur in different species, *Per* expression is obligatory. Thus, fruit flies mutant for clock genes, including *Per*, failed to develop behavioral cocaine sensitization (Andreatic *et al*, 1999).

Since one of the prominent features of clock genes, which encode a set of transcriptional modulators, is their cyclic expression, we postulate that in addition to its general permissive role (demonstrated by the absence of cocaine sensitization in *Per*-null mutants), the *rhythm* of mPER1 protein in the striatum might be involved in *diurnal* cocaine sensitization in mice. Even though all three *mPer* isoforms cycle at both the mRNA and the protein levels in the SCN, *mPer1* also shows region-specific and SCN-independent expression rhythm (von Gall *et al*, 2001; Sun *et al*, 1997; Iijima *et al*, 2002). Since SCN is thought to coordinate and regulate behavioral clocks in mammals, only a few studies have explored the SCN-independent rhythms of clock genes in the brain. Similar to our findings, Iijima *et al* (2002) observed SCN-independent *mPer* mRNA expression in the mouse striatum.

Our results demonstrate for the first time that the levels of both *mPer1* mRNA and mPER1 protein cycle in the mouse striatum. It is the PER protein and not the mRNA that may regulate gene transcription; for example, at the promoter level by interfering with the other clock protein/transcription factors (Stanewsky, 2003). Thus, the information on the rhythm of PER protein levels as opposed to mRNA levels in a brain region, such as the striatum, appears to be relevant for understanding the putative link between *Per* expression and cocaine sensitization. In naive and sham-pinealectomized mice, sensitization occurred when cocaine was injected at ZT05 (high striatal mPER1) and it did not develop if cocaine was injected at ZT20 (low striatal mPER1). Interestingly, pinealectomized mice, who also lack rhythmic striatal *mPer1* expression, developed sensitization to repeated cocaine injections at all time points, even at ZT20, when mPER1 protein levels in the striatum are low. These results indicate that the *rhythm* of mPER1 protein is as important as the *absence* (Abarca *et al*, 2002) of this protein for the suppression of cocaine sensitization. Alternatively, since our study addressed the total, but not the cell-specific *mPer1* expression in the striatum, it is possible that *mPer1* expression in certain cell populations is more affected and/or relevant for the development of cocaine sensitization. This possibility should be explored in future studies.

A critical role of the pineal gland and its products for the SCN-independent expression of *Per1* has been inferred from the effects of pinealectomy on the rhythm of *Per1* expression in the pituitary cells of hamsters and mice (Messenger *et al*, 2001; von Gall *et al*, 2002). In these experiments, not only pinealectomy but also the knockout of the melatonin MT1 receptors abolished the diurnal rhythm of the PT mPER1 protein (von Gall *et al*, 2002). Since MT1 receptors respond to both melatonin and NAS (Dubocovich *et al*, 1999), it is likely that the pineal gland-

dependent rhythms of striatal and pituitary *mPer1* expression involve these receptors, and also pineal products such as NAS and melatonin. In support of this possibility, we found evidence for the striatal expression of MT1 and MT2 receptor mRNA in the striatum. These receptors are members of a superfamily of seven-transmembrane domain G-coupled receptors (Reppert *et al*, 1995; Roca *et al*, 1996) for which specific ligands are being developed (Dubocovich *et al*, 1999), and pharmacological experiments (Sircar, 2000) could further elucidate a putative role for the striatal melatonin receptors in cocaine sensitization.

In our previous work (Uz *et al*, 2002a), we observed a dramatic difference in the occurrence of diurnal cocaine sensitization between C3H mice and C57BL mice—similar to pinealectomized mice, the latter strain lacked the diurnal pattern of sensitization and sensitized to cocaine both during the day and at night. We proposed that the absence of nocturnal suppression of cocaine sensitization in C57BL mice could have been caused by the AANAT mutation/deficiency and the inability of these mice to synthesize NAS and melatonin (Stehle *et al*, 2002; Uz and Manev, 2001; Uz *et al*, 2002a). Also Abarca *et al* (2002), who investigated the diurnal responsiveness of C57BL mice to cocaine sensitization, did not observe an absence of sensitization in these mice. These authors reported slight differences in the extent of cocaine-induced locomotor sensitization at different times during the day; the only procedure that abolished cocaine sensitization in these mice was *mPer1* knockout.

In conclusion, we demonstrated the existence of a strong correlation between the circadian pattern of mPER1 cycling in the striatum and the diurnal development of cocaine sensitization, and we provide evidence that the pineal gland plays a critical role both in striatal *mPer1* rhythm and the development of diurnal cocaine sensitization. We postulate that these pineal gland-driven rhythms are mediated by pineal products such as NAS and/or melatonin, and that they might involve melatonin receptors expressed in the striatum. Further research is needed to confirm this putative role for NAS and melatonin in regulating striatal *mPer1* gene expression and rhythm, and in the development of circadian behavioral sensitization to cocaine. A more complete characterization of these mechanisms may help us better understand the mechanisms of drug abuse and identify novel targets for the prevention and/or treatment of addictions.

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