


ORIGINAL ARTICLE

Melatonin decreases cocaine-induced locomotor activity in pinealectomized rats

Susana **Barbosa-Méndez**, Alberto **Salazar-Juárez** 

Laboratorio de Neurofarmacología Conductual, Microcirugía y Terapéutica Experimental, Subdirección de Investigaciones Clínicas, Instituto Nacional de Psiquiatría, Ciudad de México, Mexico.

Objective: Several studies have shown that the time of day regulates the reinforcing effects of cocaine. Additionally, melatonin and its MT₁ and MT₂ receptors have been found to participate in modulation of the reinforcing effects of such addictive drugs as cocaine. Loss of the diurnal variation in cocaine-induced locomotor sensitization and cocaine-induced place preference has been identified in pinealectomized mice. In addition, several studies in rodents have shown that administration of melatonin decreased the reinforcing effects of cocaine. The objective of this study was to evaluate the effect of melatonin on cocaine-induced locomotor activity in pinealectomized rats at different times of day (zeitgeber time [ZT]4, ZT10, ZT16, and ZT22).

Methods: Naïve, pinealectomized Wistar rats received cocaine at different times of day. Melatonin was administered 30 min before cocaine; luzindole was administered 15 min prior to melatonin and 45 min before cocaine. After administration of each treatment, locomotor activity for each animal was recorded for a total of 30 min. Pinealectomy was confirmed at the end of the experiment through melatonin quantitation by ELISA.

Results: Cocaine-induced locomotor activity varied according to the time of day. Continuous lighting and pinealectomy increased cocaine-induced locomotor activity. Melatonin administration decreased cocaine-induced locomotor activity in naïve and pinealectomized rats at different times of day. Luzindole blocked the melatonin-induced reduction in cocaine-induced locomotor activity in pinealectomized rats.

Conclusion: Given its ability to mitigate various reinforcing effects of cocaine, melatonin could be a useful therapy for cocaine abuse.

Keywords: Melatonin; cocaine; locomotor activity; diurnal rhythms; luzindole

Introduction

Substance use disorders (SUD) constitute an important public health problem worldwide, affecting a great number of individuals. SUD is characterized by a high propensity for relapse.¹ Several studies in humans have reported that SUD is associated with disruptions in multiple behavioral and physiological circadian rhythms, including sleep, eating patterns, corticosterone, glucose, and endocrine fluctuations.²⁻⁴ Other studies have found that chronic cocaine abusers show seasonal patterns of craving and substance abuse.⁵

Studies in animals have shown that the time of day regulates the reinforcing effects of psychostimulants such as cocaine^{6,7} and nicotine,⁸ and even of opioids such as morphine.⁹ Pioneering studies by Abarca et al.⁶ demonstrated that cocaine-induced locomotor sensitization varies according to the day/night cycle. Other authors have reported that reinstatement of cocaine-induced place preference increases during the day.^{10,11} In addition,

cocaine self-administration has been found to vary with time of day.¹²

Other studies have described diurnal and circadian variations in dopamine levels,^{13,14} dopamine transporters (DAT), tyrosine hydroxylase (TH),¹⁵ and dopamine clearance (DC)¹⁶ in the caudate, nucleus accumbens (NAcc), and prefrontal cortex (PFC) in rats.

The pineal gland is a brain structure capable of transducing environmental lighting conditions into an endogenous hormonal signal by synthesizing and releasing melatonin. This process which occurs under the control of the endogenous biological clock (hypothalamic supra-chiasmatic nuclei [SCN]), which times melatonin synthesis to occur daily in synchrony with the light/dark cycle, peaking during the dark period and declining to almost negligible levels during daytime. Melatonin plays an important role in the circadian modulation of behavioral and physiological functions.¹⁷⁻¹⁹

As mentioned above, the production and release of melatonin peak at night and fall during the day.^{18,19}

Correspondence: Alberto Salazar-Juárez, Laboratorio de Neurofarmacología Conductual, Microcirugía y Terapéutica Experimental, Subdirección de Investigaciones Clínicas, Instituto Nacional de Psiquiatría, Ciudad de México, 14370, Mexico.

E-mail: azazel_vamp@yahoo.com.mx

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However, surgical removal of the pineal gland (pinelectomy [Pnx]) results in inability to synthesize and release melatonin.^{20,21} This model of experimentally induced melatonin deficiency has been a very useful tool for studying the role of melatonin in the etiopathogenesis of several brain disorders, including anxiety, mood disorders, addictions, and Alzheimer's disease.

The MT₁ and MT₂ melatonin receptors are responsible for some of the effects of melatonin at the cerebral level, such as the inhibition of dopamine release in the retina and mesencephalic dopamine areas, the inhibition of neuronal firing in the SCN, and the regulation of testicular and ovarian function through inhibition of prolactin and testosterone secretion; they also participate in the regulation of cortisol secretion, as well as in regulation of oxytocin receptor gene expression and, consequently, uterine contractility. At the peripheral level, MT₁ and MT₂ receptors participate in cardiovascular regulation by modulating vasoconstriction and vasodilation; in regulation of the immune response, by increasing splenocyte proliferation; and in the regulation of glucose homeostasis, through inhibition of insulin secretion and upregulation of leptin production.^{17,22,23}

A number of studies have identified melatonin receptors in several regions of the dopaminergic system,^{24,25} and described diurnal variations in active MT₁ receptor levels in the NAcc and the ventral tegmental area (VTA), where such levels are higher during the night (zeitgeber time [ZT]21) and lower during the day (ZT5).²⁵

Other research has studied how melatonin and its receptors modulate the reinforcing effects of addictive drugs such as cocaine,²⁶ nicotine,²⁷ and amphetamines.²⁸ These studies found a loss of diurnal variation in cocaine-induced locomotor sensitization and conditioned place preference (CPP) in melatonin-deficient pinealectomized C3H/HeJ mice.^{10,29} Furthermore, in C3H/HeN mice, cocaine induces locomotor sensitization during the light phase, when melatonin levels are low, whereas at night, when melatonin levels are high, this effect is blunted.¹⁰ No such diurnal variation is found in C57BL/6J mice, which are considered melatonin-deficient due to disruption of an arylalkylamine N-acetyltransferase gene (the rate-limiting enzyme in melatonin synthesis).¹¹

Given the large body of evidence suggesting that melatonin regulates diurnal cocaine-induced variations^{10,11,29} and decreases the reinforcing effects of nicotine²⁷ and alcohol³⁰ in mice, this study sought to explore the effect of melatonin administration at different times of day (ZT4, ZT10, ZT16, and ZT22) on cocaine-induced locomotor activity in naïve and pinealectomized rats. We hypothesized that melatonin administration would decrease such activity.

Methods

Animals

Male Wistar rats (age 8 weeks, weight 250-280 g) were used for this study. Rats were housed in groups of four in standard plastic cages (57 x 35 x 20 cm), in a colony room

kept at a constant temperature (21 ± 2 °C) and humidity (40-50%), either on a 12:12-h light/dark cycle (400 lux of white light during the light phase and complete darkness during the dark phase) or under continuous light (L/L) (lights on 24 h/day) to induce functional Pnx. Throughout the study, to reduce disturbance to a minimum and the likelihood of inadvertent nonphotic entrainment among rats held in L/L, routine cleaning was carried out every 1 to 2 days at random times of day.

For the 12:12 light-dark (L/D) and L/L cycles, the time of day was expressed as ZT, as follows: at 8 a.m., the lights are turned on; this time point is set as ZT0. At 8 p.m., the lights are turned off; this time point is set as ZT12. ZT24 is ZT0 of the next day.

All animals had continuous access to water and rodent chow pellets, except during experimental sessions.

Drugs

Cocaine hydrochloride (COC) was kindly donated by the Mexican government, under strict regulatory controls. All drugs used in experimental animals were kept under official surveillance (COFEPRIS, LC-0004-2003). Melatonin (Sigma-Aldrich, St. Louis, MO, USA) and luzindole (Tocris Cookson, Ballwin, MO, USA) were purchased after obtaining the required regulatory permission, as per official guidelines (COFEPRIS-2016, Mexico). COC was dissolved in sterile saline (SAL) (0.9% NaCl, Sigma-Aldrich, St. Louis, MO, USA). Melatonin and luzindole were dissolved in ethanol and diluted with SAL. The final ethanol concentration was 0.1% in both vehicle and melatonin solution. The solutions were compounded extemporaneously before intraperitoneal (i.p.) administration to the animals. During the experiments, all solutions were maintained at 4 °C. Melatonin and luzindole solutions were protected from light exposure by storage in black bottles. SAL was used as control in all experiments.

To ascertain whether melatonin could prevent the effects of cocaine, it was administered 30 min before COC or SAL. To ascertain whether luzindole, a selective MT₁ and MT₂ melatonin receptor antagonist, attenuated the ability of melatonin to decrease cocaine-induced locomotor effects, it was administered 15 min prior to melatonin. The volume injected into the animals depended on their body weight (BW) in grams (100 mL).

Dose selection

Determination of the optimal dose of cocaine was based on previous studies, which reported that 10 mg/kg cocaine produces a robust increase in locomotor activity and behavioral sensitization.²³ This dose of cocaine does not cause seizures or lethality.³¹⁻³⁵

The optimal melatonin dose (30 mg/kg) was determined in accordance with previous reports indicating that 30 mg/kg melatonin decreases cocaine-induced locomotor activity in rats.³⁶ We selected a 30 mg/kg dose of luzindole because it has been shown to block melatonin receptor function in rodents.³⁷

Pinealectomy

For surgical removal of the pineal gland and sham surgery, rats were weighed, anesthetized with ketamine HCl (90 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.), and secured in a stereotaxic instrument. A sagittal opening was made on the scalp. After pushing the skin aside to expose the lambdoid suture, a disk-shaped piece of bone was gently resected with a drill to allow visualization of the transverse and sagittal sinuses. The cerebral veins were carefully deflected to minimize blood loss. Any bleeding was controlled with Gelfoam (The Upjohn Co. Kalamazoo, U.S.).

The superficial pineal gland, located below the posterior venous sinus confluence, was removed using fine forceps. After Pnx, the disk-shaped bone flap was returned to the skull and the scalp was sutured with cotton thread once temporary hemostasis had been achieved. After surgery, each animal received a single injection of penicillin (120,000 IU/kg, intramuscular [IM]) and was monitored for potential postoperative complications. In sham-operated rats (Pnx-S), all of the aforementioned procedures were performed, except for removal of the pineal gland.

At the end of each procedure, histological examination of each pineal gland was carried out to verify completeness of Pnx and absence of damage to nearby brain areas.

Melatonin enzyme-linked immunosorbent assay (ELISA)

Melatonin levels were determined in plasma samples collected from the brachial vein at ZT22 (dark phase). Briefly, 500 μ L samples of blood were collected into small heparinized vials and kept on ice until centrifugation (1,000 rpm for 5-10 min at 4 °C) to allow for extraction of plasma. Tubes containing plasma were wrapped in aluminum foil to prevent light-induced degradation, and stored at -80 °C until analysis. Melatonin levels were measured with by a commercial ELISA kit (Enzo Life Sciences Inc., New York, USA), again taking all necessary measures to prevent photodegradation. The melatonin concentration was analyzed as per the manufacturer's instructions.

Behavioral sensitization procedure

Apparatus

For assessment of locomotor activity, animals were placed in transparent Plexiglas activity chambers (50 \times 50 \times 30 cm) linked to a personal computer (PC). Each activity chamber was surrounded by a 16 \times 16 photocell beam array located 3 cm from the floor surface to scan locomotor activity (OMNIALVA, Instruments, Ciudad de México, Mexico). Photobeam interruptions were automatically quantified with OABiomed software v. 1.1 and analyzed subsequently. Locomotor activity was defined as the interruption of consecutive photobeams (OMNIALVA, Instruments, Ciudad de México, Mexico).

Procedure

Spontaneous locomotor activity was estimated with a standard protocol.³⁵ Animals were habituated to the

activity chambers in three 30-min sessions and were randomly assigned to different pharmacotherapy groups. Locomotor activity was recorded for 30 min. The rats were returned to their home cages after each experimental session had been completed.

Experimental procedures

This study used 480 male Wistar rats, which were allocated across five experimental groups. For Experiments 1 and 2, we used 64 animals that were further divided into eight experimental groups (n=8); for Experiment 3, we used 128 animals in 16 groups (n=8); for Experiments 4 and 5, we used 160 animals in 20 groups (n=8). Each experimental group received a different pharmacological treatment.

Experiment 1: Diurnal differences in cocaine-induced locomotor activity

This experiment included two phases: the light phase, which included measurements at two time points (ZT4 and ZT10), and the dark phase, which included measurements at ZT16 and ZT22. Rats in this experiment were divided into groups according to the ZT at which cocaine was given.

After 3 days of habituation, at ZT4, the SAL and cocaine groups received SAL solution (0.9% NaCl, i.p.) and COC (10 mg/kg, i.p.), respectively, for 10 days: during the light phase at ZT4 and ZT10, and during the dark phase at ZT16 and ZT22. After administration of each treatment, locomotor activity for each animal was recorded for a total of 30 min (Figure 1A). The rats tested at a given time of day were not tested again at any other time.

Experiment 2: Continuous lighting increases cocaine-induced locomotor activity

Experiment 2 evaluated the effect of continuous lighting as per the above-described protocol. The rats in this experiment were divided into groups according to the ZT when cocaine was given.

After 3 days of habituation, at ZT4, the SAL and COC groups received SAL solution (0.9% NaCl, i.p.) and COC (10 mg/kg, i.p.), respectively, for 10 days at ZT4 and ZT10, then at ZT16 and ZT22. After administration of each treatment, locomotor activity for each animal was recorded for a total of 30 min (Figure 2A). At the end of the experiment, serum melatonin levels were analyzed by ELISA as described above.

Experiment 3: Pinealectomy enhances cocaine-induced locomotor activity

Experiment 3 evaluated the effect of Pnx as per the protocol described in Experiment 1. The rats were divided into groups according to the ZT when cocaine was given. This experiment consisted of three phases: phase I, or the cocaine-impregnation phase, which lasted 5 days; phase II, or the Pnx phase, which lasted 5 days; and phase III, or the post-Pnx phase, which lasted 10 days (Figure 3A).

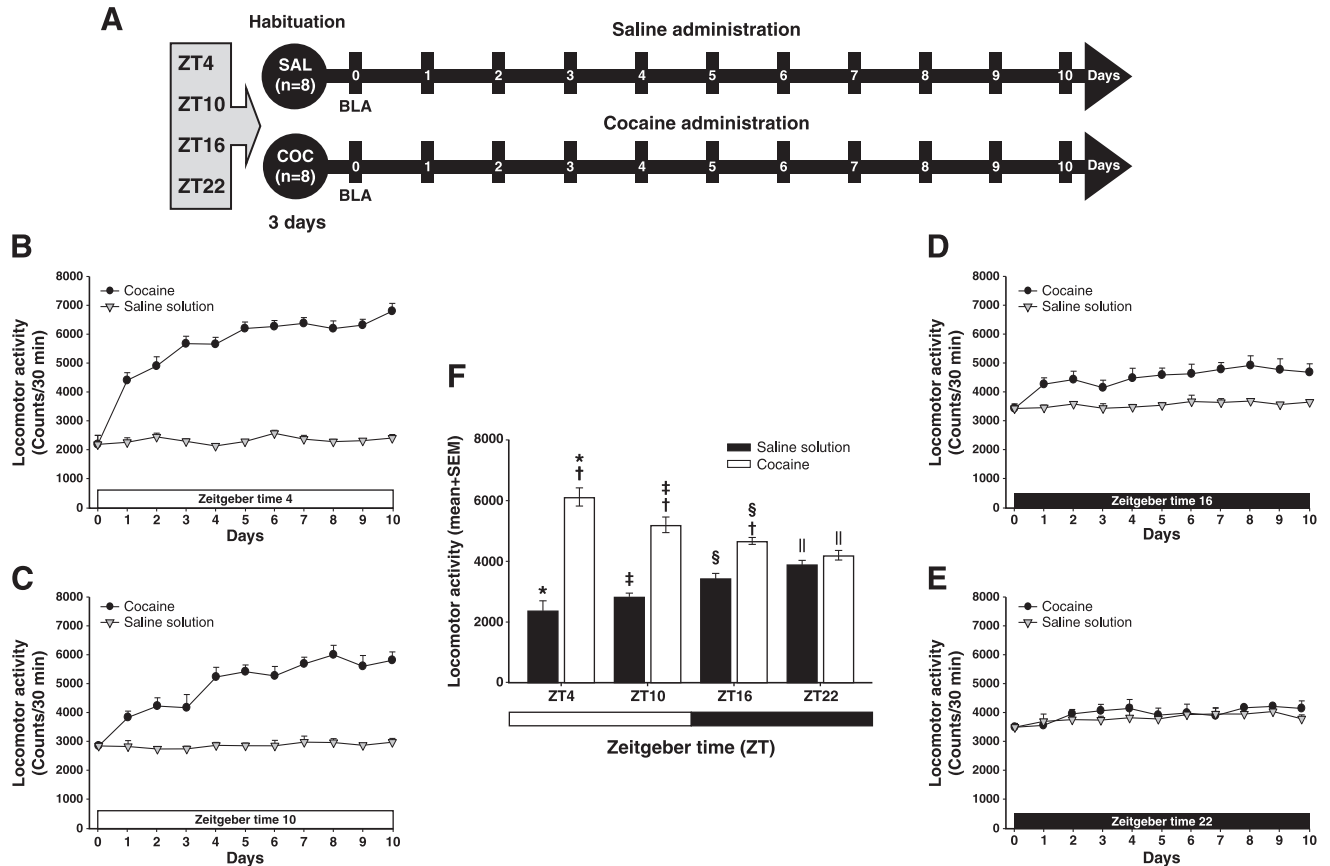


Figure 1 Diurnal variation in cocaine-induced locomotor activity. A) Experiment timeline. B-E) Cocaine-induced locomotor activity at Zeitgeber time (ZT)4, ZT10, ZT16, and ZT22. F) Mean (10 days) locomotor activity (\pm standard error of the mean [SEM]) by group ($n=8$ animals per group) at different times of day. White bars correspond to the administration of saline (SAL), and black bars, to the administration of cocaine (COC). The zero time point corresponds to basal locomotor activity (BLA). * Significant ($p < 0.01$) effects of COC on locomotor activity compared to the SAL group. † Significant ($p < 0.01$) effects of COC or SAL on locomotor activity at ZT4 compared to the COC group at ZT10. ‡ Significant ($p < 0.01$) effects of COC or SAL on locomotor activity at ZT16 compared to the COC group at ZT22. § Significant ($p < 0.01$) effects of COC or SAL on locomotor activity at ZT10 compared to the COC group at ZT16. || Significant ($p < 0.01$) effects of COC or SAL on locomotor activity at ZT22 compared to ZT4, determined by a two-way analysis of variance (ANOVA) followed by Tukey's tests.

After 3 days of habituation, during the impregnation phase, the Pnx-S (Pnx-S-SAL; Pnx-S-COC) and the Pnx (Pnx-SAL; Pnx-COC) groups received SAL (0.9% NaCl, i.p.) and COC (10 mg/kg, i.p.) for 5 days. Subsequently, during the Pnx phase, the rats underwent sham or complete Pnx. All animals were monitored for possible postoperative complications. In the post-Pnx phase, animals received SAL or COC for 10 days at ZT4 and ZT10, then at ZT16 and ZT22. After administration of each treatment, locomotor activity for each animal was recorded for a total of 30 min (Figure 3A). Pnx was confirmed at the end of the experiment by testing for melatonin (ELISA) on serum samples collected during the dark period.

Experiment 4: Melatonin reduces cocaine-induced locomotor activity in pinealectomized rats

Experiment 4 evaluated the effect of melatonin as per the protocol described in Experiment 1. The rats were divided into groups according to the ZT when cocaine was given.

This experiment consisted of four phases: phase I, or the cocaine-impregnation phase, which lasted 5 days; phase II, or the Pnx phase, which lasted 5 days; phase III, or the post-Pnx phase, which lasted 10 days; and phase IV, or the melatonin phase, which lasted 10 consecutive days (Figure 4A).

After 3 days of habituation, during the impregnation and post-Pnx phases, the Pnx-S (Pnx-S-SAL+SAL, Pnx-S-COC+COC, Pnx-S-SAL+MEL, Pnx-S-COC+MEL) and Pnx (Pnx-SAL+SAL, Pnx-COC+COC, Pnx-SAL+MEL, Pnx-COC+MEL) groups received SAL (0.9% NaCl, i.p.) and COC (10 mg/kg, i.p.). During the Pnx phase, the rats underwent sham or complete Pnx. All animals were monitored for possible post-surgical complications. During the melatonin phase, the rats received SAL or melatonin 30 min before COC or SAL for 10 days at ZT4 and ZT10, then at ZT16 and ZT22. After administration of each treatment, locomotor activity for each animal was recorded for a total of 30 min (Figure 4A). Pnx was confirmed at the end of the experiment by melatonin ELISA.

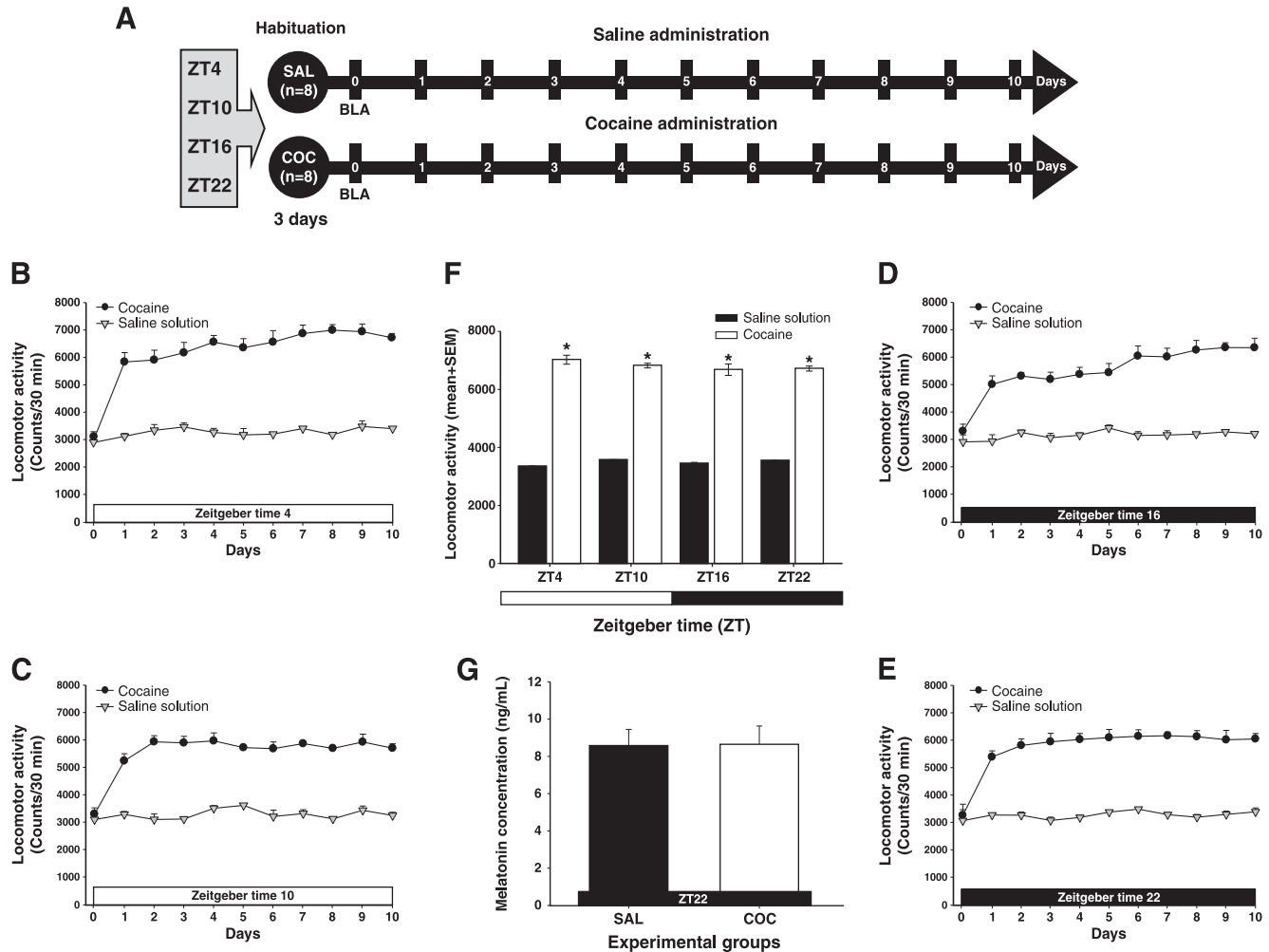


Figure 2 Continuous lighting enhances cocaine-induced locomotor activity. A) Experiment timeline. B-E) Effect of continuous lighting on locomotor activity induced by cocaine at Zeitgeber time (ZT)4, ZT10, ZT16, and ZT22. White bars correspond to the administration of saline (SAL) and black bars correspond to the administration of cocaine (COC). The zero time point corresponds to the basal locomotor activity (BLA). F) Mean (10 days) locomotor activity (\pm standard error of the mean [SEM]) by group (n=8 animals per group) at different times of day. *Significant ($p < 0.01$) effects of COC on locomotor activity compared to the SAL group, determined by a two-way analysis of variance (ANOVA) followed by Tukey's tests. Inset shows the effect of continuous light (L/L) on the mean concentration \pm SEM of serum melatonin. † Significant ($p < 0.01$) effects induced by L/L on serum melatonin levels in the SAL and COC groups determined by one-way ANOVA followed by Tukey's tests (not observed).

Experiment 5: Luzindole blocks melatonin-induced reduction of cocaine-induced locomotor activity in pinealectomized rats

Experiment 5 evaluated the effect of luzindole as per the protocol described in Experiment 4. The rats in this experiment were divided into four groups, according to the ZT at which cocaine was given. This experiment consisted of five experimental phases: phase I, or the cocaine-impregnation phase, which lasted 5 days; phase II, or the Pnx phase, which lasted 5 days; phase III, or the post-Pnx phase, which lasted 10 days; phase IV, or the melatonin phase, which lasted 10 consecutive days; and phase V, or the antagonist phase, which also lasted 5 consecutive days (Figure 5A).

After 3 days of habituation, the Pnx-S (Pnx-S^{SAL}+SAL+MEL, Pnx-S^{COC}+SAL+MEL) and Pnx (Pnx^{SAL}+SAL+MEL, Pnx^{COC}+SAL+MEL) groups received SAL or COC during the impregnation and post-Pnx phases. For the melatonin phase, the rats received melatonin 30 min before COC or SAL for 10 days. For the antagonist phase, all groups received SAL 15 min before melatonin and melatonin 30 min before COC or SAL at ZT4 and ZT10 and ZT16 and ZT22.

Rats in the Pnx-S (Pnx-S^{SAL}+LUZ+MEL, Pnx-S^{COC}+LUZ+MEL) and Pnx (Pnx^{SAL}+LUZ+MEL, Pnx^{COC}+LUZ+MEL) groups, during the impregnation and the post-Pnx phases, received SAL or COC. For the melatonin phase, animals received melatonin 30 min before COC or SAL for 10 days. For the antagonist phase, all groups received

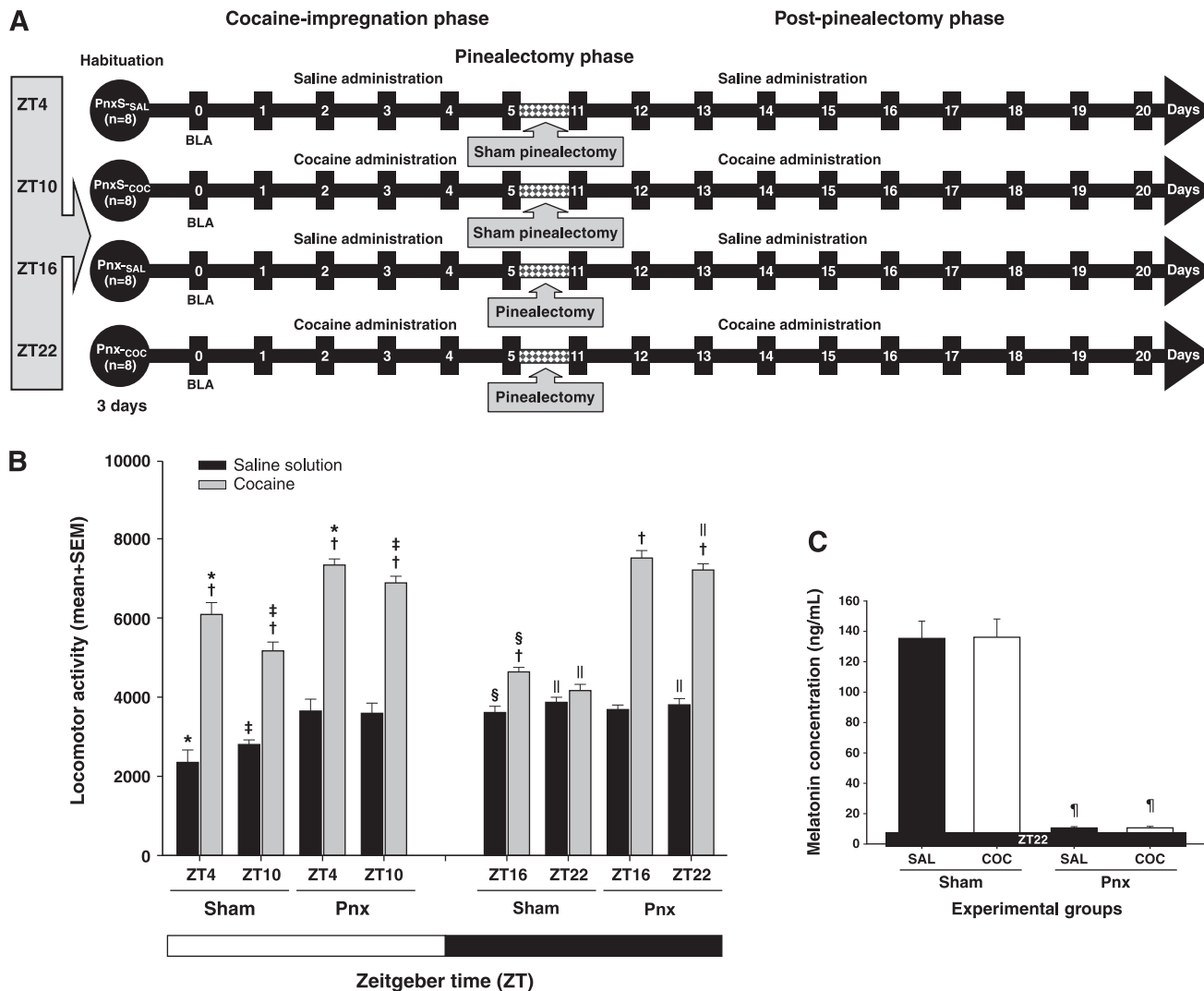


Figure 3 Pinealectomy (Pnx) increases cocaine-induced locomotor activity. A) Experiment timeline. The zero time point corresponds to the basal locomotor activity (BLA). B) Mean (10 days) locomotor activity (\pm standard error of the mean [SEM]) by group ($n=8$ animals per group) at ZT4, ZT10, ZT16, and ZT22. White bars correspond to the administration of saline (SAL) and black bars correspond to the administration of cocaine (COC). C) Effect of pinealectomy on the mean concentration \pm SEM of serum melatonin. *Significant ($p < 0.01$) effects of COC on locomotor activity compared to the SAL groups. † Significant ($p < 0.01$) effects of COC or SAL on locomotor activity at ZT4 compared to ZT10. ‡ Significant ($p < 0.01$) effects of COC or SAL on locomotor activity at ZT16 compared to ZT22. § Significant ($p < 0.01$) effects of COC or SAL on locomotor activity at ZT10 compared to ZT16. || Significant ($p < 0.01$) effects of COC or SAL on locomotor activity at ZT22, compared to ZT4, determined by a three-way analysis of variance (ANOVA) followed by Tukey's tests. ¶ Significant ($p < 0.01$) effects induced by pinealectomy on serum melatonin levels at ZT22 determined by one-way ANOVA following Tukey's tests.

luzindole 15 min before melatonin, and melatonin 30 min before COC (10 mg/kg, i.p.) or SAL, at ZT4 and ZT10, then ZT16 and ZT22. After administration of each treatment, locomotor activity for each animal was recorded for a total of 30 min (Figure 5A). Pnx was confirmed at the end of the experiment by melatonin ELISA (Melatonin ELISA Kit).

Statistical analysis

Data were expressed as mean \pm standard error of the mean (SEM). For Experiments 1 and 2, locomotor activity

in each group was evaluated with a two-way analysis of variance (ANOVA), with groups (SAL and COC) and time of day (ZT4, ZT10, ZT16, and ZT22) as the between-subjects factors. If the interaction yielded a significant F value, a post-hoc analysis of differences was performed between groups, followed by an additional Tukey's test. For Experiment 3, we used a three-way ANOVA, with groups (SAL and COC), surgery (sham and complete Pnx), and time of day (ZT4, ZT10, ZT16, and ZT22) as the between-subjects factor, followed by Tukey's test. For Experiments 4 and 5, a four-way ANOVA was carried out, with groups (SAL and COC), treatment

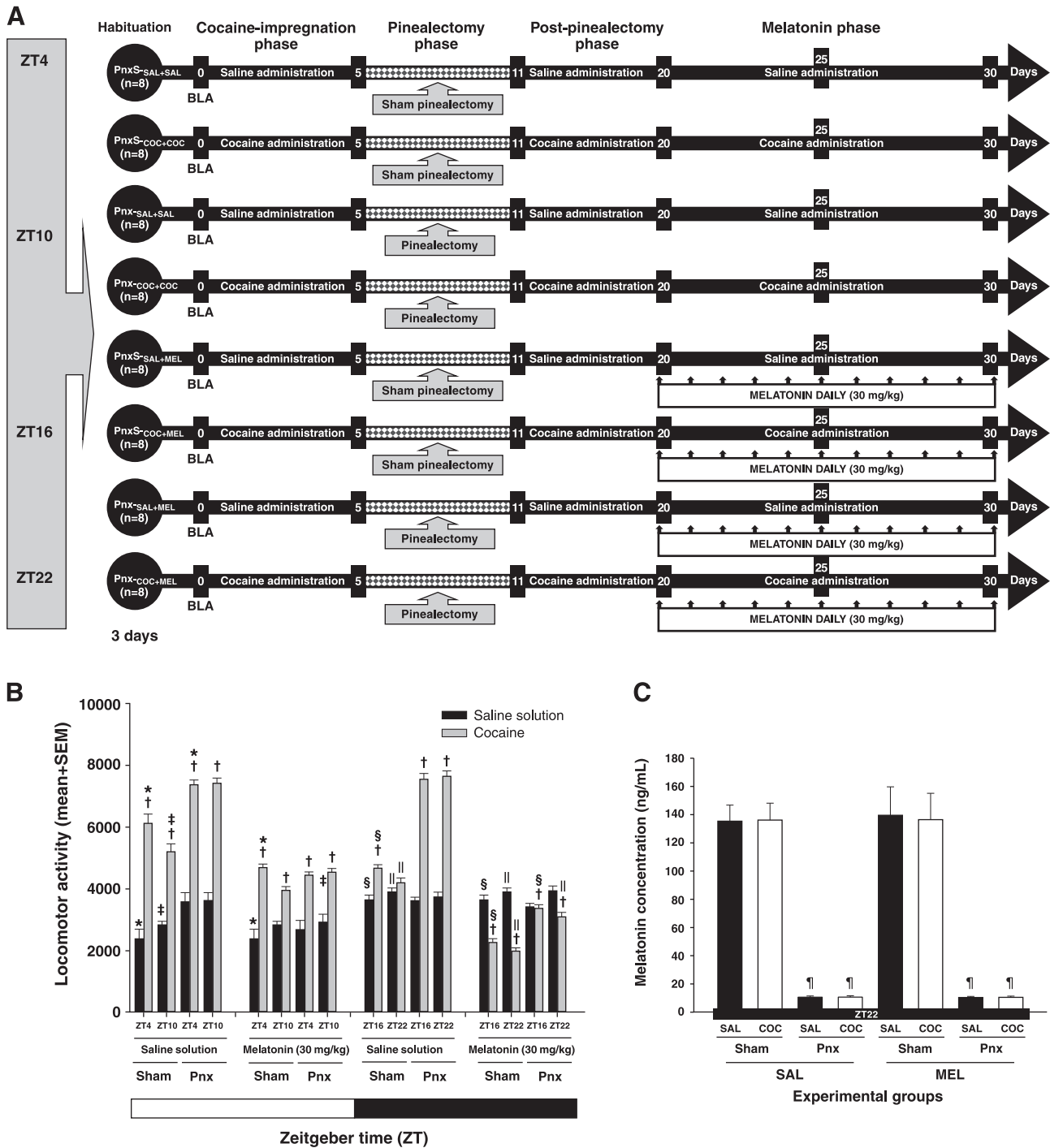


Figure 4 Melatonin decreases cocaine-induced locomotor activity in pinealectomized animals. A) Experiment timeline. The zero time point corresponds to the basal locomotor activity (BLA). B) Mean (10 days) locomotor activity (\pm standard error of the mean [SEM]) by group ($n=8$ animals per group) at different times of day. White bars correspond to the administration of saline (SAL) and black bars correspond to the administration of cocaine (COC). C) Effect of pinealectomy on the mean concentration \pm SEM of serum melatonin. * Significant ($p < 0.01$) effects of COC on locomotor activity compared to the SAL groups. † Significant ($p < 0.01$) effects of COC or SAL on locomotor activity at ZT4 compared to ZT10. ‡ Significant ($p < 0.01$) effects of COC or SAL on locomotor activity at ZT16 compared to ZT22. § Significant ($p < 0.01$) effects of COC or SAL on locomotor activity at ZT10 compared to ZT16. ¶ Significant ($p < 0.01$) effects of COC or SAL on locomotor activity at ZT22 compared to ZT4, determined by a four-way analysis of variance (ANOVA) followed by Tukey's tests. ¶ Significant ($p < 0.01$) effects induced by pinealectomy on serum melatonin levels in ZT22, determined by one-way ANOVA following Tukey's tests.

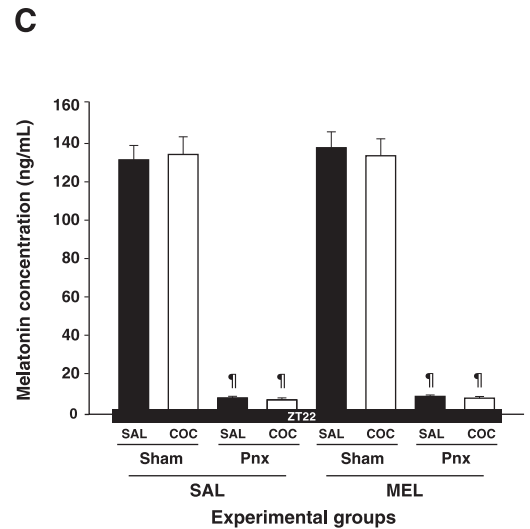
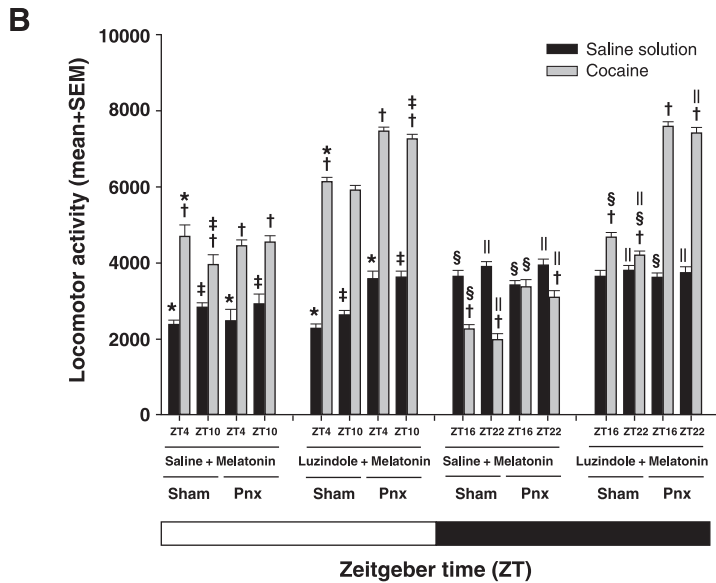
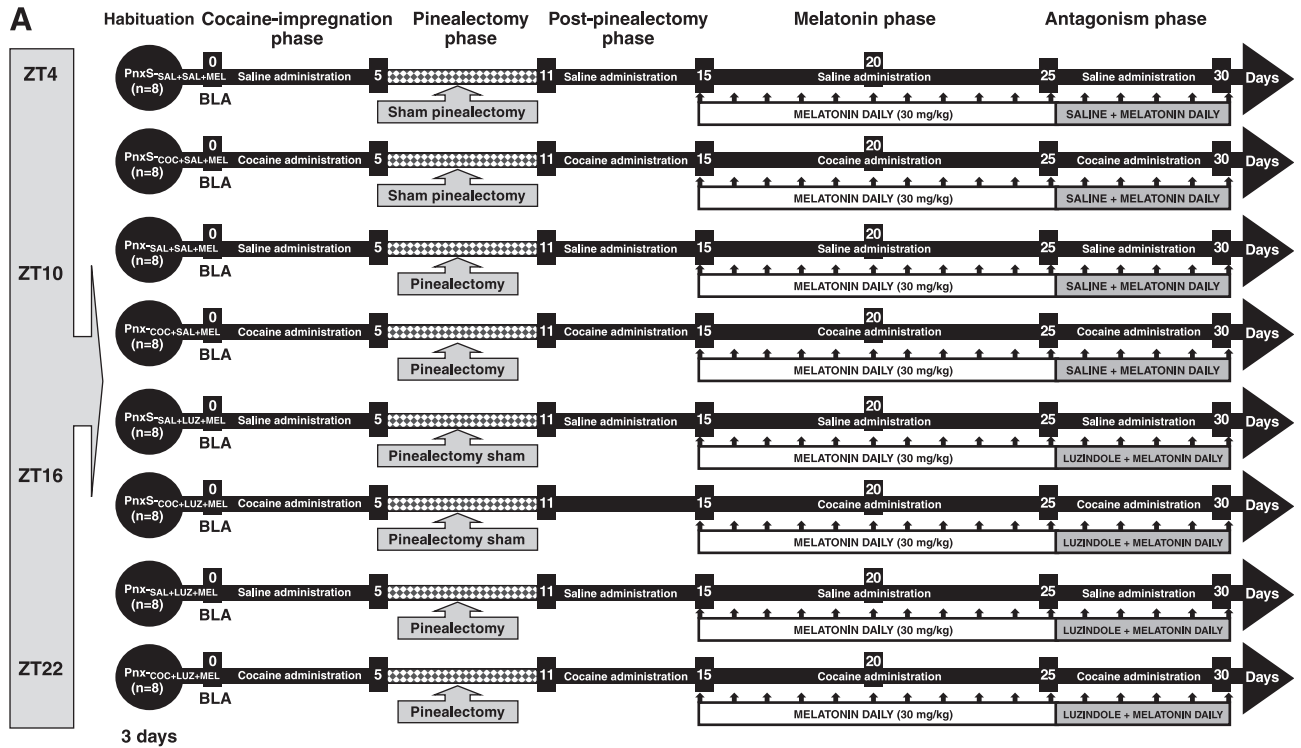


Figure 5 Luzindole decreases the effect of melatonin on cocaine-induced locomotor activity in pinealectomized animals. A) Experiment timeline. B) The zero-time point corresponds to the basal locomotor activity (BLA). Mean (± standard error of the mean [SEM]) by group (n=8 animals per group) at different times of day. C) White bars correspond to the administration of saline (SAL) and black bars correspond to the administration of cocaine (COC). C) Effect of pinealectomy on the mean concentration ± SEM of serum melatonin. * Significant (p < 0.01) effects of COC on locomotor activity compared to the SAL groups. † Significant (p < 0.01) effects of COC or SAL on locomotor activity at ZT4 compared to ZT10. ‡ Significant (p < 0.01) effects of COC or SAL on locomotor activity at ZT16 compared to ZT22. § p Significant (p < 0.01) effects of COC or SAL on locomotor activity at ZT10 compared to ZT16. || p Significant (p < 0.01) effects of COC or SAL on locomotor activity at ZT22 compared to ZT4, determined by a four-way analysis of variance (ANOVA) followed by Tukey's tests. ¶ Significant (p < 0.01) effects induced by pinealectomy on serum melatonin levels at ZT22, determined by one-way ANOVA followed by Tukey's tests.

(SAL and melatonin), surgery (sham and complete Pnx), and time of day (ZT4, ZT10, ZT16, and ZT22) as the between-subjects factor, again followed by Tukey's test. For comparison of melatonin levels, one-way ANOVA was used. Statistical significance was accepted at $p < 0.05$.

Ethics statement

The procedures were approved by the institutional animal care and bioethics committee and conducted in strict compliance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH). In order to avoid possible side effects due to lighting conditions, pharmacological treatments (clearance), and to avoid physical damage to animals, an independent group design was used in which each animal was used only once.

Results

Experiment 1: Diurnal differences in cocaine-induced locomotor activity

Figures 1B to 1E show the effect of time of day (ZT) on cocaine-induced locomotor activity. SAL animals showed an increase in locomotor activity dependent on the time of day, peaking at ZT22. In contrast, the COC animals, which received 10 mg/kg cocaine, showed a decrease in locomotor activity dependent on the time of day, peaking at ZT4.

Two-way ANOVA found significant differences in the groups vs. time-of-day interaction ($F_{3,924} = 202.085$, $p < 0.0001$). Tukey's test revealed differences in locomotor activity in the COC group compared to the SAL group, at ZT4 ($p < 0.001$), ZT10 ($p < 0.003$), and ZT16 ($p < 0.005$). However, the statistical analysis found no differences between groups at ZT22 ($p = 0.75$).

Additionally, the post-hoc test found differences in locomotor activity between the COC and SAL groups at ZT4, compared to that in both groups at ZT22 ($p < 0.001$). Statistical analysis revealed differences in locomotor activity between the two groups at ZT4 compared to ZT10 ($p < 0.004$), at ZT10 compared to ZT16 ($p < 0.002$), and at ZT16 compared to ZT22 ($p < 0.006$) (Figure 1F). This suggests that cocaine-induced locomotor activity varied according to the time of day.

Experiment 2: Continuous lighting increased cocaine-induced locomotor activity

Figures 2B to 2E show that, among animals in the SAL and COC groups subjected to continuous lighting, locomotor activity increased regardless of the time of day. The statistical analysis found significant differences (two-way ANOVA; group vs. time-of-day interaction; $F_{3,640} = 7.29$, $p < 0.0001$) in mean locomotor activity in the COC-treated group, compared to that shown in the SAL group, at ZT4 ($p < 0.001$), ZT10 ($p < 0.001$), ZT16 ($p < 0.001$), and ZT22 ($p < 0.001$) (Figure 2F). The post-hoc test, however, found no differences in locomotor activity

between the SAL and the COC groups at the times of day evaluated ($p = 0.98$).

Additionally, ELISA showed a decrease in plasma melatonin levels in animals of both groups in ZT22 (Figure 2F inset). One-way ANOVA did not find differences between the groups in this respect ($F_{1,15} = 5.607$, $p = 0.33$).

Experiment 3: Pinealectomy increased cocaine-induced locomotor activity

Figures 6A to 6D show the effect of removal of the pineal gland on cocaine-induced locomotor activity. In the Pnx-S_{SAL} and Pnx-S_{COC} groups, locomotor activity was altered depending on the time of day, as described in Experiment 1. Additionally, melatonin-deficient animals treated with SAL (Pnx-S_{SAL} group) showed an increase in locomotor activity, as described in Experiment 2. In contrast, the pinealectomized animals treated with cocaine (Pnx-COC group) showed an enhanced increase in cocaine-induced locomotor activity at ZT4, ZT10, ZT16, and ZT22.

A three-way ANOVA found differences between the group vs. surgery vs. time-of-day interactions ($F_{3,1280} = 104.56$, $p < 0.0001$). Tukey's test identified significant differences in mean locomotor activity in the Pnx-COC group, compared to the Pnx-S_{SAL}, Pnx-S_{COC}, and Pnx-S_{SAL} groups, at ZT4 ($p < 0.003$), ZT10 ($p < 0.002$), ZT16 ($p < 0.001$), and ZT22 ($p < 0.001$). Nevertheless, statistical analysis found no differences in locomotor activity in the Pnx-COC and the Pnx-S_{SAL} groups between ZT4 and ZT10 ($p = 0.94$), at ZT10 compared to ZT16 ($p = 0.88$), or at ZT16 compared to ZT22 ($p = 0.95$) (Figure 3B), respectively.

The post-hoc test did find differences between the Pnx-S_{SAL} and Pnx-S_{SAL} groups at ZT4 ($p < 0.001$) and ZT10 ($p < 0.001$), but not at ZT16 ($p = 0.89$) or ZT22 ($p = 0.99$).

Removal of the pineal glands was confirmed by melatonin ELISA on serum samples collected in the dark period (ZT22), when melatonin levels normally rise. One-way ANOVA found differences between the groups ($F_{3,31} = 9523.908$, $p < 0.0001$), and post-hoc analysis revealed differences in plasma melatonin levels in the pinealectomized groups (Pnx-S_{SAL} and Pnx-COC) compared to the Pnx-S_{SAL} ($p < 0.001$) and Pnx-S_{COC} ($p < 0.001$) groups (Figure 3C).

Experiment 4: Melatonin decreased cocaine-induced locomotor activity in pinealectomized rats

During the post-Pnx phase, rats in the Pnx-S_{SAL}+SAL, Pnx-S_{SAL}+COC, Pnx-S_{MEL}+SAL, and Pnx-S_{MEL}+COC showed alterations in locomotor activity that varied according to the time of the day, as described in the previous experiment. The Pnx-S_{SAL}+SAL and Pnx-S_{MEL}+SAL groups showed an increase in locomotor activity, as described in Experiment 3. In contrast, animals subjected to removal of the pineal gland (Pnx-S_{SAL}+COC, Pnx-S_{MEL}+COC) experienced an enhanced increase in cocaine-induced locomotor activity at ZT4, ZT10, ZT16, and ZT22.

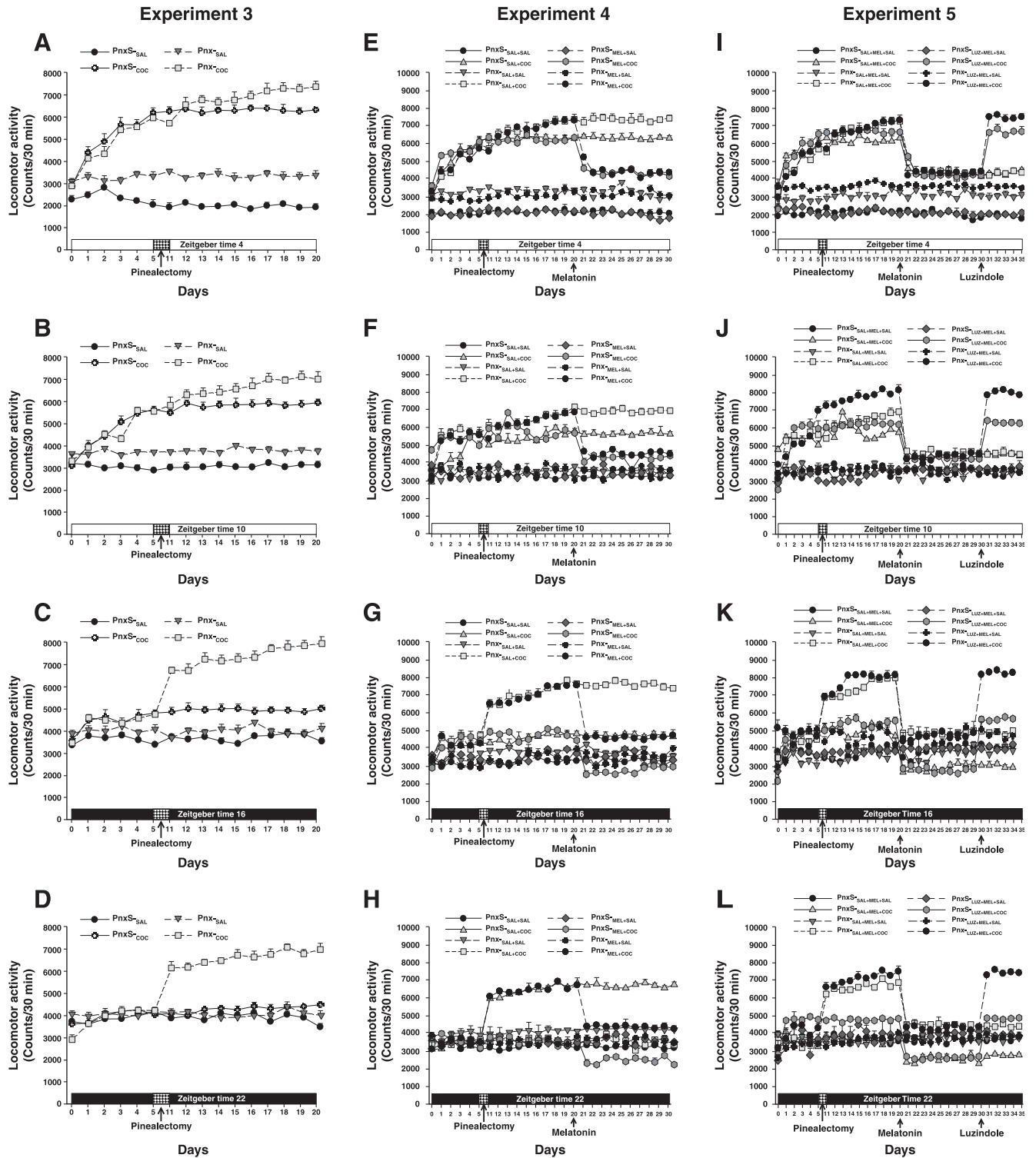


Figure 6 Role of the pineal gland in cocaine-induced locomotor activity. A-D) Experiment 3: Effect of pinealectomy on cocaine-induced locomotor activity at ZT4, ZT10, ZT16, and ZT22, respectively. E-H) Experiment 4: Effect of melatonin on cocaine-induced locomotor activity at ZT4, ZT10, ZT16, and ZT22, respectively. I-L) Experiment 5: Effect of luzindole on cocaine-induced locomotor activity in animals treated with melatonin at ZT4, ZT10, ZT16, and ZT22, respectively.

During the melatonin phase, administration of melatonin to rats in the Pnx-S^{-MEL+SAL} and Pnx^{-MEL+SAL} groups did not alter locomotor activity at any of the four time points of assessment. In contrast, daily treatment with melatonin in the Pnx-S^{-MEL+COC} and Pnx^{-MEL+COC} groups resulted in a decrease in cocaine-induced locomotor activity (Figure 6E to 6H).

Four-way ANOVA found differences between group vs. surgery vs. treatment v.s time-of-day interactions ($F_{3,2560} = 102.43$, $p < 0.0001$). Tukey's test revealed differences in mean locomotor activity in the Pnx^{-MEL+COC} group compared to the Pnx-S^{-SAL+SAL}, Pnx-S^{-SAL+COC}, Pnx-S^{-MEL+SAL}, Pnx-S^{-SAL+SAL}, Pnx^{-MEL+SAL}, and Pnx-S^{-SAL+COC} groups at ZT4 ($p < 0.003$), ZT10 ($p < 0.002$), ZT16 ($p < 0.004$), and ZT22 ($p < 0.003$). However, the post-hoc test found no differences in locomotor activity in the Pnx^{-MEL+COC} group compared to the Pnx-S^{-MEL+COC} group at ZT4 ($p = 0.72$), and ZT10 ($p = 0.97$) (Figure 4B).

In addition, the statistical analysis did not reveal differences in cocaine-induced locomotor activity in the Pnx^{-MEL+COC} group between ZT4-ZT10 and ZT16-ZT22 ($p = 0.91$).

Removal of the pineal glands was confirmed by melatonin ELISA. One-way ANOVA found differences between the groups ($F_{7,63} = 7538.107$, $p < 0.0001$). The post-hoc analysis revealed differences in plasma melatonin levels in the Pnx-S^{-SAL+SAL}, Pnx-S^{-SAL+MEL}, Pnx-COC+COC, and Pnx-COC+MEL groups compared to the Pnx-S^{-SAL+SAL} ($p < 0.001$), Pnx-S^{-SAL+MEL} ($p < 0.001$), Pnx-S^{-COC+COC} ($p < 0.001$), and Pnx-S^{-COC+MEL} ($p < 0.001$) groups (Figure 4C).

Experiment 5: Luzindole blocked melatonin-induced reduction in cocaine-induced locomotor activity in pinealectomized rats

During the antagonism phase, in the Pnx-S^{-SAL+MEL+SAL} and Pnx-S^{-SAL+MEL+COC} groups, locomotor activity depended on the time of day, as described in the previous experiment, where daily melatonin caused a significant decrease in cocaine-induced locomotor activity in pinealectomized animals (the Pnx-S^{-SAL+MEL+SAL} and the Pnx-S^{-SAL+MEL+COC}). In contrast, treatment with luzindole administered prior to melatonin to animals having undergone sham or complete Pnx (Pnx-S^{-LUZ+MEL+COC} and Pnx^{-LUZ+MEL+COC}) increased cocaine-induced locomotor activity at ZT4, ZT10, ZT16, and ZT22.

A four-way ANOVA detected differences between group vs. surgery vs. treatment vs. time-of-day interactions ($F_{3,320} = 14.410$, $p < 0.0001$). Tukey's test found differences in mean locomotor activity in Pnx-S^{-LUZ+MEL+COC} and Pnx^{-LUZ+MEL+COC} compared to Pnx-S^{-SAL+MEL+SAL}, Pnx-S^{-SAL+MEL+COC}, Pnx-S^{-SAL+MEL+SAL}, Pnx-S^{-SAL+MEL+COC}, Pnx-S^{-SAL+LUZ+MEL}, and Pnx^{-LUZ+MEL+SAL} at ZT4 ($p < 0.003$), ZT10 ($p < 0.002$), ZT16 ($p < 0.002$), and ZT22 ($p < 0.004$; Figure 5B). This suggests that the decrease in locomotor activity in animals treated with melatonin was due to its action on the MT₁ and MT₂ melatonin receptors.

Removal of the pineal glands was confirmed by melatonin ELISA. One-way ANOVA found differences between the

groups ($F_{7,63} = 9438.332$, $p < 0.0001$). Post-hoc analysis revealed differences in plasma melatonin levels ($p < 0.001$) similar to those found in the previous experiment (Figure 5C).

Discussion

Diurnal differences in cocaine-induced locomotor activity

Several studies have reported that, in rats and mice, the behavioral effects of cocaine,^{6,7} nicotine,⁸ and alcohol³⁰ depend on the time of day of administration.^{6,38} These studies have found a strong behavioral sensitization response to cocaine in the early morning, during the light phase (ZT4), and a decrease at the end of the light phase (ZT12), reaching its lowest levels at the end of the dark phase (ZT20).³⁸

Additionally, day/night variations have been described in the reinstatement of cocaine-induced CPP^{10,11} and in cocaine self-administration.¹²

These results are consistent with those of this study. Locomotor activity increased at the beginning of the light phase (ZT4) and decreased at the end of the dark phase (ZT22).

Some authors have reported that this increase in cocaine-induced locomotor activity is due to higher dopamine release.³⁹ Other researchers have suggested that dopamine plays an important role in the relationship between drug reward and circadian rhythms.⁴⁰ These studies have described diurnal fluctuations in extracellular dopamine levels in the dorsal striatum, which peak during the active phase.^{13,14} They have also described diurnal and circadian variations in DAT levels,¹⁵ DC,¹⁶ expression and function of TH,¹⁵ and monoamine oxidase A (MAO) enzyme in the VTA and NAcc in rats, with greater expression during the light phase (ZT4-6) than during the dark phase (ZT16-20).⁴⁰

This suggests that the diurnal variations in cocaine-induced locomotor activity described herein may be the result of diurnal variations in the previously described elements of the dopaminergic system.

Nonetheless, a strong link between the light cycle and behavioral responses to cocaine has been suggested.⁴¹ Sorg et al.⁴¹ reported that changes in the proportion of light during the day (photoperiod) modulated different cocaine behavioral responses. They also found that exposure of rats to short photoperiods suppressed reinstatement of cocaine-induced CPP and altered the levels of DAT and TH protein in the PFC. On the other hand, long photoperiods did not alter the reinstatement of cocaine-induced CPP.⁴¹

Several studies have found that C3H7HeN mice show cocaine-induced locomotor sensitization in the light phase, but not during the dark phase.^{10,29} This diurnal variation in cocaine-induced locomotor sensitization has been associated with diurnal fluctuations in melatonin levels.¹⁹ In C3H7HeN mice, the highest levels of melatonin occur during the dark phase and the lowest levels are observed in the light phase, when cocaine enhances locomotor activity.^{10,29} This diurnal variation, however, is not observed in C57BL/6J mice, which are considered

melatonin-deficient.¹¹ This suggests a potential link between nocturnal melatonin and the lack of cocaine-induced locomotor activity during the dark phase.¹⁷

Continuous lighting increased cocaine-induced locomotor activity

Our study found that exposure to L/L caused: 1) a loss of circadian variations in cocaine-induced locomotor activity; 2) decreased serum melatonin levels; and 3) increased cocaine-induced locomotor activity.

These results are in line with previous results indicating that exposure to L/L decreases serum melatonin levels⁴² and enhances the psychostimulant effects of cocaine, while altering different rhythms⁴³; that is, under L/L, the diurnal rhythm of cocaine self-administration is fragmented and animals consume significantly less cocaine.⁴³ In male Wistar rats, 1 month of exposure to L/L exerted a significant effect on voluntary morphine consumption, increased severity of naloxone-induced withdrawal symptoms, and was associated with upregulation of Per1, Per2, and D₁ dopamine receptor mRNA expression levels in the striatum and PFC.⁹

The circadian changes associated with continuous exposure to light, known as functional Pnx, are the result of a decrease in endogenous circulating melatonin levels.⁴² This leads to pathophysiological changes, metabolic disruption, endocrine malfunction, and increased free radicals, which, in turn cause molecular damage and abnormal behaviors.⁴² Moreover, constant light disrupts the circadian rhythms of spontaneous locomotor activity, increases anxiety- and depressive-like behaviors, and impairs short-term memory.^{42,44}

On the other hand, animals exposed to constant dark conditions show a circadian rhythm of reinstatement of cocaine-induced CPP, cocaine self-administration, and melatonin release.⁴¹ This suggests that continuous lighting increases the likelihood of consumption of such drugs as cocaine and morphine, mainly due to a decrease in plasma levels of melatonin.

Pinealectomy increased cocaine-induced locomotor activity

Several studies have described the effects of Pnx on mice.^{10,29} As mentioned above, in C3H/HeN mice, cocaine-induced locomotor sensitization and CPP decrease during the dark phase.^{11,29} This diurnal variation is eliminated with removal of the pineal gland,^{10,29} which is also the case specifically for Per1 gene expression in C3H/HeJ pinealectomized mice.²⁹ Additionally, pineal gland removal decreases plasma levels of melatonin in rats and mice.¹⁴

Our study found similar results. In pinealectomized rats, cocaine-induced locomotor activity increased at the times of the day we evaluated, and there was a loss of diurnal variation. Additionally, removal of the pineal gland also decreased serum melatonin levels, as in previous experiments.^{20,21}

Several studies have reported the putative effects of melatonin and the pineal gland on the dopaminergic system.²⁶ Melatonin reduces dopamine content,

replacement, and release, and enhances the affinity of D₂ dopamine receptors.⁴⁵ Since cocaine increases dopamine release in the NAcc and VTA³⁹ and melatonin negatively regulates the dopaminergic system,²⁴ potentiation of cocaine-induced locomotor activity in pinealectomized animals is likely due to a lack of negative regulation of the dopaminergic system by melatonin.

Melatonin decreased cocaine-induced locomotor activity in pinealectomized rats

In this study, we found that melatonin administration prior to cocaine administration in naïve or pinealectomized rats significantly decreased cocaine-induced locomotor activity at different times of day (ZT4, ZT10, ZT16, and ZT22).

In a pioneering study, Sircar³⁶ found that acute melatonin dosing of naïve Sprague-Dawley rats decreased cocaine sensitization. These results are in line with ours: naïve rats Wistar that received melatonin showed a significant decrease in cocaine-induced locomotor activity at different times of day. Still, a major difference between the Sircar³⁶ study and ours is that we did not evaluate the effect of exogenous melatonin at different times of day.

Melatonin levels measured by ELISA were lower in rats subjected to surgical removal of the pineal gland. However, even in these animals, administration of exogenous melatonin was able to decrease cocaine-induced locomotor activity. Several researchers have reported that the removal of the pineal gland does not alter the density of melatonin receptors⁴⁶⁻⁴⁸; this correlates with our finding that exogenous melatonin decreased cocaine-induced locomotor activity in pinealectomized animals, probably because of its action on the MT₁ and the MT₂ melatonin receptors in the striatum.²⁴

Various studies have described the role of the MT₁ and MT₂ melatonin receptors in the modulation of responses to drugs of abuse.²⁸ MT₁ and MT₂ gene knockdown prevents the development and expression of methamphetamine-induced locomotor sensitization and CPP in melatonin-expressing C3H/HeN and low-melatonin-expressing C57BL/6J mice.²⁸ In addition, Uz et al.²⁴ reported that the MT₁ – but not the MT₂ – receptors are necessary for cocaine-induced locomotor sensitization in rodents.^{24,25}

It was recently reported elsewhere that melatonin decreased cocaine self-administration in rats²⁶ and modulated alcohol-seeking and decreased alcohol relapse-like behaviors in rats.³⁰

Luzindole blocked melatonin-induced reduction in cocaine-induced locomotor activity in pinealectomized rats

Several studies have described the effect of luzindole (an MT₁ and MT₂ melatonin receptor antagonist) on melatonin-induced behavior.⁴⁹ Han et al.⁴⁹ reported that systemic or intracerebral administration of 25 or 50 mg/kg melatonin to KM mice prevented the expression of morphine-induced CPP; this effect was reversed by treatment with luzindole. These results are consistent with those obtained in the present study. Administration of luzindole to naïve or pinealectomized rats treated with

melatonin blocked the effect of melatonin and increased cocaine-induced locomotor activity.

As mentioned above, melatonin modulates dopamine release through its MT₁ and MT₂ receptors in the dopaminergic system.^{19,50} Such decrease – likely the effect of melatonin administration on cocaine-induced locomotor activity in pinealectomized animals in this study – was the result of the activation of MT₁ and MT₂ receptors in the striatum. This is demonstrated by the fact that use of luzindole, an MT₁ and MT₂ receptor antagonist blocked the effect of melatonin and restored the effect of cocaine on locomotor activity at the different times of the day evaluated in this study.

Drugs of abuse, such as cocaine, disrupt various behavioral and physiological rhythms.⁴ Further, administration of melatonin significantly decreases the reinforcing effects of cocaine in rodents,³⁶ probably by modulating the dopaminergic system through its MT₁ and MT₂ receptors.¹⁹

Several studies have described the role of clock genes in addiction in rodents and humans.⁵¹ Exposure to drugs of abuse such as cocaine disrupts expression of the *period*, *clock*, and *npas₂* genes, all of which are so-called core clock genes – part of a conserved transcriptional-translational feedback loop known as the molecular clock, which is responsible for generating biological rhythms in the body.^{52,53} In mice, a negative mutation in the *clock*, *per1*, or *per2* genes causes loss in diurnal and circadian rhythmicity in cocaine-induced self-administration, locomotor sensitization, and CPP.^{6,54} Additionally, other studies have shown that clock genes regulate dopaminergic function,^{2,50} while others have shown that melatonin modulates the expression of clock genes.³ This suggests that the decrease in function of the *period*, *clock*, and *npas₂* genes increases vulnerability to cocaine abuse by reducing behavioral rhythmicity, increasing dopaminergic function, and increasing drug abuse during the day. In turn, melatonin, through its receptors in the dopaminergic system, may restore the rhythmic expression of the clock genes, thus weakening the reinforcing effect of cocaine.^{25,50}

In this study, we found that melatonin decreased cocaine-induced locomotor activity in naïve and pinealectomized rats at different times of the day. Given the ability of melatonin to mitigate various reinforcing effects of cocaine at different times of day, melatonin could be a useful therapeutic agent to reduce cocaine abuse.

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Disclosure

The authors report no conflicts of interest.

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