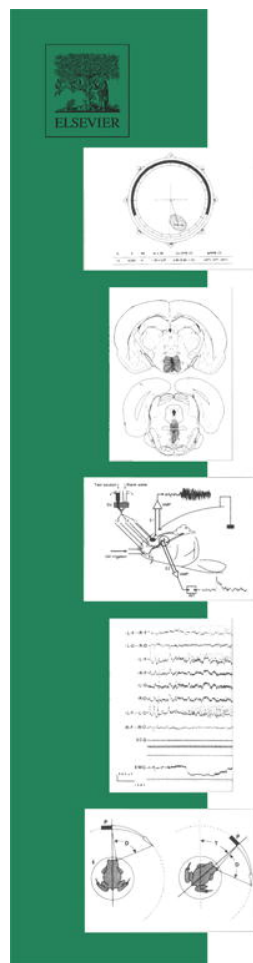


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## Pre- and post-nicotine circadian activity rhythms can be differentiated by a paired environmental cue

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### Abstract

Previous studies have shown that addictive drugs presented daily at fixed times produce circadian (oscillator-driven) anticipatory and evoked activity rhythms in rats. Other studies have shown that environmental cues paired with addictive drugs produce tolerance to drug effects and elicit craving behavior when presented without the drug. The present study tested these circadian entrainment and paired-cue conditioning effects together. This study compared the ability of daily nicotine and saline injections at different fixed times to entrain pre-injection (anticipatory) and post-injection (evoked) circadian activity rhythms in two groups of female Sprague–Dawley rats. One group (Paired) had an environmental cue (a tone) paired with the effects of the nicotine injection, and the second group (Unpaired) had the tone paired with the effects of the saline injection. The rats were housed singly for 56 days in chambers with attached wheels under constant dim light and rate-limited food access. During three separate injection phases, nicotine and saline were administered daily at different fixed times, and the tone was presented at the second injection time. Three multi-day test phases examined circadian activity (a) without injections or tone, (b) with the tone alone at normal and novel times, and (c) with the tone absent and with injections occurring at normal and at novel times. The results showed that nicotine entrained both pre- and post-injection circadian oscillators, and the nicotine-paired tone interfered with pre-injection anticipatory activity.

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*Keywords:* Nicotine; Drug abuse; Circadian rhythms; Pavlovian conditioning; Environmental cue; Locomotor entrainment; Drug anticipation

### 1. Introduction

Drug addictions are characterized by compulsive seeking and self-administration behaviors [1]. These behaviors have been established both in humans and in animal models [2,3]. Pavlovian conditioning to environmental cues that predict drug effects appears to play a significant role in the acquisition and maintenance of drug addictions [4–7]. For example, environmental cues repeatedly paired with drug administrations have been shown to produce drug-related behavior in the absence of that drug [8,9]. The phenomena of anticipatory responses, drug tolerance, withdrawal effects, and relapse behavior all appear to be related to conditioned responses to drug-paired cues [8,10,11].

Circadian rhythms also appear to play a role in drug addiction. For example, both circadian and circannual rhythms

have been observed in emergency room admissions for drug overdoses [12]. Studies with genetically altered mice have shown that the circadian clock gene *Per* has an important role in regulating behavioral responses to cocaine [13] and ethanol [14–16], and the effects of these drugs vary when administered at different times of the day [17,18]. Further, several addictive drugs also have been shown to affect the patterns of endogenous circadian rhythms. For example, a single dose of MDMA can cause long-term changes in sleep and motor activity patterns [19], and ethanol [17], morphine [20], and Phenobarbital [21] alter daily patterns of body temperature and activity.

Injections of methamphetamine and cocaine create circadian anticipatory behavior when administered on schedules with either circadian (24 h) or infradian (>24 h) periods [22–24]. This circadian drug anticipatory behavior is typically characterized by an increase in locomotor activity beginning approximately 22 h after administration, an effect that resembles circadian food anticipatory activity [25]. For both methamphetamine and food, these circadian anticipatory patterns appear to be independent of

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the light/dark cycle and the influence of the suprachiasmatic nucleus [26,27].

The present study examined behavior produced by injections of nicotine, a highly addictive psychomotor stimulant [28]. A number of studies have shown that environmental cues associated with nicotine administration are an important component of nicotine addiction in animal models [29–32]. These conditioned cues contribute to the reinstatement of extinguished self-administration and drug seeking in both humans and rats [33–35].

There is also evidence that circadian activity rhythms contribute to nicotine addiction. Several nicotine effects can be enhanced or attenuated depending on the time of administration [36,37], and nicotine clearance in humans shows circadian variation [38]. Nicotine intake also affects several endogenous circadian rhythms, including meal patterns [39,40], sleeping patterns [41], body temperature [42], heart rate [43], blood pressure [44], stress [45], and mood [46–49]. Additionally, daily nicotine injections induce the expression of the immediate early gene *c-fos* in the suprachiasmatic nucleus [50], which is a common index of circadian entrainment.

The present study asks whether (1) daily nicotine injections can entrain circadian activity rhythms and (2) the presence of a conditioned environmental cue will affect the expression of these rhythms. Based on the results of previous studies with methamphetamine and cocaine, we expected to see both pre-injection anticipatory activity (wheel running and drinking) and a post-injection activity spike persisting for at least two days when injections were withheld [22–24] in the Circadian Effects Test (Test 1) and the Probe Test (Test 3). We also expected the nicotine-paired tone, when presented without the nicotine injections in the Stimulus Effects Test (Test 2), to elicit activity increases comparable to post-injection activity spikes [7].

Rats show circadian rhythmicity in feeding, drinking, and locomotor activity, and these rhythms are entrained by two anatomically separable oscillators: a food-entrainable oscillator and a light-entrainable oscillator [51–56]. We used conditions of constant dim light and rate-limited feeding [57] to isolate circa-

dian activity rhythms associated with nicotine and to negate the influences of the zeitgebers of light/dark cycles and large meals. Under constant light, circadian activity rhythms typically free run with periods greater than 24 h [58,59], and in most female rats the estrous cycle is suspended [60]. We used female rats instead of males because of the length of the study, as wheel running in males tends to decrease with age over the time period of this study [61,62]. However, a study of cocaine-induced circadian rhythms in male rats found entrainment similar to that found in our previous studies of methamphetamine-induced rhythms in female rats [24]. We used daily saline injections at a time different than the nicotine injections to clarify locomotor effects related to handling, the injection procedure, the presence of the experimenter in the room, and the tone paired with the nicotine injections.

## 2. Methods

Sixteen adult female Sprague–Dawley rats were obtained from the rodent colony in the Department of Psychological and Brain Sciences, Indiana University Bloomington. Rats were approximately 90 days old at the beginning of the experiment. For the duration of the study, all rats were housed in Wahmann wheel boxes in constant light with continuous access to two water bottles. Water bottles were filled and cage liners were changed as necessary, usually every 4–6 days. Feeding was rate-limited, with access limited to no more than two 97 mg pellets (NOYES Rodent Food Pellet, Research Diets, Inc.) every 5 min. Data collected included water bottle licks, food pellets consumed, and wheel turns. The data were recorded continuously in five-minute bins using the Med-PC IV program (MedAssociates, Inc.). The rats were randomly assigned to one of two experimental groups (Unpaired and Paired) so that each group consisted of eight rats. All experimental procedures were approved by the Bloomington Institutional Animal Care and Use Committee.

A solution of 0.9% NaCl (VWR Pharmaceuticals) was used for all saline injections. A 1.0 mg/ml (free base weight) nicotine

Table 1  
Schedule of study activities

Study phase	No. of days	Unpaired group injections		Paired group injections		Stimulus presented
		11:00	19:00	11:00	19:00	
Acclimation	6	–	–	–	–	–
Acclimation injections	8	Saline	Saline	Saline	Saline	–
Nicotine Phase 1	14	Nicotine	Saline	Saline	Nicotine	19:05–19:25
Test 1: circadian effects	3	–	–	–	–	–
Nicotine phase 2	7	Nicotine	Saline	Saline	Nicotine	19:05–19:25
Test 2: stimulus effects						
Normal time	2	–	–	–	–	19:05–19:25
Novel time	2	–	–	–	–	11:05–11:25
Nicotine phase 3	7	Nicotine	Saline	Saline	Nicotine	19:05–19:25
Test 3: probe test						
Novel time	1	Saline	Nicotine	Nicotine	Saline	–
Circadian effects	1	–	–	–	–	–
Normal time	1	Nicotine	Saline	Saline	Nicotine	–
Circadian effects	1	–	–	–	–	–
Final baseline	4	–	–	–	–	–

solution was used for all nicotine injections and was administered at a volume of 1.0 mg/kg. The dosage level for this study was chosen based on a conditioned place preference study [63], in which this amount of nicotine conditioned place preference, but not place aversion. Nicotine hydrogen tartrate powder (Sigma Pharmaceuticals, St. Louis, MO) was mixed with 0.9% NaCl solution and dilute NaOH solution and brought to a pH of approximately 7.0. Nicotine and saline solutions were refrigerated at approximately 4 °C when not in use.

The rats were housed in the wheel boxes for a total of 56 days that included a 14-day acclimation period, 40 experimental days, and a 4 day post-injection period (Table 1). The acclimation period was used to adapt the rats to the wheel boxes and handling by experimenters, and to establish baseline activity levels. During the first 6 days of acclimation, rats were handled only to record daily body weight data. For the last 8 days of the acclimation period, each rat received a subcutaneous injection of saline at 1100 and 1900. For the duration of the study, body weights were recorded at the first daily injection time (1100). On days when injections were not scheduled (including test days), the rats were briefly handled at approximately 1500 to record body weights.

The experimental period consisted of three nicotine injection phases (NIPs), each followed by a short test phase. The first nicotine injection phase (NIP1) followed the acclimation phase, and lasted 14 days. During this phase, Group Unpaired rats received daily injections of nicotine at 1100 and daily saline

injections at 1900. Group Paired rats received nicotine injections at 1900 and saline injections at 1100. For the duration of this phase, an auditory stimulus (sonalert tone, approximately 80 db) was presented from 1905 to 1925. Injections for Group Paired began promptly at 1900 and finished by 1905 so that the onset of the tone was 0–5 min after the injection. This is the optimal interstimulus interval for nicotine-induced locomotor conditioning, as reported by Bevins, Eurek and Besheer [64]. Presentation of the cue immediately after the injection presumably allows it to pair with the effects of the drug, and not with the aversive injection procedure. The first test phase (Circadian Effects) examined circadian oscillations in the PRE and POST periods of the nicotine and saline injections over a three day period. During this test phase the tone was not presented and no injections were administered.

The second nicotine injection phase (NIP2) lasted 7 days. The injections and the tone occurred at the same times as in NIP1. The second test phase (Stimulus Effects) was four days long, and was designed to test for the effects of the tone without the injections at the normal presentation time and at a novel time. For the first two days of this phase, the tone was presented at its normal time from 1905 to 1925. For the last two days, the tone was presented at a novel time from 1105 to 1125.

The third nicotine injection phase (NIP3) also lasted 7 days and was identical in procedure to the prior injection phases. The final test phase (Probe Test) lasted four days, and examined the

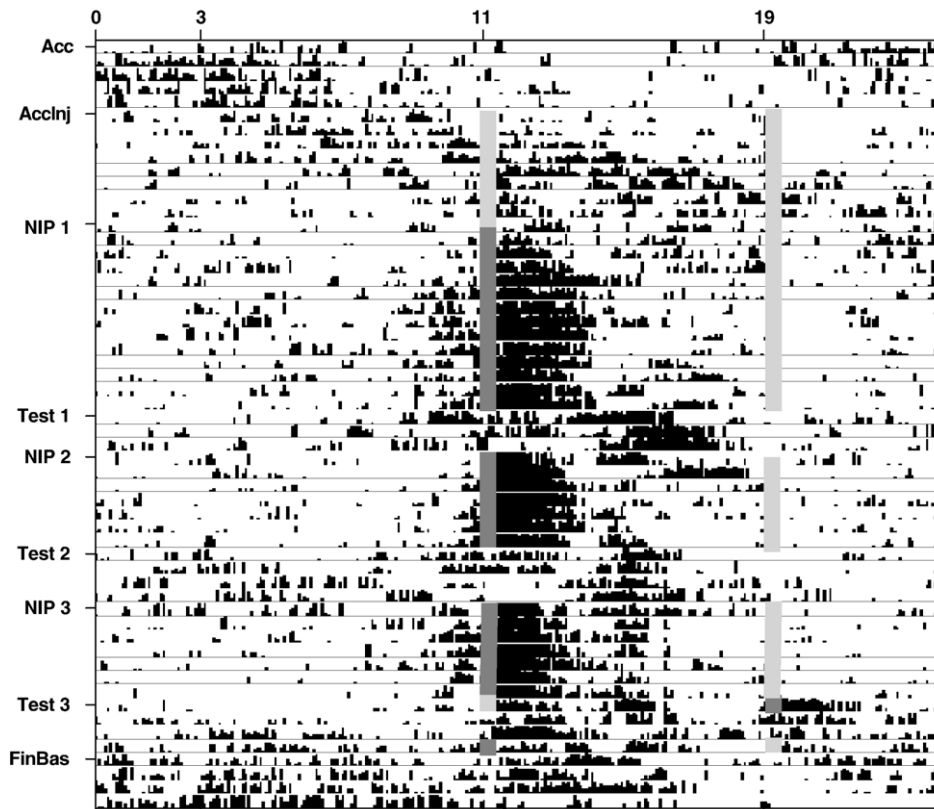


Fig. 1. Actogram of mean wheel running activity for Group Unpaired. Free-running activity occurred throughout the acclimation and acclimation injection phases (Acc and AccInj). During the nicotine injection phases (NIPs), wheel running entrained to the nicotine injection time (dark gray lines), but not to the saline injection time (light gray lines). Pre-injection anticipatory activity increases began approximately 2 h prior to the nicotine injection time. Post-injection activity spikes persisted for approximately 3 h after the nicotine injection. Both the pre- and post-injection activity increases persisted on the test days (Tests) when injections were withheld.

effects of the injections without the tone at normal and novel times. On the first day, the nicotine and saline injection times were switched for each group. On the third day, all rats received injections at the normal times. No injections were administered on the second and fourth days of this test phase.

Activity during the two hour period prior to an injection time was designated as pre-injection anticipatory activity (PRE). Activity during the three hour period immediately after an injection was designated as post-injection evoked activity (POST). Drinking and wheel running activity data were analyzed in the form of the original counts and as the percentage of total daily activity, which was calculated by dividing hourly activity by the total activity in that 24 h period. A square root transformation was performed on the wheel counts to normalize the data.

Statistical analysis was performed using SPSS version 13.0. Multivariate repeated measures tests were performed for the wheel running and drinking data. Group assignment and substance administered (nicotine or saline) were treated as between-subjects factors. Simple planned comparisons were performed to compare baseline data with subsequent days and/or phases. Pre-injection and post-injection activities were tested both as total counts and as the percentage of total daily activity. We compared data averaged over the last four days of the acclimation injection phase with data from the last four days of

the three nicotine injection phases. For each nicotine injection phase, data averaged over the last four days were compared with data from each of the test days immediately following.

### 3. Results and discussion

This section summarizes the effects of nicotine versus saline injections on wheel running and drinking during three successive injection phases (1, 2, and 3), and during the subsequent Tests 1, 2, and 3). For all statistical tests performed, similar results were obtained for all measures of entrainment (wheel counts, percentage of total daily wheel running, and drinking). Due to these similarities, only the results of the wheel count data analyses are included, except where noted.

#### 3.1. Acclimation and nicotine injection phases 1, 2, and 3

##### 3.1.1. Results

Actograms of mean wheel running activity for the 8 rats in Group Unpaired are displayed in Fig. 1, and for Group Paired in Fig. 2. Table 2 shows the period length of the activity cycles ( $\tau$  values) for the feeding, drinking, and wheel running rhythms for each phase of the study. During acclimation, rats in both groups had  $\tau$  values greater than 24 h for all measured rhythms,

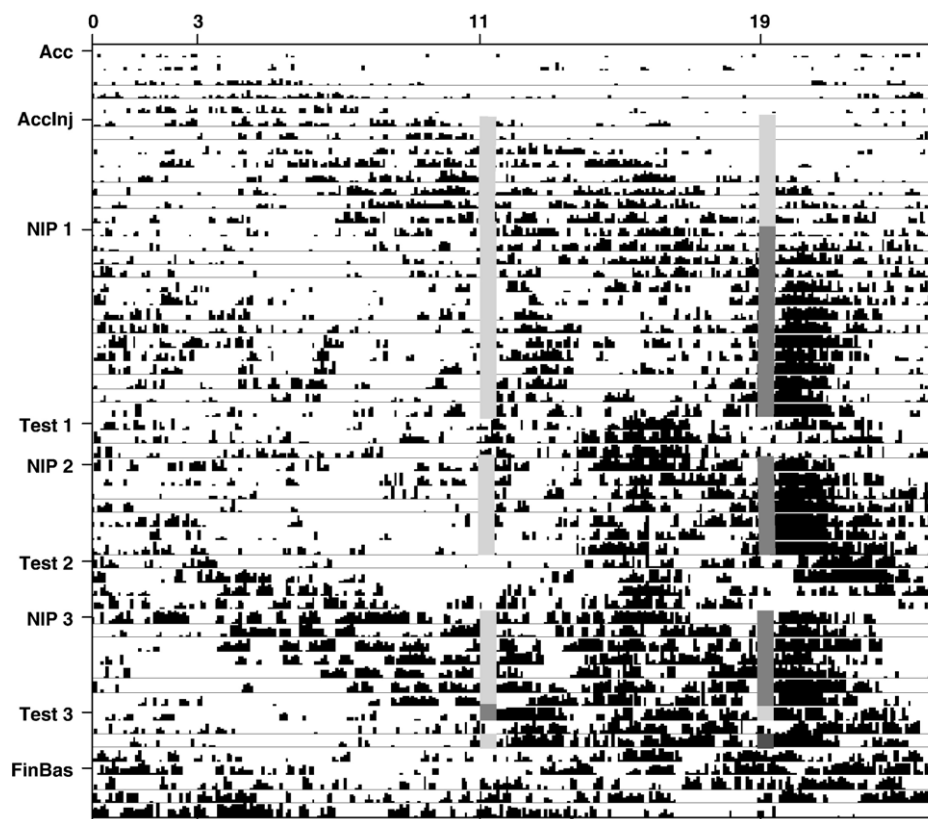


Fig. 2. Actogram of mean wheel running activity for Group Paired. Free-running activity occurred throughout the acclimation and acclimation injection phases (Acc and AccInj). During the nicotine injection phases (NIPs), wheel running entrained to the nicotine injection time (dark gray lines; saline injections are indicated with light gray lines), but free-running rhythms persisted throughout the entire study. The post-nicotine activity peak was slightly delayed in this group, but was similar in magnitude and duration to Group Unpaired, and persisted when nicotine injections were withheld on the test days (Tests). For this group, pre-nicotine activity was not greater than wheel running at other times of day.

Table 2

Period length of activity cycle ( $\tau$  value) for measured activities during study phases. Standard deviations are indicated in parentheses

Study phase	Unpaired group			Paired group		
	Wheel	Water	Food	Wheel	Water	Food
Acclimation	27.08 (2.71)	24.42 (1.03)	26.83 (2.76)	24.75 (0.69)	24.08 (1.39)	24.08 (2.57)
Acclimation injections	24.08 (3.04)	26.83 (1.38)	26.33 (1.35)	24.50 (3.39)	25.33 (2.51)	26.08 (3.13)
Nicotine phase 1	24.00 (2.85)	24.00 (2.63)	24.00 (3.28)	24.00 (2.77)	24.00 (3.64)	24.00 (3.09)
Test 1: circadian effects	24.92 (2.07)	24.25 (1.42)	24.00 (3.03)	24.33 (1.01)	24.58 (1.55)	24.67 (2.30)
Nicotine phase 2	24.00 (0.38)	24.00 (2.62)	24.00 (3.37)	24.00 (0.05)	24.00 (1.78)	24.00 (3.13)
Test 2: stimulus effects	24.17 (3.85)	27.25 (1.41)	24.00 (3.85)	24.50 (3.19)	24.75 (0.04)	24.75 (2.40)
Nicotine phase 3	24.00 (0.18)	24.00 (1.90)	23.92 (1.59)	24.00 (1.62)	24.00 (1.41)	24.00 (2.21)
Test 3: probe test	24.17 (3.57)	24.08 (2.83)	24.42 (4.16)	24.08 (3.26)	25.42 (0.95)	24.33 (3.70)
Final baseline	24.00 (1.39)	28.92 (2.29)	25.00 (1.96)	27.17 (2.41)	26.83 (1.54)	24.08 (1.31)

indicating the presence of free-running rhythms. During the nicotine injection phases,  $\tau$  values for both groups were slightly less than or equal to 24, which provides further evidence of circadian entrainment.

For the three-hour POST periods (following injections), activity levels associated with the saline injection times in both groups were significantly less than activity levels associated with the nicotine injection times (study phase:  $F(3, 26)=8.159$ ,  $p=0.001$ ; substance:  $F(1, 28)=12.267$ ,  $p=0.002$ ; phase-substance interaction:  $F(3, 26)=14.264$ ,  $p<0.001$ ). A large spike in activity occupied the first three hours after the nicotine injection in both groups, although Group Paired showed a locomotor response delay of approximately 20 min.

During the PRE period, both Group Unpaired and Group Paired showed an increase in activity that started approximately two hours prior to the nicotine injection time. However, while Group Unpaired had little activity outside of the PRE- and POST-nicotine periods, Group Paired showed a considerable amount of activity outside of the PRE- and POST-nicotine periods (see Fig. 2). Additionally, in the POST-saline period, Group Paired showed a significantly greater percentage of total daily wheel running than Group Unpaired (group-substance:  $F(1, 28)=31.191$ ,  $p<0.001$ ). A significant phase-substance interaction was also found for the PRE period ( $F(3, 26)=6.324$ ,  $p=0.002$ ).

### 3.1.2. Discussion

The results of this study showed a clear circadian activity rhythm during all nicotine injection phases. Both wheel running and drinking rhythms became entrained to the nicotine injection time for all rats. Activity in the PRE- and POST-saline periods was significantly lower than in the PRE- and POST-nicotine periods. A post-injection activity bout (POST) occurred after the nicotine injection in both Groups Paired and Unpaired throughout the study, and this bout generally lasted for 2 to 3 h.

While the two groups did not differ significantly in post-injection activity, pre-injection activity differed considerably. Group Unpaired, with no tone paired with their nicotine injections, consistently showed anticipatory wheel running and drinking prior to the nicotine injection time (PRE-nicotine). However, in Group Paired, activity in the PRE-nicotine period was not notably greater than in periods throughout the rest of the day.

### 3.2. Test 1: circadian effects

#### 3.2.1. Results

For all test phases and the final baseline phase,  $\tau$  values (period length) for both groups were greater than or equal to 24 h, indicating that, in the absence of injections, activity rhythms began to free-run (see Table 2). The percentage of total daily wheel running in the PRE and POST periods for nicotine injection phase 1 and the three days of the Circadian Effects Test are shown in Fig. 3 for Group Unpaired and in Fig. 4 for Group Paired.

The greatest amount of activity recorded on each day during Test 1 occurred at 1500, the time the rats were handled to record body weights. However, a considerable amount of activity was also recorded during the POST-nicotine periods in both groups that was greater than the activity recorded in the POST-saline periods (day:  $F(3, 26)=6.254$ ,  $p=0.002$ ; substance:  $F(1, 28)=5.205$ ,  $p=0.030$ ; day-substance interaction:  $F(3, 26)=4.518$ ,  $p=0.011$ ). Group Unpaired showed persistent activity during both the PRE- and POST-nicotine periods for all measures on days 1 and 2, but not day 3. Unlike Group Unpaired, Group Paired had little activity during the PRE-nicotine period on all three test days, but showed persistent activity during the POST-nicotine period on test days 1 and 2. Comparison of the two groups found significant differences only in the PRE-nicotine period (day-group:  $F(3, 26)=4.644$ ,  $p=0.010$ ) in this test phase.

#### 3.2.2. Discussion

During the first test phase, both the injections and the tone were withheld for three days to reveal circadian activity patterns. When nicotine was withheld, nicotine-entrained wheel running and drinking activity persisted for 2 days in the POST-nicotine period for Groups Paired and Unpaired. However, entrained activity in the PRE-nicotine period persisted only for Group Unpaired during this test phase. Group Paired did not show a notable rise in activity in the PRE-nicotine period during the injection phase, so this absence of activity is consistent with the previous pattern. Since the activity response to the nicotine injections in Groups Paired and Unpaired differed in the presence of a paired cue, this lack of anticipation in Group Paired appears to be due to a tone-induced interference with nicotine anticipatory activity.

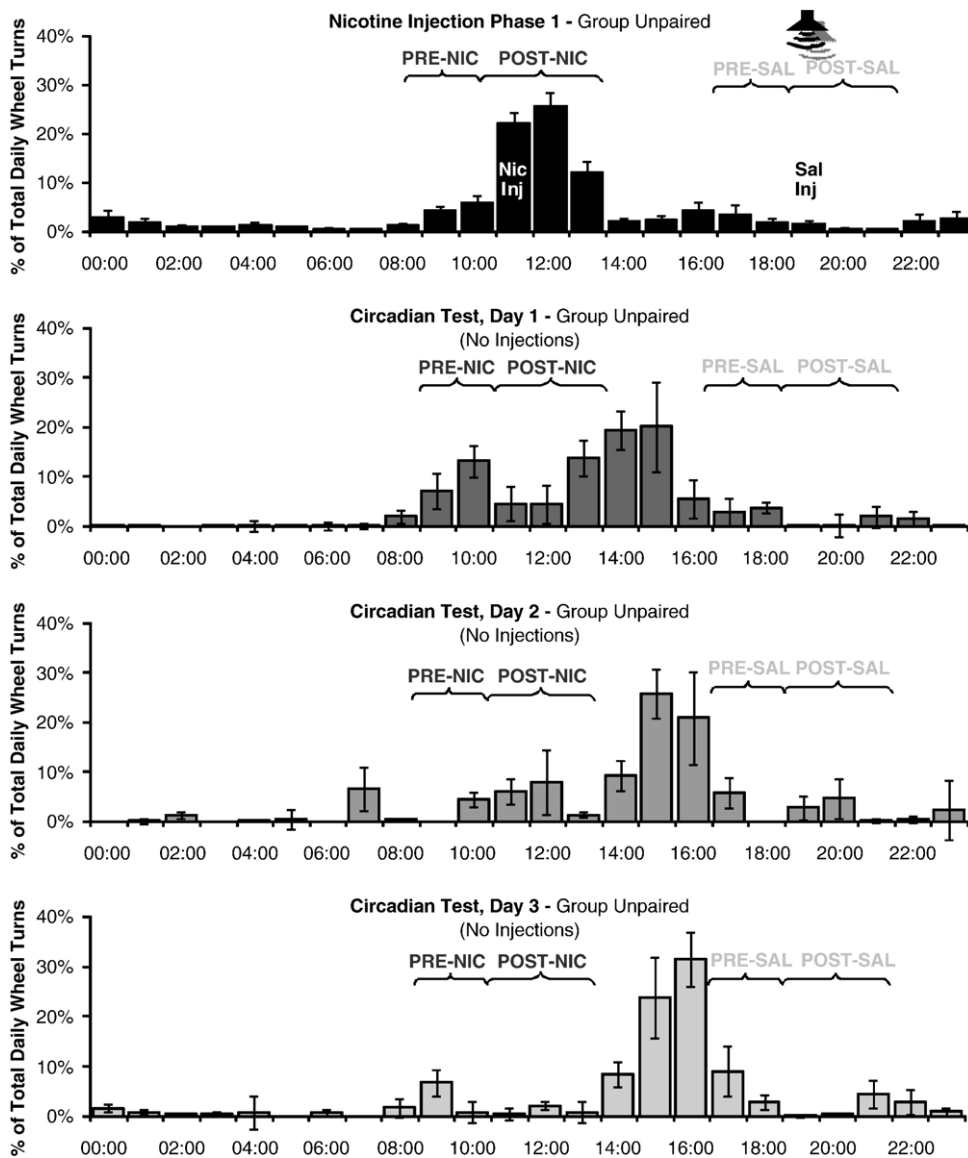


Fig. 3. Mean percentage of total daily wheel running across the 14 days of the first nicotine injection phase compared to the three days of the Circadian Effects test for Group Unpaired. During the test phase, no injections were administered and the tone was not presented, but the rats were handled briefly at 1500 to record body weights. Activity in the PRE- and POST-nicotine injection periods (PRE-NIC and POST-NIC) persisted for two days in this test. No significant activity was associated with the saline injection time (PRE-SAL and POST-SAL).

It should be noted that the greatest amount of activity recorded on the days of Test 1 (and on most test days throughout the study) occurred when the rats were handled to record body weights at a time that was not in close proximity to the nicotine and saline injection times. On test days, this handling was clearly an arousing stimulus for the rats. It is possible that the handling may have served as a predictive cue for the nicotine effects, but this is unlikely due to the lack of activity observed after handling for the saline injections throughout the study. The absence of an injection at 1100 on the test days may have had an activating effect for the rats, causing the effects of the handling at 1500 to be amplified. In future studies, it appears best that the rats not be handled at all for at least the first two non-injection days.

### 3.3. Test 2: stimulus effects

#### 3.3.1. Results

The percentage of total daily wheel running in the PRE and POST periods for nicotine injection phase 2 and the four days of Test 2 is shown in Fig. 5 for Group Unpaired and in Fig. 6 for Group Paired. Group Unpaired showed persistent activity in the PRE- and POST-nicotine periods on days 1 and 2, but no activity at these times on days 3 and 4. In the POST-nicotine period, Group Paired showed a locomotor response delayed approximately 20 min on the first three test days. No significant locomotor response was observed when the tone was presented at the novel time (the beginning of the POST-saline period) on days 3 and 4. In both the PRE and POST

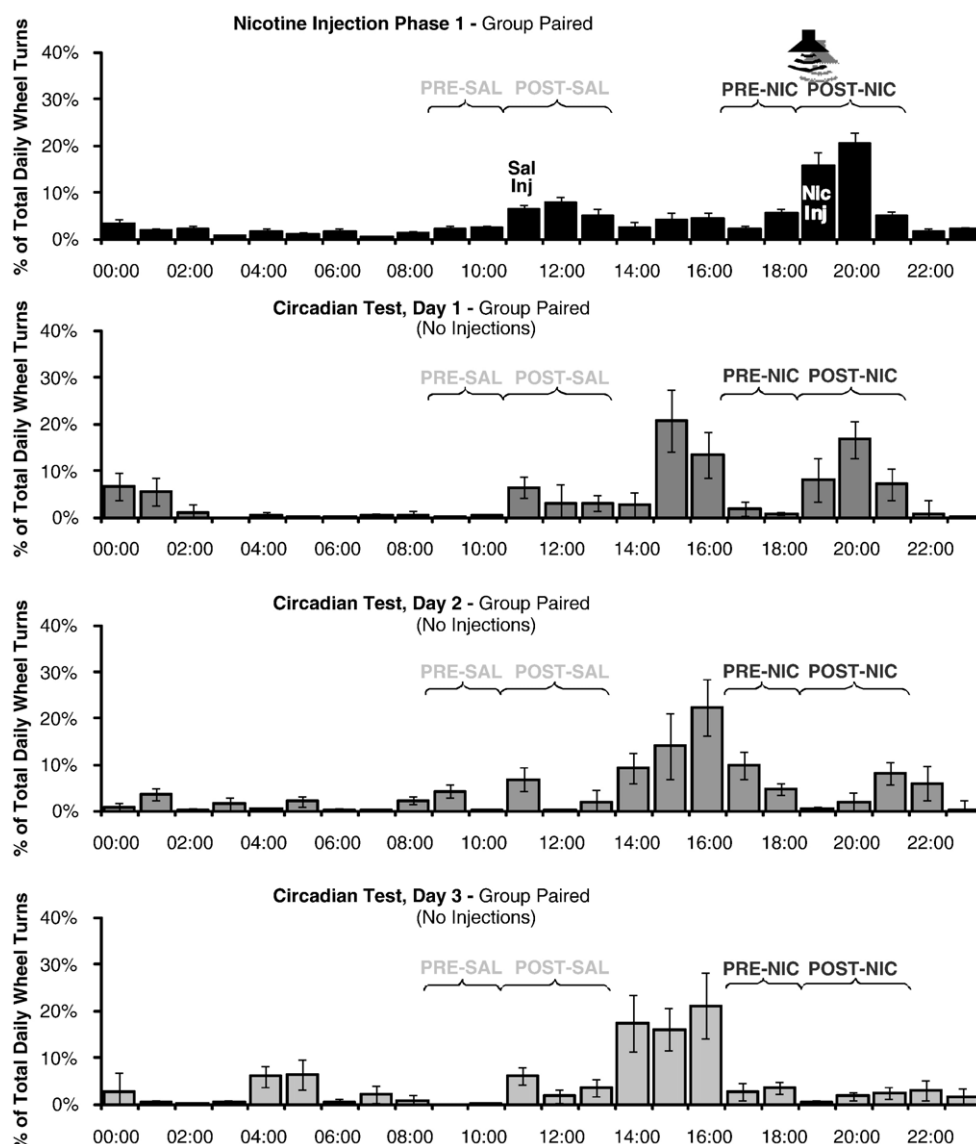


Fig. 4. Mean percentage of total daily wheel running across the 14 days of the first nicotine injection phase compared to the three days of the Circadian Effects Test for Group Paired. No injections were administered during this test phase and the tone was not presented, but the rats were handled briefly at 1500 to record body weights. Activity in the POST-nicotine injection period (POST-NIC) persisted for two days in this test. No significant activity was associated with the saline injection time (PRE-SAL and POST-SAL) or prior to the nicotine injection time (PRE-NIC).

periods, nicotine produced significantly greater activity than saline (PRE:  $F(1, 28)=6.243$ ,  $p=0.019$ ; POST:  $F(1, 28)=7.001$ ,  $p=0.013$ ), and a significant day-substance interactions (PRE:  $F(4, 25)=5.430$ ,  $p=0.003$ ; POST:  $F(4, 25)=7.637$ ,  $p<0.001$ ).

### 3.3.2. Discussion

Test 2 was designed to investigate the conditioned effects of the auditory cue. The tone was presented at its normal time for two days, then at a novel time (the earlier injection time) for an additional two days. No injections were administered during this phase.

Activity patterns in the pre- and post-injection periods in both groups were very similar to the activity patterns observed in the first test phase, although this second test phase showed a

greater peak of activity around the nicotine injection times. Group Unpaired showed persistent activity in the PRE- and POST-nicotine periods on the first two test days, and little activity at these times on the last two test days. Group Paired showed activity in the POST-nicotine period on the first two days, and also showed little activity during the PRE-nicotine period. This was consistent with the absence of activity in PRE-nicotine period in the first test phase and the second nicotine injection phase.

The results of this test showed that the effects of the tone were dependent on its relation to the nicotine administration time. Group Paired showed a robust locomotor response to the tone when it was presented at the normal nicotine injection time (POST-nicotine in Fig. 6). However, when the tone was presented at a novel time (beginning of the POST-saline period),

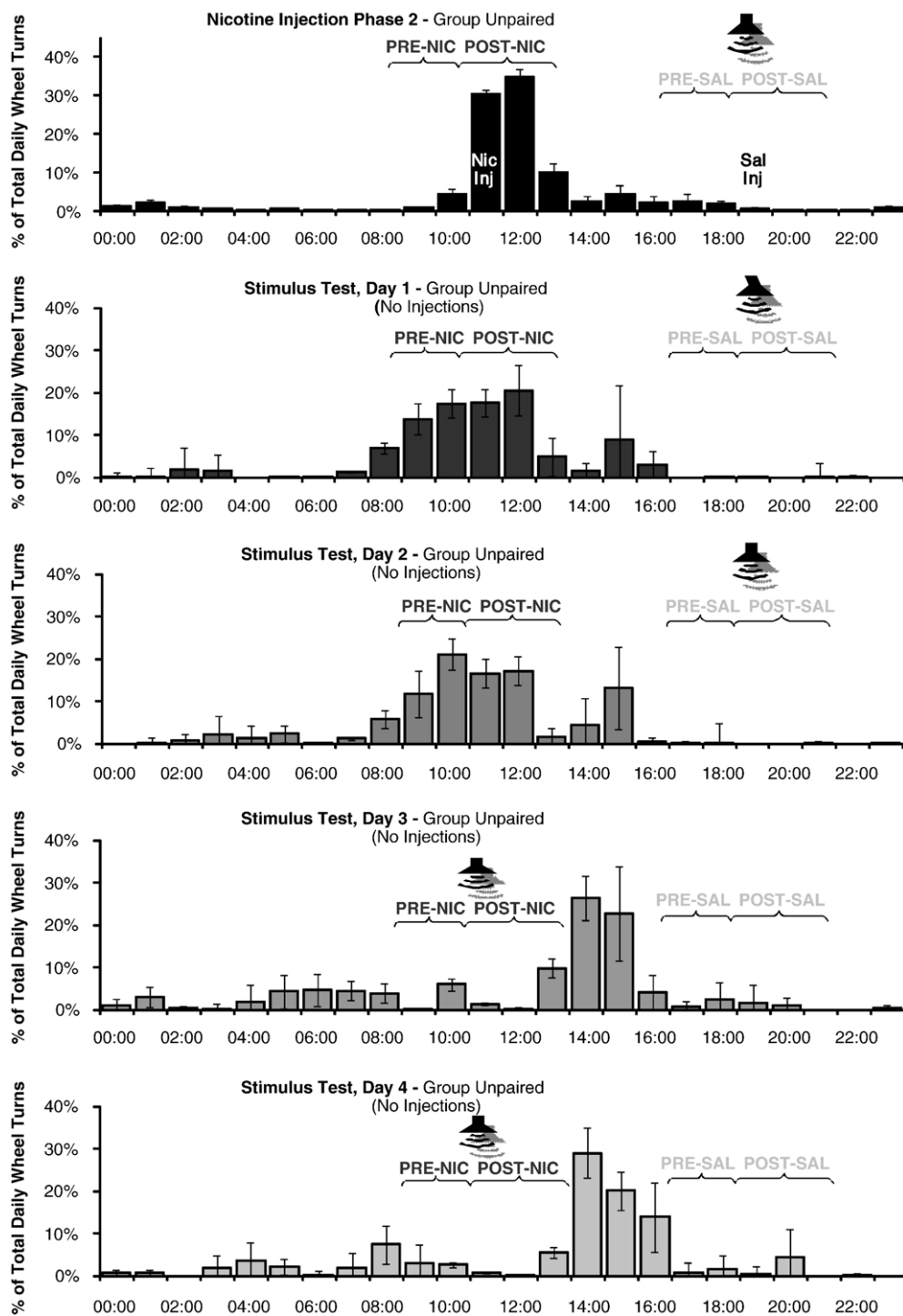


Fig. 5. Mean percentage of total daily wheel running across the 7 days of the second nicotine injection phase and the four days of the Stimulus Effects Test for Group Unpaired. The tone was presented at its normal time (1905–1925) on the first two days and at a novel time (1105–1125) on the third and fourth days. No injections were administered during this test phase, and as in the previous test phase, the rats were handled briefly at 1500 to record body weights. Activity in the PRE- and POST-nicotine periods (PRE-NIC and POST-NIC) persisted for two days. No noticeable activity was recorded in these periods when the tone was played on the last two days.

no evoked locomotor response was observed. Despite the earlier tone presentation, Group Paired also continued to show activity in the normal POST-nicotine period on day 3 of this test. This result indicates that the conditioned activity response to the paired tone was coupled to the time of administration, and cannot be evoked simply by presenting the tone at a different time of the day.

For Group Unpaired, the tone may have acted as a “negative” cue, signaling the absence of nicotine. During the nicotine injection phases, the tone was always paired with the saline injections (at the beginning of the POST-saline period) for this group. The complete lack of activity shown in response to the novel tone presentation (at the beginning of the POST-nicotine period) was indistinguishable from the amount shown in the PRE-saline and POST-saline

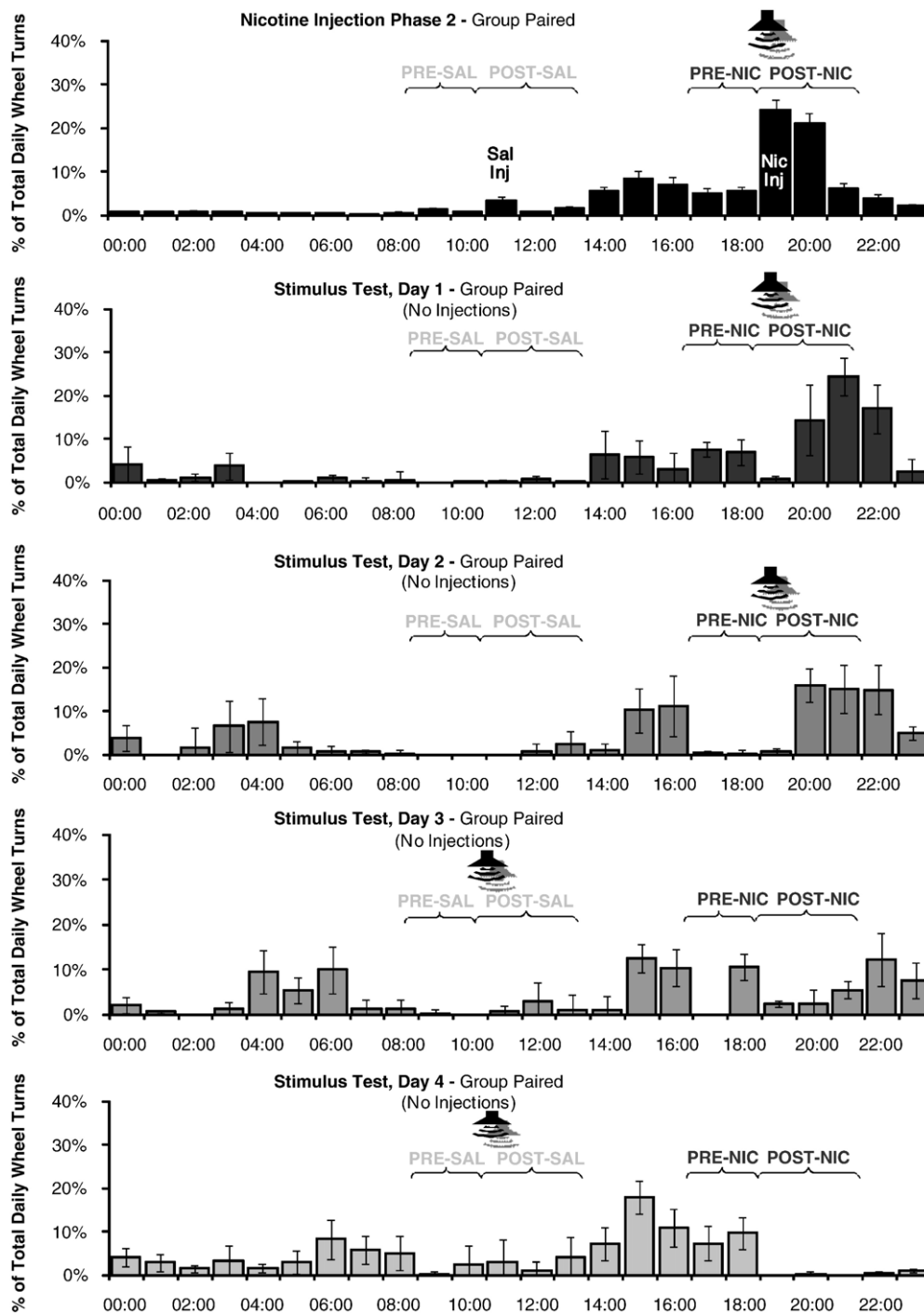


Fig. 6. Mean percentage of total daily wheel running across the 7 days of the second nicotine injection phase and the four days of the Stimulus Effects Test for Group Paired. The tone was presented at its normal time (1905–1925) on the first two days and at a novel time (1105–1125) on the third and fourth days. No injections were administered during this test phase, but the rats were handled briefly at 1500 to record body weights. Significant activity was evoked when the stimulus was played at the normal time, but not at the novel time.

periods. This occurred even though the time of the novel stimulus presentation was the normal nicotine injection time for this group. This may indicate that the cue was a negative predictor of nicotine and perhaps produced a conditioned inhibitory response not coupled to a specific time of day. However, all that we can say definitively is that it did not appear completely neutral for these rats.

### 3.4. Test 3: probe test

#### 3.4.1. Results

Fig. 7 shows the percentage of total daily wheel running in the PRE and POST periods during the third nicotine injection phase and the Probe Test for Group Unpaired, and Fig. 8 shows

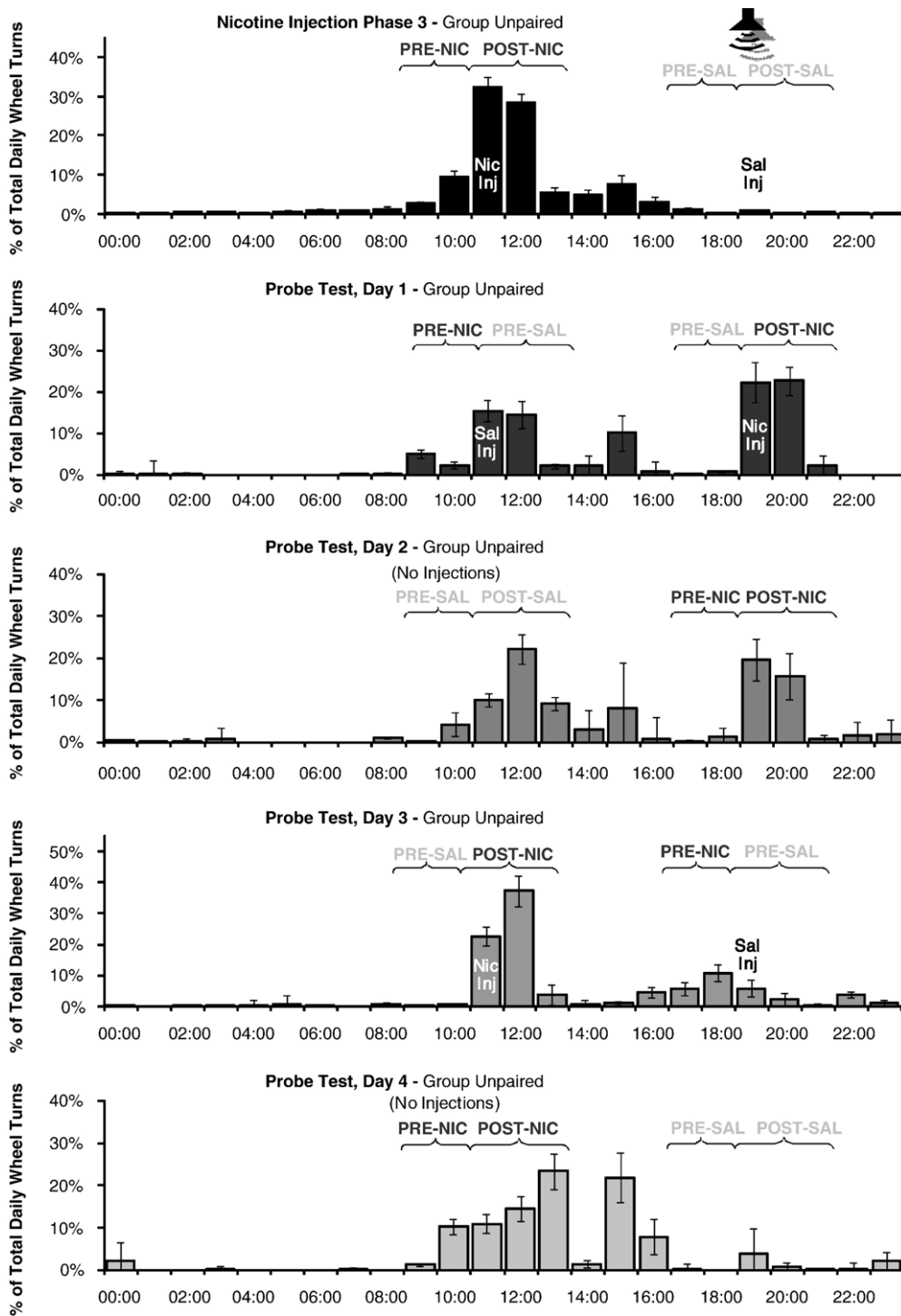


Fig. 7. Mean percentage of total daily wheel running across the 7 days of the third nicotine injection phase and the Probe Test for Group Unpaired. The nicotine and saline injection times were switched on day 1, and returned to the normal times on day 3. No injections were administered on days 2 and 4, and the tone was not presented throughout this test. On days 2 and 4, the rats were handled briefly at 1500 to record body weights. Notable activity was observed in the PRE- and POST-nicotine periods at the normal time, which was consistent with the rest of the study.

these data for Group Paired. For the PRE periods, a significant day-substance interaction was found ( $F(4, 25)=3.575, p=0.019$ ), but this interaction was not significant for the POST periods ( $F(4, 25)=2.316, p=0.085$ ).

On day 1, the nicotine and saline injection times were switched for all rats. Group Paired and Group Unpaired both showed a marked increase in wheel running activity in the new

POST-nicotine period. A considerable amount of activity was also observed in the new POST-saline period, but was not as large as the amount of activity normally associated with this time of day. Both groups also showed increased activity in the PRE-nicotine period, which preceded the saline injection on this test day. The results of the planned comparisons showed that activity in all periods on this test day were not significantly

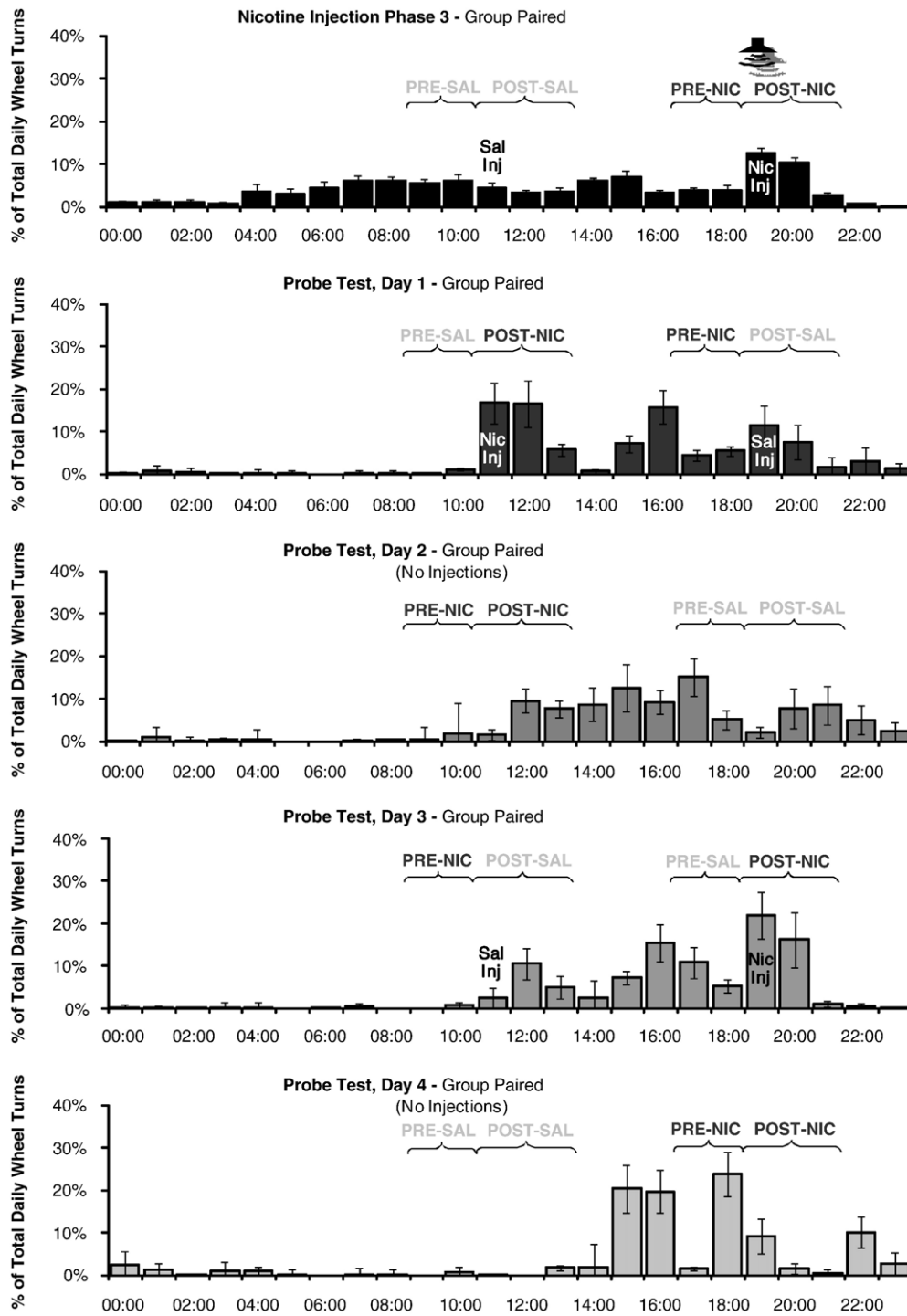


Fig. 8. Mean percentage of total daily wheel running across the 7 days of the third nicotine injection phase and the Probe Test for Group Paired. The nicotine and saline injection times were switched on day 1, and returned to the normal times on day 3. No injections were administered on days 2 and 4, and the tone was not presented throughout this test. On days 2 and 4, the rats were handled briefly at 1500 to record body weights. Activity was consistently observed in the PRE- and POST-nicotine periods at the normal time. Significant activity in this PRE period was not observed in this group during the nicotine injection phases when the tone was paired with the nicotine injection.

different from activity during the previous injection phase (nicotine injection phase 3).

On day 2, when no injections were administered, little activity occurred in the PRE-saline and PRE-nicotine periods established on test day 1. However, considerable activity was

observed in the novel saline and nicotine injection POST periods. The response to nicotine and saline in the PRE and POST periods differed significantly from the response to these substances in the previous injection phase, indicating that the pre-injection anticipation takes more than one day to reset

to a new time. This was confirmed by planned comparisons (PRE ( $F(1,28)=6.170, p=0.019$ ) and POST periods ( $F(1, 28)=4.506, p=0.043$ )).

On day 3, injections were administered again at the normal times, but without the paired tone. For both groups, activity was observed in the POST period for both nicotine and saline, but a larger amount of activity occurred after the nicotine injections. Some activity also occurred in the PRE period prior to the evening injections in both groups (the novel nicotine time for Group Unpaired, and the novel saline time for Group Paired). For the PRE period on this test day, compared to nicotine injection phase 3, planned comparisons showed a significant day-substance interaction ( $F(1, 28)=5.085, p=0.032$ ) and day-group-substance interaction ( $F(1, 28)=5.287, p=0.029$ ).

On day 4, when no injections were administered, Groups Paired and Unpaired showed little activity before or after the saline injection time, indicating again that saline does not entrain circadian activity. For both groups, significant activity was associated with the nicotine injection time in both the PRE and POST periods. Planned comparisons showed that activity in both POST periods on this test day were significantly different when compared to nicotine injection phase 3, illustrating that the circadian responses to the injections differed when the stimulus was not presented (day:  $F(1, 28)=23.011, p<0.001$ ; day-group:  $F(1, 28)=5.030, p=0.033$ ; day-substance:  $F(1, 28)=6.862, p=0.014$ ). Activity in the PRE periods for both groups on this test day also differed significantly from the nicotine injection phase for the percentage of total daily wheel running ( $F(1, 28)=5.558, p=0.026$ ) and for drinking ( $F(1, 28)=4.930, p=0.035$ ).

### 3.4.2. Discussion

The third test phase examined the effects of the time of administration and the different substances without the tone. The nicotine and saline injection times were switched on day 1. No injections were administered on day 2. On day 3, both substances were administered at the original times, and no injections were administered on day 4.

In contrast to the previous study phases, Groups Paired and Unpaired here showed similar results throughout this test phase in the absence of the tone. When the injections were administered at novel times on day 1, both groups showed activity in each POST period and in the PRE period prior to the saline injection (administered at the normal nicotine injection time). Group Paired also increased activity three hours prior to the novel saline injection time, so the activity in this period may not have been directly related to the normal nicotine injection time.

When both injections were withheld on day 2, a considerable amount of activity was recorded during the POST periods for both the nicotine and saline injections. There was little activity recorded in the PRE periods for either injection. Since the saline injections did not entrain activity throughout the study, the activity in the POST-saline period is likely due to the circadian effects of the nicotine injections two days prior. It could also possibly be coupled with the conditioning of handling cues related to the injection procedure. The lack of pre-injection anticipation for the novel nicotine injection time suggests that it takes more than one drug administration to develop an obvious level of

circadian anticipatory activity at a new time. However, given the large amount of activity observed in the POST-nicotine period, it follows that the post-injection activity rhythm needs only one administration to reset to a novel time.

Both groups showed similar responses when the two substances were administered again at the normal times on day 3. Small amounts of activity were recorded in the PRE-saline and POST-saline periods, and little activity was recorded in the PRE-nicotine period (which had previously been the novel saline injection time). As in the nicotine injection phases, the largest amount of activity was recorded in the POST-nicotine period.

On day 4, all injections were withheld again. Very little activity was recorded in the PRE-saline and POST-saline periods. Surprisingly, in both groups, a large amount of activity was recorded in both the PRE-nicotine and POST-nicotine periods. For Group Paired, this was the largest amount of pre-injection anticipatory activity observed throughout the study. The previous day (Test 3, day 3) was the only day in the study in which this group received a nicotine injection at the normal time without the paired tone. This accounts for the fact that on day 4 there was no statistically significant difference in activity between the two groups for the PRE-nicotine period. Until this point in the study, the two groups showed consistent differences in PRE-nicotine activity. The similarity in pre-nicotine activity levels shown here after a single unpredicted delivery of nicotine strongly supports our interpretation that the presence of the tone at the nicotine administration time interfered with the expression of pre-injection anticipatory activity.

## 4. General discussion and conclusions

In summary, these data indicate that the circadian locomotor activity pattern induced by a daily nicotine injection is actually divided into two parts based on two oscillator-like components: a pre-injection anticipatory activity bout and a post-injection evoked activity response, both of which persist on a circadian interval for at least 2 days when nicotine is withheld. The conditioned response to the tone is also coupled to the nicotine administration time, but the tone cannot reset the post-injection activity rhythm to a new time when presented without an injection.

Although it might seem simplest to hypothesize that the pre- and post-injection rhythms are controlled by the same circadian oscillator, the data from Group Paired (in which the injection is followed by a 20-minute tone) clearly argue that a separate oscillator controls each rhythm. In Group Paired, the post-injection evoked response appeared unchanged by the presence of the tone relative to the response shown by Group Unpaired. However, the pre-injection anticipatory bout was essentially eliminated by the reliable presence of the tone. The critical role of the tone in producing this pre-injection interference in Group Paired was confirmed in the Probe Test (Test 3, day 3) in which nicotine was administered in the absence of the tone at the normal nicotine injection time. On the following day, Group Paired showed a marked increase in pre-injection activity — a pre-injection activity level never previously shown by this group.

Although these data appear to demonstrate that nicotine injections entrain two (at least partly independent) circadian oscillator-

circuits, it is not clear whether the circadian pre-injection anticipatory timing circuit and the circadian post-injection evoked circuit are separable from the two currently acknowledged circadian oscillators: the light-entrainable oscillator (controlled by the suprachiasmatic nucleus) [65,66], and the food-entrainable oscillator (at least partly controlled by the dorsomedial hypothalamic nucleus) [52,67]. Previous studies have shown that a conditioned cue can reset the light-entrainable oscillator [68,69], although independent replication of this result has proven difficult [70].

Because the anatomical location of the putative food-entrainable oscillator was only recently established [52,67], the neurophysiological basis of the conditioning of drug entrained rhythms has not been explicitly related to food anticipation. However, it is worth noting that a study by de Groot and Rusak [71] showed a suppressive effect of a conditioned stimulus on circadian food anticipatory activity, an effect that appears similar to the suppressive effect of the tone on nicotine anticipation we reported in this study. This similarity raises the possibility, but in no way confirms that the pre-nicotine activity rhythm is mediated by the food-entrainable oscillator. Note that the circadian food-entrainable timing circuitry was available in this study because our use of a rate-limited feeding schedule prevented sufficient food intake at one time to entrain this circuitry [57].

Finally, it should be noted that repeated administrations of both higher and lower doses of nicotine than the one used in this study are known to produce sensitization and/or tolerance in a variety of physiological and behavioral responses, including locomotor activity [72–77]. Considering these data, it is very likely that sensitization and tolerance contributed to the circadian entrainment effects observed in the present study. However, despite considerable individual differences in total wheel running, the overall circadian entrainment patterns in this study were similar within each experimental group. Based on this consistency, we would expect similar entrainment patterns in experiments using other doses, sexes, and strains.

In particular, given typical assumptions about sensitization and tolerance, it would be expected that circadian anticipatory activity would be enhanced by the presence of a tone that predicts the effects of nicotine. Therefore, it is difficult to explain our finding that the presence of a tone paired with nicotine effects on one day suppressed circadian drug anticipatory activity on the following day. However, our data could be used to argue that sensitization and tolerance effects are also influenced by circadian timing cues, rather than controlled exclusively by environmental cues, as is commonly assumed.

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