Published in final edited form as:

Behav Brain Res. 2009 December 14; 205(1): 102–114. doi:10.1016/j.bbr.2009.07.002.

Fentanyl, but not haloperidol, entrains persisting circadian activity episodes when administered at 24- and 31-hour intervals

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Abstract

Administration of several drugs of abuse on a 24-hour schedule has been shown to entrain both pre-drug (anticipatory) and post-drug (evoked) circadian activity episodes that persist for several days when the drug is withheld. The present tested the entrainment effects of fentanyl, an opioid agonist with a noted abuse liability, and haloperidol, an antipsychotic dopamine antagonist without apparent abuse liability. Adult female Sprague-Dawley rats housed under constant light in cages with attached running wheels received repeated low, medium, or high doses of either fentanyl or haloperidol on a 24-hour administration schedule followed by a 31-hour schedule (Experiment 1) or solely on a 31-hour schedule (Experiment 2). The results showed that all three doses of fentanyl entrained both pre-drug and post-drug episodes of wheel running when administered every 24 hours, and the combined pre- and post-fentanyl activity episodes persist for at least 3 days when the drug is withheld during test days. On the 31-hour schedule, fentanyl produced an "ensuing" activity episode approximately 24 hours post-administration, but failed to produce an anticipatory episode 29–31 hours post-administration. In contrast, haloperidol injections failed to produce both pre-drug episodes on the 24-hour schedule and circadian ensuing episodes on the 31-hour schedule, and post-haloperidol suppression of activity appeared to mask the freerunning activity rhythm. Taken together, these results provide additional evidence that drugs of abuse share a common ability to entrain circadian activity episodes.

Keywords

Fentanyl; Haloperidol; Drug Addiction; Drug Abuse; Circadian Rhythms; Locomotor Entrainment; Drug Anticipation; Ensuing Activity; Wheel Running

Introduction

A drug user must repeatedly and voluntarily consume a drug over an extended period of time, regardless of multiple adverse consequences, to be diagnosed with substance dependence [4]. Although different types of addictive drugs produce a diverse range of physiological and behavioral effects, these drugs all appear to "hijack" the regions of the brain that mediate reward, form learned associations, and control executive decision-making [16,21,32,62,70]. In recent years, a great deal of additional evidence has emerged showing that drugs of abuse

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also affect the circadian timing systems of the brain and periphery. The present study is part of a larger project investigating the abilities of drugs of abuse to entrain episodes of circadian locomotor activity.

Circadian rhythms are biological phenomena that oscillate on an approximately 24-hour schedule to synchronize with the Earth's daily rotation [48]. In a process known as entrainment, neural clock systems or "oscillators" detect the daily occurrence of environmental zeitgebers (time cues) and send outputs that adjust the timing of behavioral and physiological events to coordinate with the timing of the zeitgeber [18,19]. If the zeitgeber is removed, the circadian rhythm soon drifts into a free-running rhythm with a period slightly longer or shorter than 24 hours. In both neural and peripheral tissues, circadian rhythms are controlled at the cellular level through a transcriptional-translational autoregulatory feedback loop of paired "clock" genes [34]. In mammals, the transcription of the heterodimers CLOCK and BMAL1 induces the translation of several subtypes of the target genes *Period* (*Per*) and *Cryptochrome* (*Cry*).

Two major systems have been identified that act as oscillators (pacemakers) that regulate endogenous circadian rhythms. The best-known pacemaker is the light-entrainable oscillator (LEO), which entrains the basic rest-activity cycle to circadian light-dark transitions [47,65]. The LEO resides in the suprachiasmatic nucleus (SCN) of the hypothalamus. The second oscillator, the food-entrainable oscillator (FEO), responds to the presentation of daily meals to entrain episodes of food-anticipatory activity that precede the presentation of the meal by approximately 2 hours [46,64]. The anatomical location of the FEO has yet to be definitively identified. This timing system appears to include both a reward-entrainable oscillator that involves the mesolimbic dopamine pathways [5] and a metabolic-entrainable oscillator that may involve the dorsomedial hypothalamic (DMH) nucleus [25,45], although several studies have shown that rats with DMH lesions continue to show food-anticipatory activity episodes [39,41]. Several additional neural and peripheral tissues have also been linked to the food-anticipatory system [13–15,40,74].

In addition to the well-known zeitgebers of light and food, a number of other stimuli have been shown to affect circadian rhythms [51]. Of particular interest to the present study are the abilities of drugs of abuse to entrain and/or disrupt such rhythms. Several drugs are known to affect daily sleeping patterns, including alcohol [9,52,63], cocaine [80], MDMA [6], and opiates [33,54,69]. Nicotine has been shown to affect circadian patterns of heart rate [30], blood pressure [2], and body temperature [58]. Cocaine administration affects the diurnal fluctuations of several hormones [78], and cocaine sensitization can only develop if the drug is presented during the active part of the rest-activity cycle [3]. Further, clinical studies have observed circadian consumption patterns in both heavy smokers [31] and alcoholics [12]. Circadian patterns have even been observed in emergency room admissions for drug overdoses and other medical issues related to drugs of abuse [20,50,60]. More recently, the expression of the mammalian *Per2* gene has been found to modulate cocaine and ethanol sensitization [1,71], and the CLOCK gene has been found to modulate the formation of cocaine-paired place preference via the mesolimbic dopamine pathway [44].

Previous studies have shown that i.p. administration of methamphetamine on a 24-hour schedule entrains episodes of wheel running, water drinking, and feeding activity in laboratory rats [36]. After several days on this fixed circadian administration schedule, rats show an increase in activity 1–2 hours prior to the administration time in the absence of discriminative environmental cues. In addition to this pre-drug anticipatory episode, post-drug evoked activity episodes are also observed. If the drug is withheld, both the pre- and post-drug episodes appear to persist for 2–3 days around the administration time. Similar entrainment of activity episodes has also been observed with daily administrations of cocaine [81] and nicotine [24].

A possible alternative to a drug-induced circadian entrainment hypothesis is that the rats learn the 24-hour inter-administration interval like any other time interval. If pre-drug anticipatory activity is solely the result of regular interval learning, then the rats should be able to anticipate inter-administration intervals that are considerably longer or shorter than 24 hours. However, if this phenomenon is actually circadian entrainment, then the rats should not be able to anticipate an interval outside the range of circadian entrainment. The range of circadian entrainment for food anticipation is estimated to be 23–29 hours [73], as few rats are able to show anticipation for meals presented at intervals shorter than 23 hours or longer than 29 hours. Interestingly, when meals are administered to rats every 31 hours, an apparent circadian “ensuing” episode of activity occurs approximately 24 hours after the meal is presented, but pre-meal anticipatory activity is not recorded immediately prior to the next meal presentation (29–31 hours post-meal) [82,83]. Similar approximately 24-hour ensuing activity (in the absence of pre-drug anticipation) has also been shown for methamphetamine administered on a 31-hour schedule [57] and for cocaine on a 33-hour schedule [81]. This strongly supports a circadian basis for drug-induced entrainment of activity episodes.

The present study investigated drug-related circadian entrainment in the case of two additional drugs, fentanyl and haloperidol, under both 24- and 31-hour schedules. We selected fentanyl, a Schedule II opiate with a relatively high abuse potential [7,11,68], as a further test of the hypothesis that the ability to entrain circadian episodes is an effect common to all drugs of abuse. We chose to administer haloperidol, a dopamine antagonist, to test the possibility that a large perturbation of dopamine system functioning, in the form of an increase (fentanyl) or a decrease (haloperidol), would be sufficient to produce circadian entrainment of activity.

Fentanyl is an opioid analgesic commonly prescribed for chronic and post-surgical pain management, usually in the form of a transdermal patch [28]. Like other opiates, fentanyl interacts with μ opioid receptors, acutely activates the mesolimbic dopamine system, and produces a robust activation of locomotor activity [26,28]. Chronobiological studies in hamsters have revealed that fentanyl also modifies the ability of the SCN to react to photic stimuli and can induce behavioral phase shifts in the rest-activity cycle when administered during the subjective day [76,77].

Haloperidol is a typical anti-psychotic that is often prescribed for the treatment of schizophrenia [29], and is usually administered orally once a day [37]. Like many other anti-psychotics, haloperidol is a dopamine receptor antagonist [29]. Specifically, haloperidol has a high affinity for D_2 receptors and a relatively low affinity for D_1 receptors [42]. Chronic administration of haloperidol elicits increased D_2 receptor expression in the striatum [38], whereas chronic administration of drugs of abuse elicit decreased D_2 receptor availability [79]. In rats, haloperidol causes locomotor inhibition, impaired feeding behaviors, ptosis, orolingual deficiencies, and catalepsy, and these effects persist for several days after administration ceases [10,23,66].

The present study tested whether the daily administration of low, medium, or high doses of fentanyl or haloperidol to separate groups of rats on circadian (24-hour) and infradian (31-hour) schedules would entrain wheel running episodes in individually housed female rats. Based on its addictive properties, we hypothesized that fentanyl would act as a zeitgeber to entrain circadian activity episodes, but that haloperidol would not. As in previous studies with methamphetamine, cocaine, and nicotine, this entrainment should take the form of a pre-drug anticipatory activity episode and a post-drug evoked response during the 24-hour injection series. Both the pre- and post-drug episodes should persist at the daily administration time for 2–3 days when the drug is withheld during the test phases. Finally, in the 31-hour injection series, fentanyl should produce ensuing activity approximately 24 hours after administration,

and pre-drug anticipatory activity should not be apparent immediately prior to the administration time (29–31 hours post-administration).

Materials & Methods

All experimental procedures followed the Public Health Service Policy on the Humane Care and Use of Laboratory Animals (rev. 2002) and were approved by the Bloomington Institutional Animal Care and Use Committee.

Subjects

A total of 64 female Sprague-Dawley rats were obtained from the rodent colony in the Department of Psychological and Brain Sciences at Indiana University Bloomington. Female rats were used instead of males because of the long length of the study, as daily wheel running in males tends to decrease with age [59,67]. Rats were approximately 90 days old at the beginning of the study, with an initial body weight range of 186–283 g. Rats were randomly divided into eight groups of 8 rats. Experiment 1 (24- and 31-Hour Cycles) used 3 fentanyl dosage groups and 3 haloperidol dosage groups, which were named Low Group, Medium Group, and High Group. Experiment 2 (31-hour Injections Only) used one dosage group per drug which was called 31-Hr Group and received the same dosage as the Medium Group in Experiment 1.

Apparatus & Conditions

For the entirety of the study, all rats were housed in cages with attached wheels (cage dimensions: 25.4 cm × 15.25 cm × 12.7 cm; wheel dimensions: 17.7 cm radius, 10.8 cm width) inside sound- and light-controlled cabinets that each housed 6 wheel boxes. A ventilation fan was used to maintain air flow and to mask outside noise. Water was available *ad libitum*, and water bottles and cage liners were changed once every week per institutional guidelines. The rats were recorded continuously for the duration of the study for wheel running, water drinking, and food consumption. Wheel turns were recorded using microswitches, and the water licks and pellets consumed were recorded with infrared sensors. Data were recorded continuously in one-minute bins using the Med PC-IV program (MedAssociates, Inc) run on an IBM PC.

Rats were kept in constant dim light (LL) that differed as a function of their location (~30 lux in the cage; ~100 lux in the wheel). Feeding was rate-limited, with food access restricted to no more than two 97-mg pellets (Test Diets, Inc.) every five minutes. The LL condition and the rate-limited feeding schedule were utilized to prevent entrainment to either the light/dark cycle or to large daily meals [17] and to easily distinguish the free-running rest-activity rhythm from the drug-induced activity episodes. It is also worth noting that the use of constant light typically suspends the female estrous cycle [22], which keeps daily baseline activity levels relatively consistent for individual rats.

Drug Solutions

Fentanyl citrate powder (Sigma Pharmaceuticals) was dissolved in 0.9% NaCl solution to concentrations of 6 µg/ml (Low Group), 12 µg/ml (Medium Group, 31-Hr Group), and 24 µg/ml (High Group). Haloperidol powder (Sigma Pharmaceuticals) was dissolved in glacial acetic acid and mixed with 0.9% NaCl solution so the acid formed 0.5 to 2% of the total solution. The pH of each solution was adjusted to approximately 7.5 with NaOH solution. Haloperidol solutions were mixed to dosage concentrations of 0.1 mg/ml (Low Group), 0.2 mg/ml (Medium Group, 31-Hr Group), and 0.4 mg/ml (High Group).

Both the fentanyl and haloperidol solutions were administered via intraperitoneal (i.p.) injections at a dosage volume of 2.5 ml/kg. All drug solutions were refrigerated at 4°C when not in use.

Study Schedules

Experiment 1: 24- and 31-Hour Cycles—Experiment 1 lasted 94 days. During the initial 22-day acclimation phase, no injections were administered, and the rats were handled and weighed three times per week at semi-random times of the day between 1000 and 1500. This phase allowed the rats to adjust to the wheel boxes and handling by the experimenters, as well as to establish free-running activity rhythms and baseline activity totals. For the 24-hour injection series, daily injections (fentanyl or haloperidol) were administered at approximately 1000 for three eight-day injection series designated FEN 1, FEN 2, and FEN 3 for fentanyl groups and HAL 1, HAL 2, and HAL 3 for haloperidol groups. Each of these injection series was followed by a three-day test phase (Test 1, Test 2, and Test 3) in which no injections were administered and the rats were not disturbed unless equipment malfunctions required it.

For the 31-hour injection series, 12 successive injections were administered over 15 days, with the first injection occurring at 1000 and the last occurring at 1500. During a 72-hour test phase following the last injection, the rats were only disturbed if required by equipment malfunctions. The study ended with a 14-day final baseline phase in which no injections were administered, but the rats were handled periodically (between 1000 and 1500) to record body weights.

Experiment 2: 31-Hour Injections Only—Experiment 2 lasted 56 days and used experimentally naïve rats. During the 22-day acclimation phase, no injections were administered, and the rats were handled 3 times per week as in Experiment 1. The 31-hour injection cycle in this experiment was identical to that of Experiment 1, with 12 injections (fentanyl or haloperidol) administered over 15 days, and the first injection occurring at 1000. A 72-hour test period followed the last injection, and the rats were disturbed as little as possible during this time. A final 10-day baseline phase followed the test phase. During this baseline phase, no injections were administered, and the rats were handled 3 times per week (between 1000 and 1500) to record body weights.

Data Analysis

The two-hour period immediately prior to an injection was designated as the PRE (pre-drug) period. The three hour period immediately following an injection was designated as the POST (post-drug) period. Additionally, for the 31-hour injection series, the five-hour period 22–27 hours after the injection was designated as the ENS (ensuing) period. The ENS period comprises the 2-hr prior to the circadian interval, and the three hours following the circadian interval. Wheel running data counts were collapsed into the PRE, POST, and ENS bins for each phase of the study as applicable. A square root transformation was performed on the total wheel counts to normalize the data, as the wheel counts (but not the percentage of daily wheel running) showed a high degree of variability among individual rats. Data were analyzed as total wheel turns for the 24-hour injection series and as the percentage of total daily activity for the analysis of the 31-hour injection series; the latter measure was calculated by dividing the activity in the relevant period (PRE, POST, or ENS) by the total activity recorded for the 24-hour day. To allow a direct comparison of the results in the 24- and 31-hour injection series, percentage data from the 31-hr injection series were also analyzed in 24-hour periods. The percentage of daily activity measure was analyzed instead of the absolute wheel counts for the 31-hour data because these analyses included rats from two separate experiments that had different basal running levels.

In Experiment 1, one rat in the fentanyl Low Group unexpectedly died on the second day of the 31-hour injection series (study day 56), and its data were therefore excluded from analyses of the 31-hour injection series. Data for this rat were included in statistical analyses for the 24-hour injection series.

Entrainment of activity episodes to the 24-hour injection series was determined by the combination of visual inspection of actograms of the wheel running data (Figure 1 & Figure 2) and using multivariate repeated measures analyses with planned comparisons to compare the average magnitudes of activity in the PRE and POST periods during the acclimation phase (Acc) and the three 8-day 24-hour injection series (FEN 1/2/3 or HAL 1/2/3). Significant increases in activity in the PRE and POST periods during the 24-hour injection series separate from the free-running rest-activity cycle were interpreted as entrainment. Drug dosage was treated as a between-subjects factor in these statistical tests.

Persistence of activity episodes in the test days was determined by a multivariate repeated measures analysis (with planned comparisons) to compare activity in the PRE and POST periods during the 24-hour injection series with activity in these periods during the test days. Specifically, the data analyzed were an average of all 24-hour injection days (24 total days across three 8-day series) and an average of each of the three test days across the 3 injection/test series (example: Test Day 1 average includes Test 1, Day 1; Test 2, Day 1; and Test 3, Day 1). A lack of a significant difference between the 24-hour injection series and the test days was interpreted as persistence of activity.

Entrainment of activity episodes to the 31-hour injection series was determined by comparing the percentage of daily activity (in each 24-hour period) in the PRE and POST periods between the 24-hour injection series and the 31-hour injection series for all rats in Experiment 1 using multivariate repeated measures analyses with planned comparisons. The dosage was treated as a between subjects factor. The same statistical test was also used to compare average ensuing activity (ENS) in the 31-hour injection series to an average of the combined activity in the PRE and POST periods on the first test day during the 24-hour injection cycle (Test Day 1 average PRE + POST). Finally, to assess the effects of initial 24-hour training on activity levels during the 31-hour injection series, an independent samples t-test (equal variances not assumed) was performed to compare average activity in the PRE, POST, and ENS periods during the 31-hour injection series between the rats in Experiment 2 and the Medium Group rats in Experiment 1, which received matching doses of fentanyl or haloperidol.

Results

Entrainment of Activity Episodes to 24-Hour Injection Cycles

During acclimation, all rats showed clear free-running activity rhythms with periods of approximately 25–26 hours (Figure 1 & Figure 2 - ACCL). As fentanyl was repeatedly administered at the same daily time during the injection series, these free-running rhythms persisted, but wheel-running also became entrained to the injection time in separate activity episodes. In Figure 1a (Low Dose Group) and Figure 1b (Medium Dose Group), this episodic entrainment is particularly evident in the second and third injection series (FEN 2 and FEN 3), and in Figure 1c (High Dose Group), circadian episodic entrainment is already apparent in the first injection series (FEN 1).

Unlike the fentanyl rats, activity episodes did not entrain to the haloperidol administration time in any of the three haloperidol dosage groups (Figure 2). As with fentanyl, free-running rhythms are visible throughout the study for the haloperidol rats, and these rhythms appear to have been either interrupted or masked by haloperidol administration (HAL 1, HAL 2, HAL 3). The active

or inactive part of the free-running rhythm appears to resume 10–12 hours after administration, presumably at the limit of the duration of action of haloperidol.

Post-Drug Evoked Response—Both fentanyl and haloperidol produced marked (but opposite) activity responses following administration of the drug. Fentanyl evoked a large bout of activity that lasted 3–4 hours followed by a much smaller bout of activity that lasted an additional 2–3 hours (Figure 3a). In the POST period (0–3 hours after the injection), the fentanyl-evoked activity in each of the three injection series (FEN 1, FEN 2, and FEN 3) was significantly higher than the acclimation activity levels, $F(3, 19) = 16.883$, $p < 0.001$. Additionally, the amount of post-drug activity showed a significant difference as a function of dosage, $F(2, 21) = 3.528$, $p < 0.05$. Fentanyl showed a “U-shaped” dose-response curve, with the medium dose (0.03 mg/kg) producing the smallest locomotor response, the low dose (0.015 mg/kg) producing the second-smallest response, and the high dose (0.06 mg/kg) producing the largest locomotor output.

In contrast, the haloperidol injections evoked a 9–12-hour episode where activity was blunted or eliminated relative to baseline levels (Figure 4a). This drug-evoked activity suppression in the POST period was significantly different from the acclimation series, $F(3, 19) = 14.091$, $p < 0.001$. There were no significant differences in activity between the three haloperidol dosage groups during acclimation or the injection series during the POST period, $F(2, 21) = 0.655$, $p = 0.530$.

Pre-Drug Anticipatory Activity—After several days of fentanyl administration on a 24-hour schedule, a noticeable rise in activity was recorded that began approximately 2 hours prior to the injection time (Figure 1, Figure 3a). This pre-drug activity bout is particularly pronounced in the Medium and High Groups, especially in the second and third injection series (FEN2 and FEN 3). For fentanyl, the pre-drug activity (PRE) in injection series 1 was not significantly different from activity in this period during acclimation, $F(1, 21) = 0.75$, $p = 0.787$, but was significantly higher than acclimation levels in both series 2, $F(1, 21) = 19.794$, $p < 0.001$, and in series 3, $F(1, 21) = 33.595$, $p < 0.001$. There were no significant differences in PRE period activity among the three fentanyl dosage groups in either the acclimation or 24-hour injection series, $F(2, 21) = 1.329$, $p = 0.286$.

In contrast to fentanyl, haloperidol administration on a 24-hour schedule did not induce a consistent anticipatory response in the PRE period (Figure 4a). Whether the rats were active or inactive in a particular PRE period appeared to depend on which phase of the free-running rest-activity rhythm fell at that time (Figure 2). PRE period activity was significantly different between the acclimation and the first injection series, $F(1, 21) = 5.360$, $p < 0.05$, but not between acclimation and the second injection series, $F(1, 21) = 1.719$, $p = 0.204$, or the third injection series, $F(1, 21) = 0.071$, $p = 0.793$. There were also no significant differences in activity between the three haloperidol dosage groups during the PRE period, $F(2, 21) = 0.631$, $p = 0.542$.

Test Phases (Persistence of Entrained Activity Episodes)—Each of the three 8-day 24-hr injection series were followed by a 3-day test phase in which injections were withheld and the rats were left undisturbed (Figure 1 and Figure 2 – Test). During these test days, rats that received fentanyl continued to show large amounts of activity in the PRE and POST periods just as they did during the 24-hour injection series (Figure 3b–3d). Overall, wheel running in the PRE period was not significantly different when the 3 Test Days were compared to the 24-hour injection series, $F(3, 19) = 3.097$, $p = 0.051$, which indicates that activity levels in the PRE period during the Test Days were comparable to the levels recorded in this period on the days when fentanyl was administered. A significant difference was found among the three

fentanyl dosage groups, $F(2, 21) = 4.572$, $p < 0.05$, with considerably higher wheel counts recorded for the High Dose Group.

The fentanyl rats also showed persisting activity in the POST period when fentanyl was withheld on the test days, particularly following the second and third injection series (FEN2 and FEN3). However, the activity levels recorded in the post-drug period on the Test Days were always lower than the levels recorded immediately following a fentanyl injection, presumably due to the lack of the direct locomotor-stimulating effects of the drug. The persisting (but reduced) activity in the POST period generally showed a steady decline across the 3 days of the test phase. Overall, wheel running in the POST period was significantly lower during the Test Days than during the injection series, $F(3, 19) = 4.512$, $p < 0.05$. However, the results of the planned comparisons showed that POST period wheel running on the first Test Day was not significantly different from post-fentanyl running during the injection series, $F(1, 21) = 2.212$, $p = 0.152$. However, the persisting activity declined steadily so that wheel running was significantly different from the injection series on Test Day 2, $F(1, 21) = 7.687$, $p < 0.05$, and on Test Day 3, $F(1, 21) = 13.402$, $p < 0.01$. There were no significant differences among the 3 fentanyl dosage groups during the POST period on the Test Days, $F(2, 21) = 2.275$, $p = 0.128$.

Unlike fentanyl, haloperidol administration did not produce a consistent pre-drug anticipatory response during the PRE period (Figure 4b–4d). Since there was no consistent pre-drug anticipation of haloperidol, it was assumed that PRE period activity should be similarly inconsistent throughout the Test Days. Overall, PRE period running was indeed not significantly different between the Test Days and the haloperidol injection series, $F(3, 19) = 0.711$, $p = 0.557$, and there was no significant difference among the three haloperidol dosage groups, $F(2, 21) = 2.401$, $p = 0.115$.

Haloperidol administration consistently evoked a long period of suppressed activity. If this suppressed activity had been entrained to the injection time, then a similar period of low activity should have been consistently recorded in the POST period on the haloperidol-free Test Days. Instead, a varied amount of activity occurred on test days during the POST period and appeared to be dependent on the phase of the rest-activity cycle that fell at that time. POST period wheel running was significantly different between the test days and the haloperidol injection series, $F(3, 19) = 9.743$, $p < 0.001$. The three dosages of haloperidol did not produce significant differences in POST period wheel running, $F(2, 21) = 0.847$, $p = 0.443$.

Entrainment of Activity Episodes to 31-Hour Injection Cycles

Dosage Effects—During the 31-hour injection series, there were no significant differences in wheel counts or the percentage of daily wheel running found among either the three fentanyl dosage groups or the three haloperidol dosage groups for any of the periods tested (PRE, POST, and ENS), range: $F(2, 21) = 0.022 - 2.053$, $p = 0.155 - 0.978$.

Post-Drug Evoked Response—During the 31-hour injection series, both fentanyl and haloperidol evoked post-drug (POST period) activity effects that were similar in duration, but not necessarily in magnitude, to the drug-evoked responses on the 24-hour schedule (Figure 5 – Figure 8). Fentanyl administration on a 31-hour schedule evoked a bout of activity immediately following the injection that lasted approximately 2 hours (Figure 5 - POST period, Figure 7), and haloperidol administration evoked a 7–12 hour episode in which activity was suppressed (Figure 6, Figure 8). For fentanyl, the percent of daily wheel running in the POST period during the 31-hour schedule was significantly lower than the percent of wheel running in this period during the 24-hour schedule, $F(1, 20) = 6.512$, $p < 0.05$ (Figure 5). In contrast, the haloperidol-evoked POST period wheel running percentage in the 31-hour injection series

was not significantly different from the 24-hour injection series, $F(1, 21) = 1.608$, $p = 0.219$ (Figure 6).

Pre-Drug Period Activity—In contrast its effects under a 24-hour schedule, fentanyl administration on the 31-hour schedule did not produce a robust pre-drug anticipatory response immediately prior to the injection time (PRE period - 29–31 hours post-injection). The percentage of daily wheel running in this time period was significantly lower during the 31-hour schedule when compared to the 24-hour schedule, $F(1, 20) = 6.630$, $p < 0.05$ (Figure 5). Haloperidol also did not produce a consistent increase in activity immediately prior to the injection time on the 31-hour schedule. Although anticipatory activity was not consistently recorded in this period on the 24-hour schedule, the percentage of daily running in the PRE period on the 31-hour schedule was nonetheless significantly lower than the 24-hour schedule for haloperidol, $F(1, 21) = 6.775$, $p < 0.05$ (Figure 6).

Ensuing Activity—Fentanyl administration on the 31-hour schedule consistently produced an ensuing activity bout that lasted approximately 4–5 hours and occurred approximately 22–27 hours after each fentanyl injection (ENS period). There was no significant difference between wheel counts in the ENS period during the 31-hour injection series and wheel counts in the PRE and POST periods on the first Test Days during the 24-hour injection series, $F(1, 20) = 1.313$, $p = 0.265$. However, the percentage of daily running in the ENS period was significantly lower than the percentage in the PRE and POST period on the first Test Days, $F(1, 20) = 6.903$, $p < 0.05$.

In contrast to fentanyl and in keeping with its effects on a 24-hr injection schedule, haloperidol administration on the 31-hour schedule did not produce an ensuing response at the circadian interval. Instead, normal activity levels resumed after the locomotor depressant effects of haloperidol ceased, generally 8–12 hours post-injection depending on dosage. The activity level then remained relatively steady until the next haloperidol injection. For haloperidol, the percentage of daily wheel running in the ENS period was significantly higher than in the PRE and POST periods on the first Test Days of the 24-hour injection series, $F(1, 21) = 17.018$, $p < 0.001$.

Effect of Initial 24-Hour Training on 31-Hour Activity—In most cases, there were no significant differences in daily wheel running patterns during the 31-hour injection series between Experiment 1, in which the rats initially received drug injections on a 24-hour schedule, and Experiment 2, in which the rats only received injections on the 31-hour schedule (Figure 5 and Figure 6). For fentanyl, the differences in the percentage of activity were not significant for the PRE period, $t(9.272) = -1.331$, $p = 0.215$, the POST period, $t(11.212) = 1.043$, $p = 0.319$, or the ENS period, $t(10.802) = 0.612$, $p = 0.553$.

For haloperidol, there was no significant difference in the percentage of wheel running between Experiments 1 and 2 for the PRE period, $t(13.831) = 0.039$, $p = 0.970$, or the ENS period, $t(8.429) = -1.094$, $p = 0.304$. However, the percentage of wheel running in the POST period was significantly higher in the rats that initially received a 24-hour haloperidol injection schedule (Experiment 1), $t(11.360) = 2.738$, $p < 0.05$.

Discussion

The results of these experiments showed that a range of fentanyl dosages administered on a 24-hour schedule readily entrain wheel-running activity to the administration time. A pre-fentanyl anticipatory activity bout emerges 1–2 hours prior to injection time, and a post-fentanyl evoked response lasts for approximately 6 hours after administration. Heightened

activity is apparent before and after the administration time for 2–3 days when fentanyl is not administered on test days.

When fentanyl is administered on a 31-hour schedule, the post-drug evoked response remains relatively intact but is blunted in comparison to the post-drug response on a 24-hour administration schedule. This indicates that fentanyl-induced circadian entrainment of activity may inflate the effects of fentanyl, and this may partly explain locomotor sensitization to long-term (24-hour) fentanyl administration. In contrast to the 24-hour fentanyl schedule, on the 31-hour fentanyl administration schedule, there is no evidence of anticipatory activity immediately prior to the injection time (at hours 29–31). Instead, an ensuing bout of activity is shown approximately 24 hours after each fentanyl injection that is similar (in the percentage of daily wheel running) to persisting activity on the first test day of the 24-hour injection series. This ensuing activity on the 31-hour injection cycle cannot be attributed to learning of the 24-hour interval, as rats that were only given injections on a 31-hour schedule (Experiment 2) showed similar activity levels in the ENS period to rats that were initially trained on a 24-hour schedule (Experiment 1).

In contrast to fentanyl, haloperidol does not produce entrainment of circadian activity episodes under these same conditions. While a post-drug suppression of activity is obvious in the actograms of rats administered haloperidol (Figure 2), there is no evidence of a consistent pre-haloperidol anticipatory episode. The expression of the post-haloperidol catalepsy appears to be due to the systemic levels of the drug, as haloperidol has been estimated to have a half-life of 14–26 hours [37]. The lack of persisting pre- and post-haloperidol episodes during the test days provides further evidence against entrainment. Activity in the pre- and post-drug periods on the test days appears to be entirely dependent on which part of the free-running rhythm falls at the time. While fentanyl administration left the free-running rhythms intact, haloperidol administration appeared to mask these rhythms, as they resumed at the expected phase 10–12 hours after the injection.

Interestingly, the post-drug evoked effects of haloperidol on a 31-hour schedule were found to vary depending on whether the rats initially received haloperidol on a 24-hour schedule (Experiment 1) or not (Experiment 2). Rats initially receiving 24-hour haloperidol injections had higher post-drug activity levels (and therefore more tolerance to the haloperidol-induced locomotor suppression) than rats that only received 31-hour haloperidol injections. These results appear to indicate that tolerance to the locomotor effects of haloperidol is facilitated by a 24-hour administration schedule. As in the 24-hour schedule, once the sedative/cataleptic effects of haloperidol waned, the free-running activity rhythm and baseline activity levels resumed until the next injection time. There was no evidence of either a pre-haloperidol anticipation (at hours 29–31) or an ensuing response on a circadian interval (at hours 22–27). However, 31-hour haloperidol administration did produce some unexpected results. Pre-haloperidol activity on the 31-hour schedule (hours 29–31) was significantly lower than pre-haloperidol activity on the 24-hour schedule (hours 22–24), and activity during the ensuing period on the 31-hour schedule was significantly greater than during the analogous periods on the 24-hour test days. These differences may reflect differential effects of administering haloperidol on schedules longer than 24 hours, or they may simply reflect different phases of the rest-activity cycle.

The masking of activity rhythms by haloperidol contrasts notably with the entrainment of activity episodes to fentanyl administration. The use of the term “entrainment” implies that the drug is setting the activity schedule, whereas masking typically means that the activity schedule is not affected despite the presence of the drug. However, fentanyl does not appear to be a “complete” zeitgeber of the rest-activity cycle, as free-running rest-activity rhythms (with reduced amounts of activity) are still readily apparent in the actograms of rats administered

fentanyl (Figure 1). So while fentanyl can entrain a large proportion of a rat's daily activity, it does not appear to have the ability to completely entrain the rest-activity rhythm as the zeitgeber of the light/dark cycle does.

The fentanyl-induced episodic entrainment observed in the present study is qualitatively similar to the activity entrainment produced by methamphetamine [36] and cocaine [81] under similar experimental conditions. Similar pre- and post-drug episodes have also been recorded for 24-hour schedules of subcutaneous nicotine injections [24] and orally self-administered alcohol [35], although these drugs have not yet been investigated on a 31-hour schedule. The characteristics of the post-drug evoked response vary considerably in magnitude and duration among these drugs. For example, post-fentanyl activity episodes last 3–4 hours, depending on dose, whereas post-methamphetamine episodes last 5–6 hours, post-cocaine episodes last 1–2 hours, and post-nicotine episodes last 2–3 hours. For fentanyl and other drugs of abuse, the expression of the post-drug activity episodes appears to be related to the presence of the drugs in the brain and/or bloodstream. Fentanyl has an elimination half-life of approximately 3.5 hours [27], which corresponds to the end of the post-fentanyl activity episode. Likewise, nicotine has an elimination half-life of 2–3 hours [8], cocaine has a half-life of approximately 30 minutes [55], and methamphetamine has a half-life of approximately 70 minutes in Sprague-Dawley rats [61].

However, given these drug clearance rates, the expression of pre-drug activity episodes does not appear to be related to systemic drug levels. Pre-drug activity episodes for all of these drugs of abuse consistently emerge 1–2 hours prior to the daily administration time, although the magnitude of the pre-drug activity bout varies with the drug dosage. Similarly, both the persistence of pre- and post-drug episodes on the 24-hour test days and the circadian ensuing activity in the 31-hour injection series do not appear to be due to the presence of the drug in the brain or periphery.

For fentanyl, the persistence of pre- and post-drug episodes into the 24-hour test days also does not appear to be related to withdrawal symptoms. The low, medium, and high doses of fentanyl administered in the present study are not associated with high levels of somatic withdrawal symptoms in rats 24, 48, and 72 hours after chronic fentanyl administration ceases [43]. Additionally, these fentanyl doses do not lead to elevated brain reward threshold levels, which are commonly correlated with the withdrawal symptoms of drugs of abuse.

The mesocorticolimbic dopamine system seems one obvious candidate for an anatomic origin of these effects. Both fentanyl and haloperidol are known to affect dopamine transmission in this system in opposing ways [28,53]. Further, this system is often cited as a common anatomical link for the motivational effects of different drugs of abuse [16]. However, circadian entrainment of wheel running is mainly associated with circadian increases in dopamine levels in the caudate nucleus of the nigrostriatal dopamine pathway, and is not associated with mesolimbic dopamine transmission [56]. In the present study, the persistence of fentanyl-entrained wheel running into the test days could be the result of entrainment of circadian rhythms of nigrostriatal dopamine transmission.

The use of the term “entrainment” to describe drug-induced circadian activity episodes implies the involvement of either the light- or food-entrainable oscillator. Evidence from the present study and previous studies more strongly favors perturbation of the food-entrainable oscillator. The pre-fentanyl anticipatory activity in this study resembles food-anticipatory activity (FAA) that emerges prior to circadian availability of a meal [46]. Further, both methamphetamine- and food-induced circadian activity episodes have been shown to persist in rats with lesions of the SCN (the light-entrainable oscillator) [49,72,75]. However, a recent study by Ángeles-Castellanos et al. suggests that entrainment to food is separable from entrainment to rewarding

stimuli [5]. These researchers found that entrainment to a daily chocolate reward was associated with *Per1* clock gene expression in the SCN and activation of several mesocorticolimbic structures, including the nucleus accumbens and the prefrontal cortex. Entrainment to a daily meal produced similar results, but was additionally associated with activation of several hypothalamic structures, including the dorsomedial hypothalamic nucleus. These results may indicate that the food-entrainable oscillator may be further subdivided into metabolism- and reward-related oscillators. If the so-called “drug-entrainable” oscillator responsible for entrainment of activity episodes to fentanyl, methamphetamine, and other drugs of abuse is equivalent to the purported “reward-entrainable” oscillator, this further supports mesocorticolimbic structures as the primary system responsible for the episodic entrainment effects of fentanyl in the present study.

Based on the results of the present experiments and previous studies in our laboratory, we propose four behavioral criteria necessary for a drug to be considered an episodic zeitgeber. When a drug is administered on a 24-hour inter-injection schedule, (1) a pre-drug anticipatory activity episode must be observed beginning 1–2 hours prior to the administration time, and (2) a post-drug evoked activity response must be apparent after the administration time. The magnitude and duration of the post-drug response will obviously vary among the different classes of drugs. On all but the first injection day, the post-drug episode consists of a combination of directly evoked drug effects and the persisting circadian activity episode entrained by previous administrations. (3) The combination of the pre- and post-drug circadian activity episodes will persist at an approximately circadian interval for multiple days when the drug is withheld. Finally, (4) when the same drug is administered on a 31-hour inter-administration schedule, ensuing activity should be visible approximately 24 hours after each injection, but no anticipatory activity is observed immediately prior to the injection time.

Acknowledgements

This research was supported by NIH/NIDA R01DA017692. The authors would like to thank Doug Toms, Mike Bailey, George Rebec, and Scott Barton for technical assistance, and two anonymous reviewers for their comments on a previous version of this manuscript.

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Figure 1.

Fentanyl groups wheel running actograms for the acclimation and 24-hour injection series. Mean wheel running for a representative rat from each dosage group is plotted separately: (a) Low Group (0.015 mg/kg), (b) Medium Group (0.03 mg/kg), and (c) High Group (0.06 mg/kg). The horizontal axis represents the time of day (midnight to 2359), and the vertical axis represents the study day with the first day of the study at the top and the last day of the study at the bottom. Fentanyl injection times are indicated with vertical gray lines. During acclimation (ACCL), free-running rhythms of approximately 26 hours were apparent for all groups (visible as “slanted” bouts). In the injection phases (FEN 1, FEN 2, FEN 3), activity episodes eventually became entrained to the injection time, and is visible as “vertical” activity

bouts before and after the administration time. This entrained activity persisted into the test phases (Test 1, Test 2, Test 3) when injections were withheld, most notably during Tests 2 and 3.

Figure 2.

Haloperidol groups wheel running actograms for the acclimation and 24-hour injection series. Wheel running is plotted separately for a representative rat from each dosage group: (a) Low Group (0.25 mg/kg), (b) Medium Group (0.5 mg/kg), (c) High Group (1.0 mg/kg). Haloperidol injection times are indicated with vertical gray lines. During acclimation (ACCL), free-running rhythms were apparent for all rats. In the injection phases (HAL 1, HAL 2, HAL 3), a post-drug sedative response was apparent for 10–12 hours after administration. However, this post-drug response did not persist into the test phases (Test 1, Test 2, Test 3) when injections were withheld. No pre-drug anticipation of haloperidol was observed in this study.

Figure 3.

Mean wheel counts in hourly bins for the three fentanyl dosage groups. Average wheel running is depicted across the 24 study days when fentanyl injections were administered (FEN1, FEN2, and FEN 3 – see Figure 1) in Figure 3a. The pre-drug anticipatory activity bout and the post-drug evoked response are visible before (PRE) and after (POST) the administration time (0) in all dosage groups. Figures 3b, 3c, and 3d depict the average wheel running on each test day

averaged across the three tests of the study (e.g. Test 1, day 1; Test 2, day 1; Test 3, day 1) when injections were withheld. Heightened activity persisted around the fentanyl injection time (0) for 2–3 days in all fentanyl dosage groups.

Figure 4.

Mean wheel counts in hourly bins for the three haloperidol dosage groups. Average wheel running is depicted for the 24 study days when haloperidol injections were administered (HAL1, HAL2, and HAL3 – see Figure 2) in Figure 4a. The post-drug evoked response is visible after (POST) the administration time (0) in all dosage groups. A pre-drug anticipatory activity bout was not present prior to the administration time (PRE). Figures 4b, 4c, and 4d

plot the average wheel running on each test day averaged across the three tests of the study (e.g. Test 1, day 1; Test 2, day 1; Test 3, day 1) when injections were withheld. Activity on the test days was not consistent throughout the study for rats administered haloperidol, and appeared to be dependent on where the free-running activity cycle fell on each particular day.

Figure 5.

Mean hourly wheel counts for rats administered fentanyl during the 31-hour injection series (12 injections over 15 days). Figure 5a depicts rats in the three different fentanyl dosage groups that initially received injections on a 24-hour schedule (see Figure 1 and Figure 3). Figure 5b shows rats that only received fentanyl on a 31-hour schedule. Post-drug activity episodes (POST) on the 31-hour schedule were similar in duration to post-drug episodes on the 24-hour schedule, but had a significantly lower magnitude. Pre-drug anticipatory episodes were not present immediately prior to the injection time (PRE) on the 31-hour schedule, but a 4–6 hour bout of ensuing activity was consistently recorded 23–26 hours after the injection (ENS) on the 31-hour schedule. No significant differences were found between rats that initially received 24-hour fentanyl injections (a) and rats that only received 31-hour fentanyl injections (b).

Figure 6.

Mean hourly wheel counts for rats administered haloperidol during the 31-hour injection series (12 injections over 15 days). Figure 6a depicts rats in the three different haloperidol dosage groups that initially received injections on a 24-hour schedule (see Figure 2 and Figure 4). Figure 6b shows rats that only received haloperidol on a 31-hour schedule. Depending on dosage, post-drug suppression (POST) of wheel running activity lasted 6–12 hours, which was comparable to the 24-hour injection series. No consistent, separable bouts of activity or inactivity were recorded in either the PRE period (29–31 hours post-injection) or the ENS period (23–26 hours post-injection) for rats that received haloperidol. As with fentanyl, no significant differences were found between rats that initially received 24-hour haloperidol injections (a) and rats that only received 31-hour haloperidol injections (b).

Figure 7.

Fentanyl groups wheel running actograms plotted on a 31-hour scale for the 31-hour injection series and baseline phases preceding and following the injection series. Mean wheel running for a representative rat from each fentanyl dosage group in Experiments 1 and 2 is plotted separately: (a) Low Group (0.015 mg/kg), (b) Medium Group (0.03 mg/kg), (c) High Group (0.06 mg/kg), and (d) 31-Hr Group (0.03 mg/kg). The horizontal axis represents the time relative to the injection, and the vertical axis represents the 31-hour “day” with the earliest day at the top and the last day of the study at the bottom. Fentanyl injection times are indicated with thick vertical gray lines (at hour 0), and 24 hours after the injection time is marked with a thin gray line. On the 31-hour schedule, pre-fentanyl activity episodes were not observed (hours -2 to 0), but ensuing activity episodes were observed approximately 22–27 hours after each fentanyl injection.

Figure 8.

Haloperidol wheel running actograms plotted on a 31-hour scale for the 31-hour injection series and baseline phases preceding and following the injection series. Mean wheel running is plotted separately for a representative rat from each haloperidol dosage group in Experiment 1 and Experiment 2: (a) Low Group (0.25 mg/kg), (b) Medium Group (0.5 mg/kg), (c) High Group (1.0 mg/kg), and (d) 31-Hr Group (0.5 mg/kg). Haloperidol injection times are indicated with vertical gray lines (at hour 0), and a thin gray line marks 24 hours after the injection time. In contrast to fentanyl, neither pre-haloperidol anticipatory episodes nor ensuing episodes were observed on the 31-hour injection schedule.