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Diurnal rhythms in quinpirole-induced locomotor behaviors and striatal D2/D3 receptor levels in mice

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Abstract

Dopaminergic drugs, including the D2/D3 agonist quinpirole, produce lasting changes in the brain that lead to altered behavioral responses. The action of these drugs is dosing time-dependent; in fruit flies, behavioral response to quinpirole shows a marked circadian variability. Here we demonstrate diurnal rhythm-dependent variations both in quinpirole-induced locomotor behaviors and in striatal D2 and D3 protein levels in mice. We found opposing diurnal rhythms in striatal D2 and D3 protein levels, resulting in a high D2/D3 ratio during the day and a low D2/D3 ratio at night. Protracted quinpirole treatment differentially altered striatal D2/D3 rhythms depending on the time of injection (i.e., day or night). When quinpirole-induced locomotor activity was analyzed for 90 min, we found hypomotility after the first day or nighttime drug injection. By the seventh injection, daytime quinpirole treatment produced clear hyperactivity while nighttime quinpirole treatment continued to induce a significant initial hypoactivity followed by a hyperactivity period. Our data indicate that quinpirole-induced long-term alterations in the brain include dosing time-dependent changes in dopamine receptor rhythms. Therefore, we propose that diurnal mechanisms, which participate in drug-induced long-term changes in the dopamine system, are important for the development of dopaminergic behaviors.

Keywords: Behavioral sensitization; Tolerance; Striatum; Dopamine receptor rhythm; Circadian; Inbred mice

1. Introduction

Much like other neurotransmitter systems, the dopaminergic system is influenced by circadian mechanisms: dopamine release and synthesis have diurnal rhythms (Lemmer and Berger, 1978; O'Neill and Fillenz, 1985; Doyle et al., 2002). Ligand-binding studies show that dopamine receptors have circadian characteristics (Wirz-Justice, 1987; Naber et al., 1980; Kafka et al., 1983). Circadian susceptibility to the behavioral effects of dopaminergic drugs, such as SKF-38393 and apomorphine, has been reported (Martin-Iverson and Yamada, 1992; Nagayama et al., 1978; Nasello et al., 1995). Recently using the D2/D3 receptor agonist quinpirole, Andretic and Hirsh (2000) demonstrated circadian modulation of dopamine receptor-mediated behavioral responses in the fruit fly, possibly explained by 'clock gene' *Period*-driven dopamine receptor rhythms. In the same vein, we reported that cocaine-induced behavioral sensitization is dosing time-dependent (Uz et al., 2002), and that the mechanisms responsible for this dependence involve circadian genes (i.e., Period) and pineal melatonin (Uz et al., 2003; Akhisaroglu et al., 2004). Thus, it is possible that the long-term effects of dopaminergic drugs can be influenced by clock gene-mediated circadian mechanisms.

Since information on circadian alterations of dopamine receptor protein levels in the mammalian brain is not yet available, in this study we investigated whether striatal D2 and D3 receptor levels demonstrate rhythms and further, whether these rhythms influence the development of dopamine receptor-mediated locomotor behaviors in mice treated with quinpirole. It is possible that circadian alterations of dopamine receptor contents may determine the dosing time-dependent action of dopaminergic drugs; i.e.,

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they may influence the behavioral/therapeutic action of such pharmacological treatments. Furthermore, our results may provide new candidate mechanisms (i.e., clock gene-mediated circadian mechanisms) to better understand how dopamine receptors and the development of the behaviors mediated by these receptors are regulated in mammals.

2. Experimental methods

2.1. Animals and drug treatment

2.1.1. Animal characteristics

Male 8-week-old C3H/HeJ mice (n=90) weighing 25–30 g were purchased from Jackson Laboratories (Bar Harbor, ME, USA). 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 am; Zeitgeber time 00 [ZT00]). The experimental protocol was approved by the Institutional Animal Care Committee.

2.1.2. Drug dosing details

Quinpirole (Sigma Chemical, St. Louis, MO, USA) was dissolved in sterile physiological saline (0.9% NaCl) and administered intraperitoneally (1 mg/kg; i.p.) in an injection volume of 0.05 ml/25 g body weight every third day or night. The control group received vehicle, sterile physiological saline intraperitoneally. The time of injection was ZT05 for daytime and ZT21 for nighttime. Nighttime injections were administered under dim red light. The quinpirole dose (1 mg/kg), treatment frequency (every 72 hours), and the length of treatment (7 injections) were adapted from previously published work (Szechtman et al., 1994; Wang et al., 2000).

2.1.3. Protein measurement procedures

For the protein measurements, animals were sacrificed either at the end of the drug treatment (72 h after the last injection) or every 4 h over 24-h period for circadian rhythm measurements by decapitation following pentobarbital (50 mg/kg, i.p.) anesthesia. Tissue samples were frozen immediately on dry ice and later kept in −80 °C for the protein measurements.

2.2. Quantitative Western immunoblotting

2.2.1. Basal circadian levels of D2 and D3 proteins

To quantitate the basal levels of D2 and D3 proteins, striatal samples were excised from 1-mm-thick coronal brain slices [Bregma 1.54 mm through 0.74 mm (Paxinos and Franklin, 2001)] using a 12-gauge syringe needle as described elsewhere (Terwilliger et al., 1991) over 24-h-period every 4 h starting at ZT01 (n=18; three animals per time point; at night samples were collected under dim red light).

2.2.2. Quinpirole-induced changes on D2 and D3 protein levels

In another set of experiments, quinpirole-induced changes on receptor protein levels were measured; samples were taken in a similar way as described earlier 72 h after the last (seventh) day or nighttime quinpirole injection, either at ZT05 (daytime) or ZT21 (nighttime). Sample size was 12 for both day and nighttime groups (n=24). Tissue samples were homogenized in a buffer containing 20 mM Tris–HCl, 2 mM ethylene glycol-*bis* (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid, 5 mM ethylenediamine tetraacetic acid, 1.5 mM pepstatin, 2 mM leupeptin, 0.5 mM phenylmethylsulfonyl-fluoride, 0.2 units/ml aprotinin, and 2 mM dithiothreitol. After reading the protein content of the tissue homogenate, 20 to 40 μg samples and gel loading solution (50 mM Tris–HCl [pH 6.8], 4% β-mercaptoethanol, 1% sodium dodecyl sulfate [SDS], 40% glycerol, and a trace amount of bromphenol blue) were mixed and boiled for 3 min. They were loaded onto a 15% (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 milli-amperes (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 either D2 (anti-mouse; 1:500) or D3 (anti-rabbit; 1:1000) antibodies (Santa Cruz Biotechnology) 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 horseradish-peroxidase-linked secondary antibodies (anti-mouse and anti-rabbit IgGs; 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, Piscataway, NJ, USA).

2.2.3. Measurement of β-actin levels

To normalize the signal for D2 and D3 proteins in all measurements, 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). Both D2 and D3 proteins produced the expected size band at 48 kD while the β-actin band appeared at 42 kD (see Fig. 2 for sample blots). The optical density of the D2 and D3 protein 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, USA).

2.3. Locomotor activity measurements

The measurements were conducted as previously described (Uz et al., 2002, 2003). Briefly, mice (n=48, 12 mice

per group for each time point) that had not been previously exposed to the testing monitors were placed individually in mouse activity cages (Cage Rack Photobeam Activity Measurement System, San Diego Instruments, San Diego, CA, USA) equipped with computer-monitored photobeam frames for a 30-min adaptation period and their baseline locomotor activity was measured. Immediately after this 30-min interval, animals were injected with either quinpirole (i.p.; 1 mg/kg) or vehicle as a control group and returned to their activity cages for an additional 90-min period. The movement of each animal was recorded as the number of beam interruptions every 5 min and reported as locomotor activity (ambulation). Times of injections were daytime: ZT05 (10 am) and nighttime: ZT21 (2 am). Locomotor activity measurements were started 30 min before the injections to be able to record baseline activity. The experiment room was illuminated by normal fluorescent light (about 150 lux (lx) at the bench level) operated with a light timer; the nighttime drug administrations were done in dark using dim red light (0.1 lx at the bench level). After each experimental session, animals were returned to their home cages.

2.4. Statistical analysis

Two sample *t*-test (two-tailed) was used to compare the drug and control groups. Optical density values of protein samples were expressed as a percentage of the corresponding saline-treated control (calculated as ratios, mean±S.E.M.). The ambulatory counts of saline-treated mice were subtracted from the corresponding ambulatory counts of quinpirole-treated mice and are expressed as mean±S.E.M. Type I errors are properly controlled by fixing them at commonly accepted level of α=0.05. Differences are deemed statistically significant when p value<0.05. Small p-values suggest that there is a significant difference (see Results section for the p values). The power of the tests were determined using a commercial software (Minitab, Version 13) where the sample size, standardized effect size, and the alpha level were taken into consideration. We assumed unequal variances and used the pooled standard deviation in the power calculations. We found that the attained power is adequate (i.e., over 0.90) for the all *t*-tests we run.

3. Results

3.1. Diurnal rhythm of striatal D2 and D3 receptor protein levels

Naïve mice were sacrificed at 4-h intervals to obtain striatal samples for quantitative Western blot assays of D2 and D3 protein levels. We found diurnal variations in D2 (Fig. 1A) and D3 (Fig. 1B) protein levels (corrected by corresponding β-actin signals).

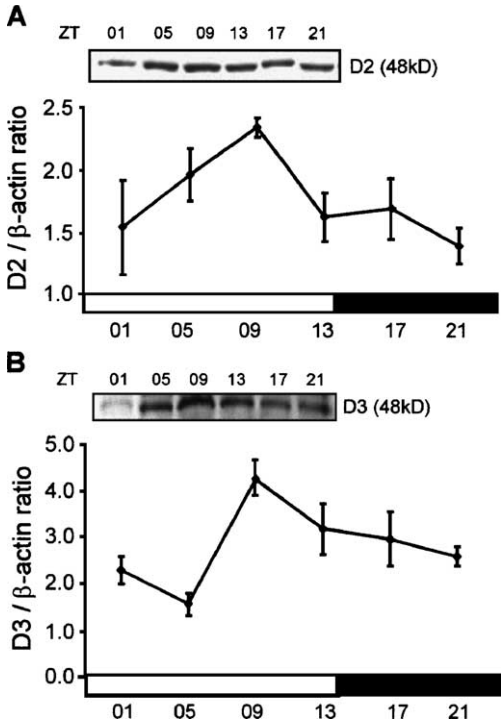

Fig. 1. Diurnal rhythms of D2 and D3 dopamine receptor protein levels in the mouse striatum. Mice (3 per group) were sacrificed every 4 h starting at Zeitgeber time (ZT) 01 (ZT00 is defined as lights on). Striatal samples were processed for Western blotting. Demonstrative Western blot images in panels (A) and (B) show striatal 24-h-rhythms of D2 and D3 signals, respectively. For the quantitative assay, D2 (A) and D3 (B) signals were normalized by their corresponding β-actin signal. The results (mean±S.E.M.) are shown as ratios of each protein to the corresponding β-actin values.

3.2. Differential effect of daytime vs. nighttime quinpirole on striatal D2 and D3 protein levels

Striatal samples were collected 72 h after the final quinpirole injection (seventh injection) either during the day (ZT05, for the daytime treatment group) or at night (ZT21, for the nighttime treatment group). Protracted quinpirole had a significant effect on D2/D3 balance; the daytime injections resulted in lower daytime D2/D3 ratio compared to saline-injected group whereas the nighttime injections led to higher nighttime D2/D3 ratio (Fig. 2). This differential effect on D2/D3 balance was achieved concomitantly by diametrically opposite changes in the content of D2 vs. D3 protein levels (Fig. 2A and B), i.e., daytime quinpirole injection reduced D2 [$t(10)$=5.54, p=0.0003] and elevated D3 [$t(10)$=−2.42, p=0.04] levels, whereas nighttime quinpirole elevated D2 [$t(10)$=−4.26, p=0.002] but reduced D3 [$t(10)$=4.48, p=0.001] levels.

3.3. Locomotor activity changes induced by quinpirole

Before injecting vehicle or quinpirole, the mice were left for 30 min in a test cage and their ambulatory activity was measured (adaptation period). The ambulatory counts for the

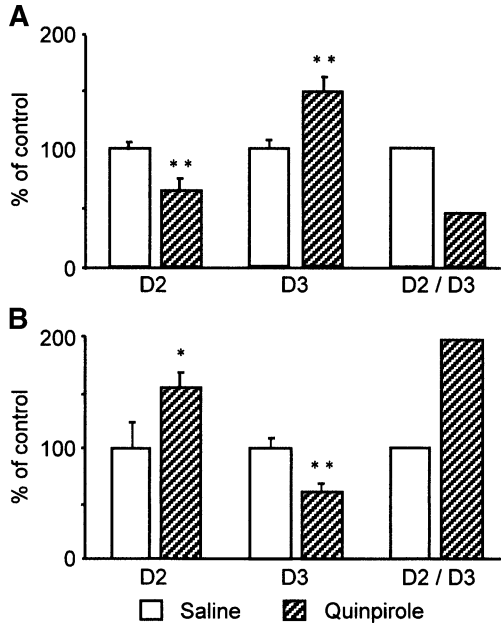

Fig. 2. Differential effects of protracted daytime vs. nighttime quinpirole treatment on striatal D2 and D3 dopamine receptor protein levels. Striatal brain samples were collected 72 h after the last daytime (panel A) or nighttime (panel B) quinpirole injection and assayed and calculated as described in Fig. 1. The results are expressed as a percentage of the corresponding saline-treated control. For D2 and D3 ratios, the results are shown as mean±S.E.M. (n=6/time point). Open bars=saline-treated control (100%); hatched bars=quinpirole; *p<0.05; **p<0.01 vs. control (t-test).

adaptation period (i.e., baseline locomotor activity) did not differ between the day and nighttime groups or in subsequent exposures throughout the duration of the experiment (data not shown). After each vehicle or quinpirole injection (i.e., every third day or night), ambulatory counts were measured for 90 min. Analysis of quinpirole-induced locomotor behavior for 90 min after each injection revealed a characteristic pattern (Fig. 3). Therefore, we divided this 90-min period into three 30-min blocks (I, II, and III in Fig. 4). Interestingly, both daytime [$t(22)$=8.70, p=0.001] and nighttime [$t(22)$=13.08, p=0.0001] initial quinpirole injections produced significant hypomotility in the first 30-min block (Fig. 4A). This hypomotility lasted longer after the first nighttime quinpirole than first daytime quinpirole as evidenced by significant hypomotility in the second 30-min block of nighttime injected mice [$t(22)$=6.13, p=0.0003]. After the seventh (i.e., last) injection, daytime quinpirole produced clear hyperactivity in all three 30-min blocks. In contrast, after the seventh nighttime quinpirole injection, there was still significant hypoactivity in the first block [$t(22)$=4.84, p=0.001], which was followed by significant hyperactivity in the second 30-min block [$t(22)$=−5.73, p=0.0001] (Fig. 4B).

4. Discussion

The main findings of this study are: (a) striatal D2 and D3 receptor protein levels express a marked diurnal rhythm; (b) repeated quinpirole administrations produced a lasting (72 h) alteration in striatal D2 and D3 protein levels and in turn markedly changed striatal D2/D3 ratios; and (c) repeated administration of quinpirole at an initial high D2/D3 ratio (i.e., daytime) resulted in locomotor hyperactivity (i.e., sensitization), whereas quinpirole administrations at an initial low D2/D3 ratio (i.e., nighttime) resulted in hypomotility and tolerance.

Studies with [^{3}H]-spiroperidol binding suggest that D2 receptors present ultradian and circadian rhythms (Wirz-Justice, 1987; Naber et al., 1980). In rats maintained under a controlled 12-h light–dark cycle (lights on 7 am), [^{3}H]-spiroperidol binding in the striatum shows two peaks, at 2 am and 2 pm (Naber et al., 1980). Our Western blot data confirmed the presence of diurnal rhythms in striatal D2 and D3 proteins. Similar to our findings of higher D2 levels during the day vs. night, Viyoch et al. (2001) found higher striatal D2 mRNA levels during the day than at night. Thus, our results support the possibility that diurnal changes in the content of striatal dopamine receptors may determine the

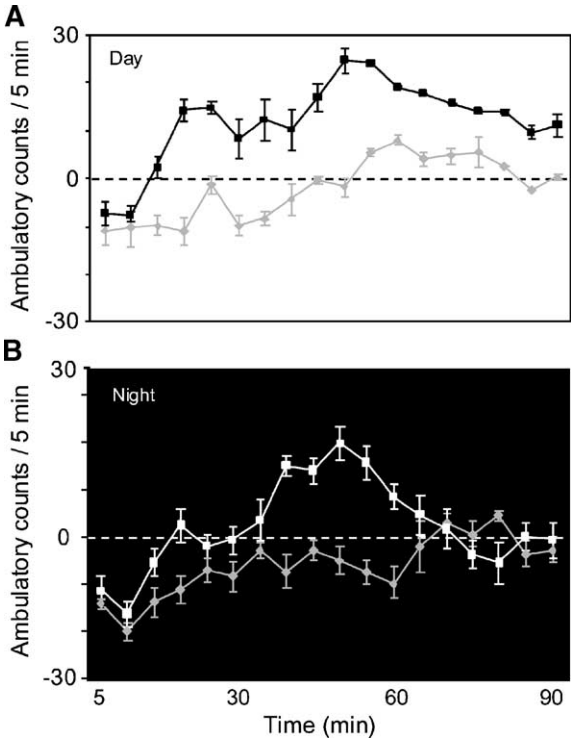

Fig. 3. Detailed analysis of locomotor activity patterns induced by quinpirole: the effects of treatment schedule and time. Locomotor activity was measured 72 h after the first or last (seventh) injection of quinpirole. The ambulatory counts of saline-treated mice were subtracted from the corresponding ambulatory counts of quinpirole-treated mice. Results (mean±S.E.M.; n=12) are expressed as cumulative 5-min values for both day (panel A) and night (panel B). A single quinpirole injection (gray line, panel A) caused predominant hypoactivity both during the day and at night (control value shown as "0," dashed lines). After the seventh quinpirole injection (black line, day; white line, night) the initial hypoactivity was followed by hyperactivity, which was more prominent and persistent in daytime experiments (panel A).

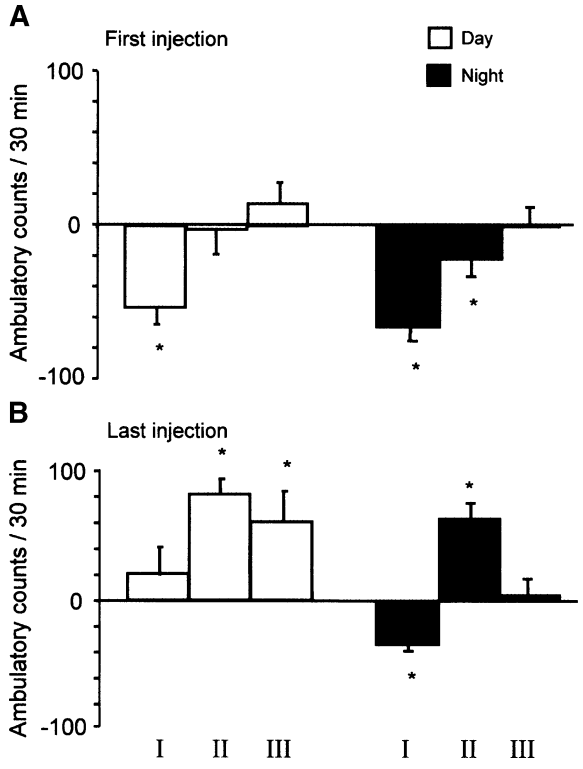

Fig. 4. Quinpirole causes a biphasic effect on locomotor activity-effect of diurnal rhythm and repeated injections. An analysis of the 90-min behavioral pattern divided into three 30-min blocks (I, II, and III) is shown in panels A (after the first injection) and B (after the last, seventh injection). The ambulatory counts of saline-treated mice (mean±S.E.M.; n=12) were subtracted from corresponding ambulatory counts of quinpirole-treated mice. Please note the changes in locomotion from hypoactivity to hyperactivity after protracted daytime quinpirole injections and the preservation of biphasic effects (i.e., initial hypoactivity followed by hyperactivity) after nighttime drug injections. *p<0.01 compared to corresponding saline-treated controls (control shown as 0 counts; t-test).

dosing time-dependent behavioral actions of dopaminergic drugs.

Furthermore, our findings indicate that circadian mechanisms do not affect all dopamine receptors in the same direction. Thus, although both D2 and D3 receptors show diurnal variations, these variations are not synchronous and as a result, the D2/D3 ratios present a marked diurnal rhythm. Hence, we propose that the balance between various dopamine receptors as exemplified here by D2/D3 ratios, is an important parameter regulated by circadian mechanisms and ultimately responsible for determining the susceptibility levels of the brain in producing a specific drug-dependent behavioral output.

Previous studies established that dosing time significantly influences the type of behavioral effects of psychostimulants such as cocaine, amphetamine, and methylphenidate (Martin-Iverson and Iversen, 1989; Gaytan et al., 2000; Uz et al., 2002, 2003). Similarly, dosing time-dependent behavioral effects were observed with dopaminergic drugs, such as SKF-38393 (D1 agonist), PHNO, and RU 24213 (D2 agonists), and apomorphine (D1 and D2 agonist; Tirelli and Jodogne, 1993; Martin-Iverson and Yamada, 1992; Nagayama et al., 1978; Nasello et al., 1995), and with a dopamine receptor antagonist haloperidol (Viyoch et al., 2001).

Our assay of locomotor activity encompassed a 90-min post-injection interval. Detailed analysis of the pattern of locomotor activity within this interval, i.e., in blocks of 30-min periods, revealed significant differences between day and nighttime responses to quinpirole. The effect of the first daytime quinpirole injection was biphasic: initial hypoactivity (first 30-min block) followed by hyperactivity (last 30-min block). This is similar to previous findings in rats (Szechtman et al., 1994; Szumlinski et al., 1997). With repetitive daytime injections, mice developed tolerance to hypoactivity accompanied by sensitization to hyperactivity. Thus, the most prominent final effect of repetitive daytime quinpirole administration was behavioral sensitization. This is consistent with the literature, in which all data were obtained from daytime experiments (Einat and Szechtman, 1993).

On the other hand, nighttime quinpirole administration produced a more pronounced hypoactivity. Although tolerance developed to hypoactivity with repetitive nighttime injections, it was not as fast or as complete as with daytime injections (e.g., hypoactivity was still significant in the first 30-min block after the seventh injection, Fig. 4C).

Similar dosing time-dependent development of tolerance was observed in experiments with a dopamine receptor (predominantly D2) antagonist haloperidol (Viyoch et al., 2001). Hence, a greater development of tolerance to catalepsy was observed after day rather than after nighttime haloperidol administration. On the other hand, in rats exposed to a continuous infusions of a selective dopamine D2 agonist PHNO [(+)-4-propyl-9-hyroxynapthoxazine], tolerance developed to the motor stimulant effects during the day, and behavioral sensitization developed at night (Munro and Martin-Iverson, 2000). While daytime tolerance was reversed to sensitization by 2-iodo-melatonin (a melatonin analog), removal of melatonin by pinealectomy did not affect nighttime locomotor sensitization (Munro and Martin-Iverson, 2000; Ruzich and Martin-Iverson, 2000).

Our results point to an interesting correlation between diurnal differences in striatal D2/D3 ratios and the dosing time-dependent development of behavioral tolerance and sensitization by quinpirole. Moreover, the feedback effect of repetitive quinpirole administration on striatal D2 and D3 levels also was dosing time-dependent. We found remarkable tolerance to quinpirole-induced hypomotility with daytime injections, i.e., starting at a high D2/D3 ratio in the striatum. Along with the development of tolerance (and consequently sensitization), daytime D2 levels and D2/D3 ratio decreased. It is possible that decreased daytime D2 levels led to tolerance to locomotor hypoactivity; in fact, Kelly et al. (1998) reported that D2-deficient mice lack quinpirole-induced locomotor suppression.

In support of the importance of D2/D3 balance in quinpirole tolerance/sensitization, Chiang et al. (2003) found that the co-treatment of D3 antagonists (i.e., U99194A and GR103691) inhibit the development of amphetamine-induced locomotor sensitization. It has been proposed that the balance between dopamine receptor-mediated actions is an important factor in the development of dopaminergic behaviors, such as locomotor sensitization (Beyer and Steketee, 2002; Richtand et al., 2001; Capper-Loup et al., 2002), and that the negative effect of D3 on both D1 and D2 receptors might be an important factor in the development of dopamine receptor-mediated behavioral sensitization (Richtand et al., 2001).

Similar to our results on quinpirole-induced changes in D2/D3 protein ratios, it has been reported that chronic quinpirole treatment down-regulates D2-specific binding whereas it up-regulates D3 binding (Subramaniam et al., 1992; Stanwood et al., 2000). Thus, the differential development of quinpirole-induced tolerance/sensitization during the day and at night may require a circadian rhythm-determined D2/D3 balance. Since we observe differential effects of quinpirole on both D2 and D3 receptor levels depending on the injection time, it is possible that the striatal rhythms of both D2 and D3 receptor levels may present phase shifts after the long-term drug treatment. This may explain the changes in D2/D3 ratio we observed in our experiments (Fig. 2).

Recent work on *Drosophila* introduced "clock genes" as an important contributor to dopaminergic behaviors (Andretic and Hirsh, 2000; Andretic et al., 1999). Hence, a mutation in the clock gene *Period* abolished behavioral sensitization to cocaine in flies (Andretic et al., 1999) and mice (Abarca et al., 2002). Recently, using pinealectomized mice, we found that striatal *Period1* (*Per1*) rhythm requires intact *N*-acetylserotonin (NAS)/melatonin rhythms and that striatal *Per1* rhythm is critical for the development of cocaine-induced locomotor sensitization (Uz et al., 2003). The regulatory role of melatonin on *Per1* rhythms was confirmed using melatonin receptor MT1 knockout mice (von Gall et al., 2002).

Since clock genes act as transcription factors, they can regulate a second group of genes, possibly through E-box motifs located on the promoter region of these genes (Kyriacou and Rosato, 2000). Recently, such regulations (i.e., E-box-mediated) have been suggested for the expression of the vasopressin gene (Ghorbel et al., 2003). Interestingly, D2 and D3 receptors contain putative E-box sequences in their promoter regions. It is possible that pineal melatonin-driven *Per1* rhythm (Uz et al., 2003), which regulates the rhythms of striatal dopamine receptors, may also regulate the diurnal behaviors observed in this study. Since melatonin receptors are expressed in the mammalian brain, including the striatum and substantia nigra (Laudon et al., 1988; Uz et al., 2003), it is possible that melatonin receptors such as MT1 participate in the putative control of D2/D3 receptor ratios.

The role of pineal melatonin in dopaminergic behaviors has previously been proposed (Zisapel, 2001; Abilio et al., 2003). Intrastriatal and intranigral injections of melatonin changed the locomotor activity of rats in a dose-dependent manner (Paredes et al., 1999). It has been also reported that melatonin and NAS may regulate dopaminergic neurotransmission by presynaptically blocking dopamine release and/or by inhibiting dopamine receptor (i.e., D1 receptor) activation through postsynaptic melatonin receptors (Dubocovich, 1983; Alexiuk and Vriend, 1993; Iuvone and Gan, 1995). Hence, we suggest that further research is needed to better understand the circadian mechanisms involved in the NAS/melatonin-and clock gene-driven regulation of dopamine receptor rhythms and behaviors such as locomotor tolerance and sensitization.

Acknowledgments

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