REPORT

Behavioral characteristics and pharmacological manipulations of a nicotine-entrainable circadian oscillator

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Chronic daily administration of nicotine and other drugs of abuse has been found to entrain pre- and post-drug circadian locomotor activity episodes that oscillate on a 24-h schedule and persist for several days after administration ceases. This drug-entrainable oscillator system could conceivably lead to circadian rhythms of drug seeking and drug use in human drug addicts. The present study (1) characterizes the ability of daily nicotine administration to entrain circadian wheel-running activity episodes in rats across a range of doses, lighting schedules, and food access; and (2) tests whether pre- and post-nicotine episodes can be altered through pharmacological manipulations. Adult female rats were housed in wheel boxes for 35–60 d, and both wheel-running and feeding-related behaviors were measured continuously. Following acclimation, nicotine or saline was administered for 16–24 d, and the rats were left undisturbed for several test days to observe the persistence of nicotine-entrained activity. The results showed that nicotine dose-dependently entrains wheel-running activity, and the highest dose of 1.0 mg/kg produces robust pre- and post-nicotine circadian activity episodes under constant, fixed, and variable light/dark schedules. In the pharmacological manipulation experiment, nicotine-entrained rats were administered one of seven pharmacological treatments (varenicline, mecamylamine, acamprosate, topiramate, naltrexone, SB-334867, or bupropion) in place of the nicotine injection for 2 d, and the rats were not disturbed for four subsequent days. Most of the treatment drugs significantly reduced post-nicotine activity episodes, but only three treatments affected pre-nicotine episodes: the μ- and κ-opioid receptor antagonist naltrexone, the orexin-1 receptor antagonist SB-334867, and the AMPA (2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid)/kainate antagonist topiramate. These results show that chronic daily nicotine administration is a robust zeitgeber that dose-dependently entrains a nonphotic oscillator system that includes opioid, orexin, and glutamate pathways.

Keywords: Addiction, circadian rhythms, episodic entrainment, locomotor activity, naltrexone, nicotine, SB-334867, topiramate

INTRODUCTION

Circadian rhythms are internally driven oscillations that synchronize biological processes with the 24-h rotation of the Earth (Moore-Ede, 1986). Most physiological and behavioral phenomena show circadian rhythmicity, including locomotor activity, sleep, body temperature, hormone and transmitter release, and feeding-related processes (Bell-Pedersen et al., 2005). Endogenous circadian timing systems require both a zeitgeber and an oscillator. A zeitgeber is a special environmental time cue that synchronizes internally driven circadian rhythms with the external environment in a process known as entrainment (Aschoff et al., 1975). The daily transitions between light and dark are the most prominent zeitgeber, but several nonphotic zeitgebers have been identified, including large daily meals (Mistlberger & Rusak, 1987) and social cues (Mrosovsky et al., 1989). An oscillator is an internal timing mechanism that responds to the zeitgeber and regulates the timing of cellular, molecular, and behavioral events (Edmonds & Adler, 1977).

There is considerable evidence that drugs of abuse can act as zeitgebers. Chronic daily intraperitoneal (i.p.) or subcutaneous administration of methamphetamine (Kosobud et al., 1998; Pecoraro et al., 2000), cocaine (White et al., 2000), fentanyl (Gillman et al., 2009), and nicotine (Gillman et al., 2008, 2010) have been shown to entrain anticipatory and evoked circadian episodes of
locomotor activity. The drug-anticipatory or pre-drug episodes typically emerge approximately 2 h prior to the daily injection time in the absence of any photic or pavlovian cues. The drug-evoked or post-drug episodes are expressed after the daily administration time and last 3–6 h depending on the drug and dosage. Most importantly, these pre- and post-drug episodes continue to be expressed around the administration time for 2 or more days after the drug is withheld. This indicates that these are internally driven rhythms that are not a result of pavlovian conditioning or drug withdrawal symptoms. Additionally, when these drugs are administered on a 31- or 33-h schedule that is longer than the circadian range of entrainment, activity episodes are expressed approximately 24 h after each drug administration (Gillman et al., 2009; Pecoraro et al., 2000; White et al., 2000). This provides conclusive evidence that the oscillator system affected by drugs of abuse runs on a 24-h (i.e., circadian) schedule and is not the result of pavlovian cues predicting the administration schedule.

It is not currently known how these drug-entrained circadian activity episodes are manifested in human drug addicts. However, there is a great deal of evidence that circadian rhythms play a role in drug addiction. The “clock” genes Period and CLOCK, which are involved in a transcriptional-translational feedback loop that regulates endogenous circadian rhythms at the cellular level (Ko & Takahashi, 2006), have been linked to addictive behaviors in both human and rodent models (Abarca et al., 2002; Akhisaroglu et al., 2004; Falcón & McClung, 2009; McClung et al., 2005; Spanagel et al., 2005). Circadian rhythms of drug use have been reported in heavy smokers (Chandra et al., 2007; Jarvik et al., 1993) and alcoholics (Danel et al., 2003), and admissions to emergency rooms for drug overdoses cycle on a daily schedule (Morris, 1987; Raymond et al., 1992). Further, cocaine craving has been shown to steadily rise in the hours leading up to cocaine use (Preston et al., 2009). Collectively, these results suggest that drug-entrained circadian activity episodes may represent a circadian-based drug craving or motivational drug-seeking behavior.

Previous studies of drug-entrainable circadian activity episodes have not yet confirmed whether these episodes are mediated independent of the light-entrainable oscillator system that is controlled by the suprachiasmatic nucleus (SCN) of the hypothalamus and activated by retinal photoreceptors (Morin & Allen, 2006). Most studies of drug-entrainable episodes to date have used constant lighting conditions, which cause light-entrainable rhythms to “free-run” at periods slightly longer or shorter than 24 h due to the absence of the light-entrainable zeitgeber (i.e., the transition from light to dark). It is important to determine whether nicotine-entrained circadian episodes can occur at any phase of the rat’s rest/activity cycle or whether these episodes only occur at a specific time of day or a specific activity phase. To test this, the present study examined whether repeated daily nicotine injections could continue to entrain circadian activity episodes when a light/dark transition occurs in the rat’s environment. The present study also examined whether the zeitgeber qualities of nicotine are dose dependent and affected by the amount of food the rat is allowed to eat at one time. Collectively, these experiments will confirm whether the nicotine-entrainable oscillator is a unique nonphotic oscillator or merely piggybacking on the light- and/or food-entrainable oscillator systems.

Whereas both the pharmacological characteristics of nicotine (Benowitz, 2008) and the anatomical mechanisms of the light-entrainable oscillator system (Morin & Allen, 2006) are well characterized, the anatomical and physiological mechanisms of nonphotic oscillators have been notoriously difficult to ascertain (Antle & Silver, 2009; Davidson, 2009; Miltberger, 2009). In addition to the studies mentioned above, the present study also screened the effects of seven pharmacological treatments prescribed for nicotine and other drug addictions on pre- and post-nicotine circadian episodes. These treatment drugs were chosen based on their pharmacological profiles targeting cholinergic, dopaminergic, glutamatergic, opioid, and orexin pathways, and most of the chosen treatments have been shown in clinical studies to reduce craving for nicotine and/or other drugs of abuse. The results will show whether nicotine-induced circadian episodes can be pharmacologically perturbed and will provide a foundation for future work into the anatomical characteristics of this system.

MATERIALS AND METHODS

This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Indiana University Bloomington under protocol no. 09-010 and adhered to international ethical standards (Portaluppi et al., 2010).

Subjects

Subjects were 200 female Sprague-Dawley rats obtained from the rodent colony in the Department of Psychological and Brain Sciences at Indiana University Bloomington or from Harlan Laboratories (Indianapolis, IN). Female rats were used because previous studies of drug-entrained circadian episodes have generally used female rats (Gillman et al., 2008; Kosobud et al., 2007), and because male rats generally show a more marked decline in wheel running with age (Peng et al., 1980). The rats were 70–120 days old at the beginning of each experiment, with a body weight range of approximately 170–250 g. Eight rats were assigned to each experimental group. A total of 25 groups were tested across four experiments examining episodic entrainment to multiple doses of nicotine and how nicotine-entrained...
episodes were affected by different food access conditions, different lighting conditions, and pharmacological treatments for drug addiction.

**Apparatus**

All rats were individually housed in either MedAssociates (St Albans, VT; 8 counts per wheel rotation) or Whamann (1 count per wheel rotation) cages with attached wheels for the duration of each experiment. The cages were kept in light- and sound-attenuated cabinets equipped with a ventilation fan to maintain airflow and mask outside noise. Each cabinet housed eight wheel cages (two per shelf). The rats were continuously monitored throughout the study for wheel running, water drinking, and feeding activities. Water was available ad libitum, and water bottles and cage bedding were changed once every week as per institutional guidelines immediately after injection administration. The numbers of wheel counts were recorded with microswitches, and water bottle licks, head entries in the feeder, and the numbers of food pellets consumed were recorded with photobeam sensors. Data were recorded continuously in 1-min bins using the Med PC-IV program (MedAssociates).

**Study schedules and experimental conditions**

A variety of experimental paradigms were used for the four experiments in this study. The timelines of these paradigms are shown in Figure 1. All experiments included an initial acclimation phase and two or three nicotine (or saline control) injection series that were each immediately followed by a 3–6-d test phase. All experimental solutions were administered via dorsal subcutaneous injections except where noted.

**Dosage range experiment**

Two separate paradigms were used in this experiment. The three lower nicotine doses (0.15, 0.3, and 0.6 mg/kg) were tested on the schedule used in previously published experiments that has been shown to produce fentanyl-induced circadian activity episodes (Gillman et al., 2009). The highest nicotine dose (1.0 mg/kg) was tested on a schedule similar to one that has previously been shown to produce nicotine-entrained episodes at that dosage level (Gillman et al., 2008). During acclimation, rats were handled three times per week between 10:00 and 13:00 h to record body weights. Nicotine (or saline) injections were administered once every 24 h during the injection series. During the test phase,
no injections were administered and the rats were not disturbed unless required by equipment malfunctions.

Food access for all rats in this experiment was rate-limited to no more than two 97-mg pellets (NOYES pellets; Test Diets, St. Louis, MO) per 5-min period. Food pellets were accessible from a food receptacle in the cage equipped with an infrared photodetector. The dispensation of the food pellets was controlled by the Med PC-IV program, which was programmed to dispense two pellets if 5 min had elapsed since the previous pellets were dispensed and if there were no pellets currently present in the receptacle. This rate-limited food access was used to prevent entrainment to a large daily meal, which has been shown to act as a zeitgeber for circadian food-antici-
patory locomotor and body temperature rhythms (Mistlberger, 1994, 2009). Under the rate-limited feeding schedule, rats readily consume their daily nutritional requirements, but they are unable to consume pellets fast enough and in sufficient quantities to constitute a meal that is large enough to entrain circadian food-antici-
patory rhythms. In adult female rats, food-antici-
patory activity generally emerges when meal size is 5 g or larger in a 2-h period (Mistlberger & Rusak, 1987).

All rats in this experiment were kept under constant light (LL) that varied as a function of the location within the cage. Constant light was used to prevent entrainment to a light/dark cycle. In adult female rats housed under LL conditions, the estrous cycle is usually suspended, with approximately half of the rats in a persistent estrous state, and the remaining rats show either persistent diestrous or altered cycling patterns (Campbell & Schwartz, 1980; Fitzroy Hardy, 1970; Kledzik & Meites, 1974; Schwartz, 1982). Light intensity was ~45 lux in the wheels and ~275 lux in the cage where the food hopper and water bottle were located. Light intensity in the room outside the cabinet where injections were performed and body weights were recorded was ~215 lux. These light intensities are considered to be “bright” light but are lower than the 300 lux intensity that reliably produces arrhythmia in adult rats (Cambras et al., 1998). Light-entrainable physiological and behavioral rhythms will free-run under constant light, and the period of these free-
running rhythms lengthens as the light intensity increases (Daan & Pittendrigh, 1976). Therefore, under the present study conditions, the free-running light-entrainable rhythms should show periods of approximately 25–26 h and be easily distinguishable from the drug-entrained circadian episodes, which should show 24-h periodicity.

Lighting conditions experiment
This experiment included two groups: FXD-VAR and VAR-FXD based on their lighting schedules. The two groups were isolated in separate cabinets (8 rats per cabinet) to prevent exposure to the other group’s light/dark schedule. Overhead lights in the room housing these cabinets were kept off at all times, and the researchers used red lights to illuminate all experimental and maintenance procedures. Group FXD-VAR were initially kept on a fixed (FXD) 10:14 light/dark cycle, with light presented from 02:00 to 12:00 h daily. Group VAR-FXD were initially kept under a variable (VAR) light/dark schedule. Within each 24-h day, 10 continuous hours of light and a variable amount of dark (2–26 h) were presented. The light cycle onset could occur as early as midnight (00:00 h) or as late as 1400 h. Light onset times were chosen semirandomly so that this time was never the same for two consecutive days.

Both groups FXD-VAR and VAR-FXD were kept on their initial light/dark schedules (fixed and variable, respectively) until the last day of test 2 (day 4). On this day, the light/dark schedules for the two groups were switched, so that group FXD-VAR was placed on a variable schedule and group VAR-FXD was placed on a fixed schedule. Each group remained under the new light/dark schedule for the last 12 d of the study.

In this experiment, the daily nicotine administration time was shifted between the three injection series so that injections would fall at different phases of the fixed light/dark cycle. As in the dosage range experiment, food access for these rats was rate-limited at all times.

Food access experiment
This experiment included two groups: RT-AD and AD-RT based on their food access. Constant lighting was used throughout this experiment. Group RT-AD were initially fed on a rate-limited (RT) schedule. Group AD-RT had food available ad libitum (AD) at the beginning of the study. Both groups RT-AD and AD-RT were kept on their initial food access conditions (rate-limited and ad libitum, respectively) until the last day of test 2 (day 4). On this day, the food access for the two groups was switched so that group RT-AD was placed on ad libitum feeding and group AD-RT was given rate-limited feeding. Each group remained on the new food access condition for the remaining 12 d of the study.

Pharmacological treatments experiment
In this experiment, each nicotine (or saline) injection series was followed by a 2-d treatment series and a 4-d test phase. During the treatment series, the selected pharmacological treatment was administered in place of the nicotine (or saline) injection at the same daily administration time. During the subsequent 4-d test phase, all injections were withheld and the rats were not disturbed.

Four of the saline control groups in the pharmacological treatments experiment were tested in an abbrevi-
ated 28-d paradigm due to temporal constraints and drug availability. These groups received the acampro-
sate, topiramate, naltrexone, and SB-334867 treatments. In this abbreviated paradigm, the acclimation saline injections and the second injection, treatment, and test phases were omitted so that the rats received only a
single 16-d saline injection series followed by a 2-d treatment series and a 4-d test phase.

Drug solutions and dosages
All drug and vehicle solutions were administered via dorsal subcutaneous injections at a dosage volume of 1.0 mL/kg except for three lower nicotine dosage groups in the dosage range experiment, which were administered via intraperitoneal (i.p.) injections at a dosage volume of 2.5 mL/kg. All solutions were refrigerated at approximately 4 °C when not in use.

Nicotine injections
Nicotine hydrochloride powder (Sigma Pharmaceuticals, St. Louis, MO) was dissolved in 0.9% NaCl solution to a concentration of 1.0 mg/mL (free base weight). The pH of the solution was adjusted to approximately 7.4 using NaOH solution. Nicotine was administered at a dosage level of 1.0 mg/kg for all rats except the three lower dosage groups in the dosage range experiment, which received doses of 0.15, 0.3, and 0.6 mg/kg. The 1.0 mg/kg dosage of nicotine has previously been shown to entrain robust pre- and post-drug circadian activity episodes in adult female rats under constant light and rate-limited feeding without adverse effects (Gillman et al., 2008, 2010). Adult rats will show conditioned place preference to nicotine when it is administered within the range of 0.4–1.4 mg/kg (Le Foll & Goldberg, 2005).

Treatment injections
A single dose of each drug was administered in the treatment experiment. Whenever possible, treatment doses were selected based on previous studies that have shown that particular dose to have an effect on the behaviors or physiological effects produced by high doses of nicotine. If these data were not available, the selected treatment doses have been shown to be sufficient to alter the effects of another drug of abuse in the absence of toxic effects. Dosage information for the mecamylamine, varenicline, acamprosate, and naltrexone treatments is listed in the Supplementary Materials.

Naltrexone, a μ-opioid receptor antagonist, is prescribed primarily for the treatment of alcoholism but has also shown some efficacy for smoking cessation when combined with nicotine-replacement therapy (Krishnan-Sarin et al., 2003; O’Malley et al., 2006). Naltrexone hydrochloride (Tocris; Bristol, UK) was dissolved in 0.9% NaCl solution to a concentration of approximately 10 mg/mL and was administered at a dosage level of 10 mg/kg. This dosage has been shown to decrease responding for food under a fixed interval schedule when administered in combination with 1.0 mg/kg nicotine (Corrigall et al., 1988).

The selective OX1 antagonist SB-334867 (Tocris) was dissolved to a concentration of approximately 10 mg/mL in a vehicle that consisted of 88% sterile water, 2% dimethylsulphoxide (Sigma), and 10% 50 mM 2-hydroxy-β-cyclodextrin. SB-334867 was administered at a dosage level of 10 mg/kg and stirred prior to each daily administration time. In rats, this dosage has been shown to extinguish cocaine-seeking behavior (Boutrel et al., 2005) and to reduce the consumption of high-fat food pellets (Nair et al., 2008). A lower dose of SB-334867 (4 mg/kg) has been shown to significantly reduce both intravenous (i.v.) nicotine self-administration and lever-pressing for nicotine rewards in a progressive ratio schedule (Hollander et al., 2008).

Topiramate, an AMPA (2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid) and kainate receptor antagonist, has shown efficacy both for alcoholism treatment and for smoking cessation (Florez et al., 2008; Kampman et al., 2004; Reid et al., 2007). Topiramate powder (US Pharmacopeia, Rockville, MD) was suspended in 0.9% NaCl solution to a concentration of approximately 50 mg/mL and mixed in a warm water bath. Topiramate was administered at a dosage level of 50 mg/kg and vigorously stirred prior to each daily administration time. This dosage has been shown to attenuate the release of dopamine, norepinephrine, and serotonin in the nucleus accumbens produced by the administration of 0.4 mg/kg nicotine (Schiffer et al., 2001).

Data analysis
Episodic entrainment
As established in a previous study (Gillman et al., 2008), the 2-h period immediately prior to an injection was designated the PRE (pre-injection) period, and the 3-h period immediately following an injection was designated as the POST (post-injection) period. Wheel counts were averaged into PRE and POST bins for each phase of the study (acclimation, nicotine injection series, test 1, etc.).

Entrainment of circadian activity episodes to chronic daily nicotine administration was determined by the combination of visual inspection of actograms of the wheel-running data (Figures 2, 3 and Figure S1), calculation of the period lengths of the activity cycles for relevant study phases, and with statistical comparisons of wheel counts among different study phases. The period lengths of the activity cycles (τ) were calculated with chi-square periodograms using Clocklab (Actimetrics, Wilmette, IL). Statistical analyses were performed using multivariate repeated-measures analysis of variance (ANOVA) with planned comparisons to compare the average magnitudes of activity in the PRE and POST periods during the acclimation phases with activity at the same times during the nicotine (or saline) injection series. Entrainment was interpreted as significant increases in activity in the PRE and POST periods during the 24-h injection series that were separate from the free-running rest/activity cycle. Drug dosage, light schedule, food access, or treatment
FIGURE 2. Episodic entrainment to daily injections of 1.0 mg/kg nicotine compared with saline. Double-plotted wheel-running (black tick-marks) actograms for rats kept in LL in the 1.0 mg/kg nicotine (A) and saline (B) groups in the dosage range experiment. Nicotine injection times are represented with transparent gray bars, and saline injections are represented with transparent white bars. Two wheel-running rhythms are visible in each actogram: the free-running light-entrainable activity rhythm is visible as a diagonal line of activity with a period of approximately 25–26 h, whereas activity centered around the nicotine and saline injections shows a period of approximately 24 h. (C) Mean hourly wheel counts during the last 4 d of the first 16-d injection series for the 1.0 mg/kg nicotine (black bars) and saline (gray bars) groups in the dosage range experiment. Pre-injection activity episodes are visible beginning approximately 4 h prior to the injection time (hour 0). (D) Persistence of wheel counts in the first 96 h after the last nicotine or saline injection in the first 16-d injection series. The calculated period lengths of the activity cycles (τ) are listed in the legend and were approximately 24 h in both groups. (E and F) Summary of the statistical comparisons of mean wheel-running episodes throughout the 50-d study in the PRE period 22–24 h after each injection time (E) and the POST period 0–3 h after each injection time. Mean wheel counts that are significantly higher than acclimation wheel running. Both the 1.0 mg/kg nicotine and saline groups showed pre- and post-injection episodes that were significantly higher than acclimation wheel running. Both the pre- and post-nicotine episodes persisted for at least 2 d, whereas pre-saline activity did not significantly persist into the test days. Therefore, episodic entrainment was significant for the 1.0 mg/kg nicotine dose but not for saline. n = 8 per drug group.
FIGURE 3. Episodic entrainment to 1.0 mg/kg nicotine under different lighting conditions. (A–C) Single-plotted actograms for a representative rat showing wheel running (black ticks) under constant light (LL) (A), a fixed 10:14 light/dark (LD) cycle (B), and a variable (continued)
administered were used as a between-subjects factor in these statistical tests.

Persistence of activity episodes in the test days was determined with a multivariate repeated measures ANOVA (with planned comparisons) to compare activity in the PRE and POST periods during the nicotine injection series with activity in these periods during the test phase. A lack of a significant difference between the 24-h injection series and the test days was interpreted as persistence of activity.

**Food access**

In addition to the episodic entrainment analyses listed above, the effects of the rate-limited vs. ad libitum food access on total food consumption were also assessed. A multivariate repeated-measures analysis was performed to compare the average number of food pellets consumed (meal size) per 2h during the acclimation and nicotine injection series. This analysis compared average hourly feeding in the PRE period, 0–2 h post-injection, 2–4 h post-injection, and for the 2-h average for the rest of the day between the rate-limited and ad libitum food conditions. Additionally, the effects of changing between the two food access conditions were assessed by subtracting food consumption during nicotine series 3 (after the food access switch) from food consumption in nicotine series 2 for the PRE, POST 0–2 h, POST 2–4 h, and rest-of-day periods.

**Treatment effects**

The percent change in activity from the nicotine (or saline) injection series was used to examine the effects of the treatments on pre- and post-drug episodes. This measure was calculated by subtracting the amount of activity in either the PRE or POST period on each of the individual treatment and baseline days from the mean amount of activity in that period on the last 4 d of the nicotine injection series. This difference value was then divided by the injection series amount to obtain the percent different in activity. As the PRE period used for each injection was calculated on the following day (i.e., 22–24 h later), there was one fewer baseline day of data available for the PRE period than for the POST period. Multivariate repeated-measures analyses with planned comparisons were then performed to compare the percent difference in activity on the individual treatment and baseline days with the percent difference in activity during the nicotine injection series, which was equal to zero in all cases. Therefore, percent differences on the treatment and baseline days that were significantly different from zero (no change in activity) were interpreted as a significant effect of the treatments on the activity levels.

One rat from the nicotine-naltrexone group and one rat from the saline-bupropion group were excluded from the analyses in the pharmacological treatments experiment because their activity counts and percent difference values for all activity measures were extreme outliers compared with the other seven rats in their treatment group (more than 3 interquartile ranges from the group mean).

**RESULTS**

**Dosage range experiment**

Chronic daily nicotine administration entrained wheel-running activity episodes in a dose-dependent manner (Figure 2, Figure S1). Under constant light, two distinct oscillations were observed in the wheel-running activity: a light-entrainable rhythm with a period of approximately 25–26 h and nicotine-entrainable episodes with periods of approximately 24 h (Figure 2A and C, Figure S1A–D). During the first nicotine injection series, wheel counts in the PRE period 0–2 h prior to the daily nicotine injection were significantly higher than acclimation levels for the 1.0 mg/kg dose, $F(1, 7) = 7.902, p < 0.05$ (Figure 2E). The 0.6, 0.3, and 0.15 mg/kg doses failed to produce significant pre-nicotine episodes in any of the injection series, $F(4, 4) = 2.652, p = 0.184$ for the 0.15 mg/kg group, $F(3, 5) = 3.484–4.584, p = 0.067–0.106$ for the other groups (Figure S1E). This was also true for post-nicotine episodes: wheel running in the POST period 0–3h after the nicotine injection time was significantly higher than acclimation levels for the 1.0 mg/kg dose, $F(1, 7) = 17.513, p < 0.01$ (Figure 2F), but not for the lower doses, $F(3, 5) = 2.388–4.126, p = 0.081–0.185$ (Figure S1F).

As stated in the Materials and Methods section above, episodic entrainment requires both that (1) the pre- and post-drug activity episodes be significantly higher than acclimation levels and (2) that these episodes persist for at least 2 d after the cessation of drug administration. In the 1.0 mg/kg nicotine dosage group, both the pre- and the post-nicotine episodes showed significant

Continued
persistence for 6 d, $F(1, 7) = 0.063{-}4.774, p = 0.065{-}0.810$ (Figure 2D). Under the same conditions as the 1.0 mg/kg dosage group, repeated saline administration produced significant pre- and post-injection episodes, $F(1, 7) = 15.766{-}25.193, p < 0.01$ (Figure 2), with slightly higher wheel counts than the 1.0 mg/kg nicotine group, but the pre-saline episodes failed to show persistence and were significantly lower than the injection series levels following the first test day, $F(1, 7) = 6.750, p < 0.05$. Therefore, according to the statistical criteria, saline administration did not produce significant episodic entrainment.

Unlike the 1.0 mL/kg volume, intraperitoneal saline administration at the 2.5 mL/kg injection volume did not produce significant wheel-running episodes in the PRE period, $F(1, 7) = 0.002{-}2.842, p = 0.136{-}0.968$, but POST period wheel counts were significantly higher than acclimation levels, $F(1, 7) = 5.795{-}19.013, p < 0.05$, in the second and third injection series (Figure S2). Post-saline episodes persisted for two test days, $F(1, 7) = 0.492{-}1.558, p = 0.252{-}0.506$, but were significantly lower on the third test day, $F(1, 7) = 10.166, p < 0.05$. In summary, both the 1.0 mL/kg subcutaneous saline (Figure 2) and the 2.5 mL/kg i.p. saline controls (Figure S2) showed some evidence of circadian activity but failed to meet the statistical criteria for entrainment.

Further, an analysis of wheel running in the 19 h of the day outside of the PRE and POST periods showed that daily wheel running in both the 1.0 mg/kg nicotine and saline groups steadily increased throughout the study, $F(1, 14) = 8.004{-}32.323, p < 0.05$ (Figure S3). This may partially account for the higher PRE and POST period wheel counts during the saline injection series when compared with acclimation levels. Additionally, the rats in the saline group consistently showed higher wheel counts than the rats in the nicotine group, although this was not a significant difference, $F(1, 14) = 1.797, p = 0.201$. Wheel running shows a high degree of individual variability, so the higher saline group wheel counts are likely due to individual baseline differences and not to an effect of the saline injections.

In summary, repeated daily administration of 1.0 mg/kg nicotine produces robust pre- and post-drug circadian activity episodes of wheel running that are not reproducible with lower nicotine doses or daily saline injections.

**Lighting conditions experiment**

The 1.0 mg/kg dose of nicotine was repeatedly administered under constant light (LL), a fixed 10:14 light/dark cycle (in both the light and dark phases), and a variable light/dark cycle described in the Materials and Methods section (Figure 3). Significant episodic entrainment was observed under all lighting conditions. Pre-nicotine episodes were significantly higher than relevant acclimation levels under LL, variable LD, and both the light phase and dark phase of the fixed LD cycle, $F(1, 28) = 96.020, p < 0.001$. These significant episodes also showed persistence for at least 3 d, $F(1, 28) = 0.909{-}1.525, p = 0.227{-}0.766$. Post-nicotine episodes were also significantly higher than acclimation levels under all lighting conditions, $F(1, 28) = 150.069, p < 0.001$. In the overall analysis of the four lighting condition groups, these post-nicotine episodes did not show significant persistence on the test days, $F(1, 28) = 9.819{-}69.553, p < 0.01$, but there were significant lighting condition–test day interactions on the first two test days, $F(3, 28) = 3.223{-}3.327, p < 0.05$, which implies that the fixed dark cycle and variable cycle groups likely showed significant persistence for 2 d.

Although nicotine produced significant episodic entrainment under all lighting conditions, the behavioral output varied among the different paradigms. In both LL and the light phase of the fixed LD cycle, wheel counts were greatly attenuated when compared with the variable LD and the dark phase of the fixed LD cycle (Figure 3D). These effects were reflected as a significant study phase–lighting condition interaction effect in the pre- and post-nicotine episodes when compared with acclimation, $F(3, 38) = 11.525{-}21.763, p < 0.001$, and in the persistence of the post-nicotine episodes into the test days as listed above (Figure 3F and G). Additionally, wheel running in the light phase of the fixed LD cycle showed a bimodal distribution of activity with an initial activity peak occurring approximately 1 h after nicotine administration that was consistent with the other lighting condition groups and a second activity peak occurring approximately 5 h after nicotine administration (Figure 3D). Since the time of the second peak was 2 h after the onset of the dark phase, this second activity peak is most likely light-entrained activity that is unrelated to the nicotine injection or a combination of light- and nicotine-entrained activity.

**Food access experiment**

Under constant light, the 1.0 mg/kg nicotine dose was also investigated under both ad libitum and the rate-limited food access condition described above (Figure S4). In short, neither food access condition appeared to significantly affect nicotine-induced episodic entrainment. Interestingly, average meal size under both food access conditions never reached the 5 g per 2 h threshold needed to produce circadian food-anticipatory activity (Figure S4A). However, the amount of food consumed after the injection time significantly increased throughout the study, and meal size at this time of the day was significantly higher than acclimation levels during the Saline Injection Series and both Nicotine Injection Series, $F(1, 14) = 9.214{-}42.814, p < 0.01$. Additionally, changing between the two food access conditions significantly lowered post-nicotine feeding, $F(1, 14) = 9.678, p < 0.01$, differentially affected feeding in the period 2–4 h after the nicotine injection (significant interaction: $F(1, 14) = 22.729, p < 0.001$), and...
significantly elevated feeding during the rest of the day, $F(1, 14) = 7.281, p < 0.05$.

**Pharmacological treatments experiment**

In the final experiment, the 1.0 mg/kg dose of nicotine was administered under constant light until episodic entrainment was established. This paradigm was then used to screen the effects of several pharmacological treatments on pre- and post-nicotine circadian activity episodes (Figure 4, Figure S6). Treatment drugs were administered in place of the nicotine (or saline) injection for two consecutive days, and this short treatment series was then followed by four test days in which no injections were administered and the rats were not disturbed. Locomotor changes recorded on the treatment days were interpreted as effects on the entrained activity episodes, whereas locomotor changes on the test days were interpreted as effects on the persistence of the circadian activity episodes.

Most of the treatments significantly reduced post-nicotine wheel running on both the treatment and the test days. In contrast, pre-nicotine circadian episodes were unaffected by the treatments, with the exceptions of naltrexone, SB-334867, and topiramate (Figure 4, Figure S6). Effects of the remaining treatments are summarized in Figure S5. As with the other treatments, naltrexone, SB-334867, and topiramate all significantly reduced nicotine-induced wheel running in the POST period on at least one of the treatment days, $F(1, 6) = 43.280–543.629, p < 0.01$ for naltrexone, $F(1, 7) = 7.136–45.073, p < 0.05$ for the other drugs, and on at least three of the test days, $F(1, 6) = 12.281–$.

**FIGURE 4.** Effects of naltrexone, the OX1 antagonist SB-334867, and topiramate on pre- and post-nicotine and -saline episodes. Percent changes in wheel counts ± standard error are shown for the PRE period (A, C, and E; 22–24 h after each injection time) and POST period (B, D, and F; 0–3 h after each injection time) on the treatment and test days as compared with the nicotine and saline injection series. The administered treatments are labeled at the top of the individual graphs. Percent changes in wheel running that are significantly different from zero: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All three treatments significantly reduced both pre- and post-nicotine wheel running but did not significantly affect pre-saline wheel running. $n = 4–8$ per treatment group.
Following the first treatment day, not show a significant change in pre-nicotine activity. Following the second and third test days, F(1,7) = 13.634–29.647, p < 0.05 on the first 2 d, F(1,7) = 0.21–0.501, p = 0.502–0.890 on the last 2 d. SB-334867 generally reduced post-saline activity, but this reduction was only significant on one of the treatment days, F(1,7) = 11.499, p < 0.05, and on two of the four test days, F(1,7) = 6.652–8.968, p < 0.05. Topiramate treatment did not significantly change post-saline wheel running on any of the treatment or test days, F(1,7) = 0.076–3.694, p = 0.096–0.791.

Naltrexone treatment induced the strongest overall reduction in pre-nicotine wheel running and was the only treatment that significantly reduced PRE period wheel counts following both naltrexone treatment days, F(1,6) = 7.475–28.337, p < 0.05. Both SB-334867 and topiramate failed to significantly alter pre-nicotine wheel running on either of the treatment days, F(1,7) = 0.083–1.644, p = 0.241–0.781. However, SB-334867 treatment induced the most robust decline in PRE period activity during the test phase, as rats that received SB-334867 treatment were the only group to show significantly lower PRE period wheel counts following all test days, F(1,7) = 8.692–35.321, p < 0.05. Both the topiramate and naltrexone treatment groups showed significant decreases in pre-nicotine wheel running following the second and third test days, F(1,7) = 7.140–95.751, p < 0.05 for topiramate, F(1,6) = 9.990–145.232, p < 0.05 for naltrexone, but did not show a significant change in pre-nicotine activity following the first treatment day, F(1,7) = 0.018, p = 0.896 for topiramate, F(1,6) = 4.585, p = 0.076 for naltrexone. In contrast, none of these treatments significantly altered pre-saline activity on either the treatment or test days, F(1,7) = 0.023–4.010, p = 0.085–0.883.

In summary, naltrexone, SB-334867, and topiramate all significantly reduced both pre- and post-nicotine circadian activity episodes, whereas the other treatments did not affect pre-nicotine episodes (see Supplementary Materials and Figure S5). Naltrexone treatment reduced pre-nicotine episodes on the greatest number of days, but SB-334867 was the only treatment drug that reduced pre-nicotine wheel running following the treatment days. These results show that both pre- and post-nicotine circadian activity episodes can pharmacologically manipulated and appear to utilize different pathways than saline-induced activity.

**DISCUSSION**

**Behavioral characteristics**

The results of this study confirm not only that nicotine is a robust nonphotic zeitgeber for circadian locomotor episodes, but also show that nicotine entrains an oscillator system that is largely independent of the light-entrainable oscillator. The zeitgeber properties of nicotine appear to be dose-dependent, as the three lower doses of nicotine (0.15–0.6 mg/kg) failed to produce significant episodic entrainment even after 24 injections. The highest dose of nicotine, 1.0 mg/kg, reliably produced episodic entrainment after 16 d of administration. Nicotine has a half-life of approximately 2–3 h (Benowitz, 1996; Gries et al., 1996; Kyeremat et al., 1988), so it is unlikely that these dosage differences are due to drug clearance. As stated above, nicotine induces conditioned place preference within the range of 0.4–1.4 mg/kg (Le Foll & Goldberg, 2005), so the 0.15 and 0.3 mg/kg doses may have been insufficiently rewarding and/or motivating to produce episodic entrainment. Given that 1.0 mg/kg is a relatively high dose of nicotine for a rat, particularly compared with the amount of nicotine a human smoker absorbs from a cigarette (Patterson et al., 2003), it is possible that the lower nicotine doses would eventually produce episodic entrainment if administered for a longer period of time. In this and other studies of drug-induced circadian episodes (Gillman et al., 2008, 2009, 2010; Kosobud et al., 1998; Pecoraro et al., 2000), the magnitude of both pre- and post-drug episodes and the duration of the persistence usually increases over time.

In contrast to nicotine, saline administered in the same paradigm produces large activity bouts that are significantly higher than acclimation, but these episodes do not persist when saline is taken away. Therefore, nicotine can be considered a nonphotic zeitgeber whereas saline cannot. The failure of the lower nicotine doses to show entrainment does not appear to be due to the different injection volumes used in the dosage range experiment, as the saline controls for both injection volumes showed similar results with some evidence of circadian activity present, but not enough to be considered entrainment.

We were surprised by the strength of the saline effects seen in these studies. Although the activity episodes induced by saline failed to meet the statistical criteria for entrainment, saline injections did induce considerable amounts of both pre- and post-injection wheel running. We have had mixed results with saline controls in the past, with some studies showing no entraining effects of saline injections (Gillman et al., 2008; Kosobud et al., 1998; Pecoraro et al., 2000), and others like the present study showing mild or moderate effects. We do not know at this time whether the primary basis for this effect is the saline, the handling, the injection itself, or a combination of these factors. However, despite the saline effects in the present study, we remain confident that nicotine’s zeitgeber properties are not the same as the saline-induced effects seen in the present study. This is most strongly illustrated by the results of the dosage range experiment (Figure 2 and Figure S1), but is also supported by the differential effects of the treatments on saline- vs. nicotine-induced episodes in

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the pharmacological treatments study (Figure 4 and Figures S5 and S6).

Further, as stated above, the rats in the saline group showed higher daily wheel counts than nicotine group rats outside of the PRE and POST periods throughout the study (Figure S3), so some of the saline effects in the present study are likely due to individual wheel-running variability. However, the high saline group wheel counts in the PRE and POST period during the injection series may indicate that the act of picking up a rat and administering an injection of a benign substance stimulates both evoked and anticipatory locomotor activity. This is particularly notable because injections of low doses of nicotine (Figure S1) and injections of the nonselective dopamine receptor antagonist haloperidol (Gillman et al., 2009) do not entrain circadian activity episodes and do not show pre- and post-injection activity bouts. These results appear to indicate that locomotor activation is a critical component of drug-induced entrainment, and the ability of a drug of abuse to act as a zeitgeber depends on its ability to stimulate locomotor activity.

Nicotine does not appear to consistently act as a zeitgeber for light-entrainable locomotor activity rhythms. If this were the case, all daily wheel-running activity would become entrained to the nicotine injection time. However, most of the actograms in the present study (Figures 2 and 3) clearly show two distinct oscillations: the nicotine-entrained episodes and the free-running or light-entrained locomotor rhythms. The sole exception occurred when nicotine was administered in the dark phase of a fixed LD cycle (Figure 3B and C). In that case, the majority of daily activity was centered around the nicotine injection time. Given these differential results, it cannot be concluded from the present study whether nicotine entrains activity independent of the light-entrainable oscillator controlled by the suprachiasmatic nucleus of the hypothalamus (Antle & Silver, 2009). There is likely some overlap between the light-entrainable and nicotine-entrainable oscillators, as the lighting conditions experiment showed that nicotine-entrained episodes are greatly blunted under constant light and under the light phase of a fixed light/dark cycle, both of which are conditions in which rodents show low activity levels due to the actions of the light-entrainable oscillator.

One caveat that should be noted is that saline controls were not included in the lighting conditions experiment due to temporal and financial constraints. The addition of saline controls in this experiment would strengthen the conclusions that could be drawn about the differences between nicotine- and saline-induced activity episodes. If, like nicotine, pre- and post-saline activity increased under fixed and/or variable light/dark cycles, this would provide evidence that episodic entrainment results from some component of the injection procedure such as the stress induced by handling. Conversely, if pre- and post-saline activity is decreased or unchanged under these lighting conditions, it would strengthen the hypothesis that nicotine itself entrains circadian activity episodes.

Finally, the nicotine-entrainable oscillator may also overlap or affect components of the food-entrainable oscillator (Mistlberger, 2009). Nicotine does not appear to be as strong a zeitgeber as a large daily meal, as food-anticipatory activity typically encompasses a much larger portion of a rat’s daily activity and usually appears within a week (Mistlberger, 2009; Mistlberger & Rusak, 1987). In the food access experiment, nicotine-entrained episodes for rats given rate-limited food access were not significantly different from episodes in rats given ad libitum access. Since meal size under ad libitum conditions never reached large enough amounts to produce circadian food-anticipatory activity, it cannot be determined whether nicotine directly affects or is affected by the food-entrainable oscillator system from the results of the present study. Future research should examine nicotine-entrained episodes under restricted feeding conditions that reliably produce food-anticipatory activity.

**Pharmacological characteristics**

The results of the pharmacological treatments experiment show that nicotine-induced circadian activity episodes can be pharmacologically manipulated via opioid, orexin, and/or glutamatergic pathways. Treatment with 10 mg/kg naltrexone, a μ-opioid receptor antagonist, produced the greatest overall reduction in PRE period wheel running on the treatment and test days. At 30 mg/kg, naltrexone produces modest reductions in rodent locomotor activity (Schaefer & Michael, 1985), and that may explain a portion of this effect. However, chronic nicotine administration has been shown to enhance the transmission of endogenous opioids in addition to stimulating dopamine and glutamate transmission (Margioris et al., 1992). Stimulation of endogenous opioid pathways is believed to mediate some of the rewarding effects of nicotine (Corrigall, 2009; Corrigall et al., 1988) and may explain a portion of the efficacy of naltrexone in smoking cessation studies.

The rewarding effects of nicotine have also been shown to be at least partially mediated by the neuropeptide orexin via the orexin-1 (OX1) receptor (Corrigall, 2009; Hollander et al., 2008). In the present study, treatment with 10 mg/kg of the OX1-receptor antagonist SB-334867 produced significant reductions in wheel-running activity on all four test days, although there was no significant change in wheel running on the SB-334867 treatment days. SB-334867 doesn’t significantly affect locomotor activity at the dose used in the present study, but higher doses reduce locomotion, rearing, grooming, and most feeding behaviors (Rodgers et al., 2001). Treatment with the glutamate AMPA/kainate antagonist topiramate also failed to alter wheel running on the treatment days, but did produce significant reductions in wheel running on two of the four test
days. Topiramate doesn’t appear to affect locomotor activity across a wide range of doses, including the dose used in the present study (Tatsuta et al., 2007). Given the differential results shown on the treatment and test days, it appears that the expression and persistence of pre-nicotine episodes may be at least partially mediated by separate mechanisms.

The results of these treatment manipulations also clearly show that pre- and post-nicotine circadian activity episodes are mediated by distinct neuropharmacological mechanisms. Although both pre- and post-nicotine episodes were significantly reduced by naltrexone, SB-334867, and topiramate, post-nicotine episodes were also significantly reduced on at least one treatment and/or test day by administration of the weak nicotinic acetylcholine receptor agonist varenicline, the nicotinic antagonist mecamylamine, the glutamate N-methyl-d-aspartate (NMDA) antagonist acamprosate, and the dopamine reuptake inhibitor bupropion (see Supplementary Materials and Figure S5).

The treatment manipulations also had substantially different and in many cases opposite effects on saline-induced episodes when compared with their effects on nicotine-induced episodes. For example, pre-saline episodes were generally increased in response to naltrexone, SB-334867, and topiramate treatment, whereas pre-nicotine episodes were generally reduced by these treatments. Thus, it appears that although nicotine and saline injections both stimulate wheel-running activity in adult female rats, the mechanism by which this occurs appears to be distinct for each substance.

As stated earlier, it should be noted that the present study was limited to single, high doses of the treatment drugs. Therefore, many of the treatments that did not significantly affect nicotine-induced circadian activity episodes may have significant effects if administered at higher or lower doses. Future studies will need to address these limitations and examine a range of doses for all of the substances used in the present research to fully determine the pharmacological profile of nicotine-entrained circadian activity episodes.

CONCLUSIONS

In this and previous studies, nicotine and other drugs of abuse have been shown to entrain robust pre- and post-drug circadian activity episodes when repeatedly administered at a consistent time of day, and these episodes persist for several days after drug use ceases. Pre-drug episodes may represent a particularly useful behavioral target for the treatment of drug addiction, as they may manifest as a circadian-based drug craving, anticipation, and/or seeking in human drug addicts. Both pre- and post-nicotine circadian episodes can be pharmacologically manipulated by treatments prescribed for nicotine and other drug addictions.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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