Entrainment to a Long Daily Cycle Blocks Behavioral Sensitization to Cocaine

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ABSTRACT

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Behavioral sensitization refers to the increase of the behavioral activating effects of a drug following its repeated administration. Here I examined the effects of varying the length of the daily cycle on the expression of sensitization to cocaine. Rats were entrained to light cycles consisting of a variable dark phase and a fixed (0.5h), light phase (i.e., T cycles). One group was placed on a 24-h T cycle (T24) and another was placed on a 26-h T cycle (T26). Each group received cocaine (10 mg/kg) or saline for five days, at the same local time daily. Thus, injections were given at the same circadian time (CT) in the T24 but not the T26 group. Only the T24 group expressed sensitization. To assess whether injections given at varying CTs blocked sensitization in the T26 group, in a subsequent experiment the timing of the injections during pretreatment was delayed each day by two hours. Thus, the T26 but not the T24 group received injections at the same CT. Once again, the T26 group failed to express sensitization whereas the T24 group showed robust sensitization. In a third experiment, all rats were pretreated under a T26; following pretreatment half were switched to T24. Only animals switched to T24 expressed sensitization. Thus, entrainment to a long cycle prevents the expression rather than the induction of sensitization. These results open up a previously under-appreciated perspective on the influence that temporal features of the environment have on the behavioral effects of a drug.

Acknowledgements

First and foremost, I would like to thank my supervisor, Dr. Arvanitogiannis, as he has been an inspiration as well as an indispensable source of knowledge. It is through his persistent intellectual and moral support that this project was brought to fruition. I would also like to extend my gratitude to Dr. Amir and Dr. Waddington-Lamont for making my first research experience a decisive one. A special thanks goes to Dave Munro and Steve Cabilio for their constant technical assistance, as well as to Frank Rogan for always being there when most needed. In addition, this experience would not have been as gratifying had it not been for my colleagues and friends Patrick Augustyniak, Leora Yetnikoff, Rebecca Bassili and Carla Lipscombe. Last but not least, I would like to thank my family and friends for their constant love and support, especially to my love Jean-Philippe, my dad, my mom, John and my grandparents. This has been a truly enriching experience!

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The grave individual and societal consequences of drug addiction have led researchers to examine how drugs act on the brain to produce the behavioral manifestations that occur during addiction. The development of addiction is characterized by a progressive and sustained increase in the incidence and intensity of drug intake. Research using animals has led to the working hypothesis that the escalation in drug use results, at least in part, from drug-induced neural adaptations (Hyman & Nestler, 1996; Vezina, 2004) that increase responsiveness to certain drug effects (Deroche, Le Moal, & Piazza, 1999; Lorrain, Arnold, & Vezina, 2000; Robinson & Berridge, 1993; Stewart & Badiani, 1993; Vezina, 2004).

Repeated exposure to psychostimulant drugs, such as amphetamine and cocaine, produces enduring increases in their rewarding and incentive properties (Deroche, Le Moal, & Piazza, 1999; Grimm, Hope, Wise, & Shaham, 2001; Lorrain, Arnold, & Vezina, 2000). Accompanying the increase in the incentive motivational value of these drugs is the enhancement of their behavioral activating effects. This phenomenon, known as behavioral sensitization, develops gradually and persists months after the termination of drug treatment (Robinson & Berridge, 1993). Remarkably, the expression of behavioral sensitization has been shown, under certain circumstances, to depend on the

environment in which the drug is given. Most research has examined the role of spatial aspects of the environment in the expression of sensitization. Little is known, however, about the impact that temporal aspects of the environment have on sensitization. This thesis examines the effects of different entrainment cycles on the expression of behavioral sensitization to cocaine.

The neurobehavioral actions of psychostimulant drugs

An acute injection of a stimulant drug is followed by a host of behavioral and neurochemical effects. The reinforcing effects of stimulant drugs have been associated with their actions on the midbrain dopamine (DA) system, arising from ventral tegmental area (VTA) neurons and projecting to nucleus accumbens (NAcc) and prefrontal cortex (Kalivas & Duffy, 1990; Vezina, 2004; Wise & Bozarth, 1987). Specifically, stimulant drugs increase released synaptic DA, thus, extracellular DA concentrations, by blocking the dopamine (DA) reuptake system (Vezina, 2004). The rise in synaptic DA in response to an acute drug administration parallels and is thought to underlie the concomitant increase in behavioral activation (Kalivas & Duffy, 1990). In addition to acute psychomotor and neuropharmacological effects, stimulant drugs produce long-term changes in DA function and DA-mediated behavior (Kalivas & Duffy, 1990; Vezina, 2004; Wise & Bozarth, 1987).

Repeated exposure to stimulant drugs results in increased responsiveness of DA neurons to subsequent drug challenge. This increased responsiveness is manifested as enhanced drug-induced DA release in the NAcc (Kalivas & Duffy,

1990; Vezina, 2004; Wise & Bozarth, 1987). In parallel to neurochemical sensitization, repeated drug use produces plastic changes in DA-regulated circuits and even alters the morphology of neurons. Both amphetamine and cocaine treatment have been shown to structurally modify neurons in the NAcc and prefrontal cortex (Li, Acerbo, & Robinson, 2004; Robinson & Kolb, 1997). These neuronal alterations include lengthening of the dendrites as well as increases in the number of dendritic spines. Evidence for this is detectable months following the drug treatment (Robinson & Kolb, 1997).

Accompanying these structural and neurochemical modifications, repeated stimulant exposure results in enduring increases of the drug's incentive motivational properties as shown by increases in the propensity for drug-seeking and drug-taking behaviors. For example, psychostimulant pretreated rats have been shown to work harder to obtain the drug at a later time (Lorrain, Arnold, & Vezina, 2000). Specifically, compared with the control group, amphetamine pretreated animals will lever press more times to obtain each successive infusion of the drug when given the chance to self-administer amphetamine on a progressive ratio schedule of drug reinforcement (Lorrain, Arnold, & Vezina, 2000). In addition to the enhanced incentive value of psychostimulant drugs, repeated drug exposure sensitizes the behavioral activating effects of the drug (Robinson & Berridge, 1993). For instance, drug pretreated animals given a challenge injection of the drug exhibit heightened locomotor activation as compared to animals that receive the drug for the first time.

There is clear evidence that the increased behavioral effects of psychostimulant drugs after their repeated administration ensue from sensitization of the DAergic system (Kalivas & Duffy, 1990; Koob & Bloom, 1988; Lorrain, Arnold, & Vezina, 2000; Wise & Bozarth, 1987). However, in some occasions, the behavioral effects of the drug may be modulated by the environmental factors surrounding drug administration (Anagnostaras & Robinson, 1996; Badiani, Anagnostaras, & Robinson, 1995; Badiani, Camp, & Robinson, 1997; Jodogne, Marinelli, Le Moal, & Piazza, 1994). Below I summarize how different environmental factors interact with the effects of the drug on the brain to affect behavior.

Context-specific sensitization

Sensitization consists of two distinct stages: induction and expression. The induction or development of sensitization refers to the drug-induced neural adaptations that progressively bring about changes in behavior following exposure to stimulant drugs. The expression of behavioral sensitization relates to the heightened neurochemical and behavioral indices typically observed in response to a challenge injection of the drug in rats previously treated with the drug, as compared to saline pretreated animals. Although the action of stimulant drugs on their pharmacological targets in the brain is responsible for sensitization, the environment surrounding drug administration may, under certain circumstances, regulate both the expression and the induction of sensitization.

The induction of sensitization has been shown to depend on the environment in which the animals were previously exposed to the psychostimulant drug. Contrary to rats exposed to a stimulant drug in a novel environment, animals pretreated in their home cage fail to express robust behavioral sensitization (Anagnostaras & Robinson, 1996; Badiani, Anagnostaras, & Robinson, 1995; Badiani, Camp, & Robinson, 1997). The difference in the drug-induced behavioral response between the "home" and "novel" situations can only be eliminated by pretreating the home pre-exposed rats with higher doses of the drug (Browman, Badiani, & Robinson, 1998a, 1998b). This indicates that identical drug quantities may result in different neuroadaptive changes in the brain depending on the environment surrounding the psychostimulant drug's administration.

In addition to the influence of the environment on the induction of sensitization, the environment may also modulate or even prevent altogether the expression of sensitization (Anagnostaras & Robinson, 1996; Jodogne, Marinelli, Le Moal, & Piazza, 1994; Vezina & Stewart, 1984). That is, under certain circumstances, the expression of behavioral sensitization may come under the control of the environment. A case in point, is the failure to observe sensitization in animals that have been pretreated with the drug in a particular environment and then tested for sensitization in a different environment (Anagnostaras & Robinson, 1996; Jodogne, Marinelli, Le Moal, & Piazza, 1994; Vezina & Stewart, 1984). Even though previously exposed to the drug, and hence subjected to the drug's pharmacological actions, animals tested for the expression of sensitization

in a drug-unpaired environment react to a challenge injection of the drug similarly to drug-naïve animals. Interestingly, if the drug-pretreated animals are subsequently returned for testing in the drug-paired environment, they show robust sensitization (Anagnostaras & Robinson, 1996). Thus, the contextual stimuli surrounding drug intake are an important determinant of whether or not sensitization is expressed. Although there is no arguing about the significance of contextual cues on the development and expression of sensitization, little is yet known about the role of other environmental cues.

Time signals can also modulate sensitization

Animals make extensive use of not only physical but also temporal characteristics in the environment to plan their actions more effectively. After all, many opportunities and risks which animals face do not occur randomly with respect to time of day. The ability to time changes in the physical environment is intrinsically mediated by the circadian system. The biological clock at the core of this internal timekeeping system is vital in the quest for survival. It is through this self-sustained endogenous clock that species are able to coordinate both physiological and behavioral responses to environmental demands. Although the notion that the environment can influence the behavioral effects of a drug is widely accepted, very little attention has been given to the role that timing signals may have in modulating the effects of the drug on its presumed pharmacological targets in brain circuitry.

Timing signals have recently been implicated as important modulators of the neurobehavioral effects of drugs. For example, Arvanitogiannis, Sullivan, & Amir (2000) showed that the expression of behavioral sensitization to amphetamine can come under the control of time cues. A challenge injection of amphetamine produced a greater sensitized response when the injection was given at the same time as during pre-exposure to the drug. For this experiment, rats housed under a 12-h light-dark cycle (LD) were treated either with saline, early during the light part of LD (morning), and amphetamine, late during the light part of LD (evening), or with saline on both of these times. For testing, the saline control and amphetamine pre-exposed groups were divided into subgroups so that half of the rats from each group were tested in the morning and the other half in the evening. Only rats pre-exposed to amphetamine and tested for sensitization at the same time as during pre-exposure were found to be sensitized when compared to saline control rats.

Another recent study examined the effect on sensitization of receiving the drug consistently at the same time of day, as opposed to receiving the drug at inconsistent times across days (White, Feldon, Heidbreder, & White, 2000). The consistent timing was in relation to the 24-hour circadian cycle. Both groups got consistent periods between injections, but the inconsistent group received injections that were separated by 33 hours. Thus the injections were inconsistent with respect to the time in the light/dark cycle. When these groups were given a test for sensitization, only the 24-hour group showed sensitization. However, the failure of the 33-hour group to show sensitization is difficult to interpret. In this

group, not only were the injections inconsistent but also outside of the range of entrainment of the circadian clock (Jud, Schmutz, Hampp, Oster, & Albrecht, 2005; Madrid et al., 1998). These two issues may be somewhat confounded. As a result, it would have been better to have had a group that received inconsistent injections with respect to time of day, but within the range of entrainment of the circadian clock.

The first experiment reported in this thesis was designed so as to isolate the impact of an inconsistent schedule of drug administration on sensitization.

Experiment 1

This experiment assessed the effects of varying the timing of drug injections on sensitization. As such the experiment resembled that of White et al., (2000). However, although in the present experiment the schedule of drug administration was either regular or variable, the interval between injections was in each case held within the range of circadian entrainment.

This was accomplished by entraining rats to two different light cycles consisting of a long, variable, dark phase and a short, fixed (30 min), light phase (i.e., T cycles). One group was placed on a 24-h T cycle (T24) while another group was placed on a 26-h T cycle (T26). Both of these cycles are well within the limits of entrainment of the circadian clock (Jud, Schmutz, Hampp, Oster, & Albrecht, 2005; Madrid et al., 1998), but because injections were given every 24 hours, the two entrainment schedules differed in the opportunity they provided for predicting the time of the next drug administration. Thus, injections were consistent and predictable in the group entrained to the 24-h T cycle. Conversely, injections were always given at different circadian times in the group entrained to the 26-h T cycle.

If the expression of behavioral sensitization is blocked by inconsistent times of drug delivery, only the T24 drug group should exhibit sensitization on the test day.

Method

Subjects

The subjects were twenty-four experimentally naïve male Wistar rats from the Charles River breeding farms (St-Constant, Quebec). They weighed 375-450 g at the start of the experiment. Food and water were available ad libitum. The experimental procedures followed the guidelines of the Canadian Council on Animal Care. All experimental procedures had the approval of the Animal Care Committee, Concordia University, and all efforts were made to attend to the wellbeing of the animals used.

Apparatus

Upon arrival, the rats were housed temporarily in plastic cages (43.2 x 20.3 x 21.6 cm) with pine wood shavings lining the floor. At the beginning of the experiment all animals were transferred to separate plastic cages (50 x 26.8 x 36.4 cm) with wire mesh floors and equipped with a running wheel (34.5 cm in diameter, Nalgene, Rochester, New York). Waste pans filled with sawdust that could be removed for easy cleaning were located beneath the cages. Each cage was placed in a lightproof black melamine cabinet (66 x 66 x 44 cm) equipped with a ventilation system and a 4 W fluorescent light used to simulate a specific light-dark cycle. An IBM TCM50 512 computer controlled the lighting for each box. Activity data were transmitted from the running wheels to the computer via

a magnetic microswitch. Activity rhythms were recorded and analyzed using ClockLab software.

Once entrained, animals were transported in plastic buckets covered with black garbage bags from the running wheels to the locomotor activity boxes. The locomotor activity boxes were located in an adjacent dark testing room. Locomotor activity was measured in wooden boxes (43.2 × 22.2 × 30.5 cm) with Plexiglass front panels and wire-mesh floors. Two infrared photocells were evenly spaced along the longitudinal axis of the chamber (4.8 cm above the floor and 14.7 cm apart). The photocells were connected to a PC computer via an electrical interface that enabled the recording of locomotor activity.

Drug Administration

Cocaine hydrochloride (Medisca, Quebec) was dissolved in 0.9% saline. Animals were injected with 10 mg/kg of cocaine during pretreatment and 5 mg/kg of cocaine on the test day. Injections were administered intraperitoneally (i.p.).

Procedure

Acclimatization. All rats underwent a one-week acclimatization period during which they were handled daily. Throughout this week animals were housed in pairs in the animal colony and were kept on a 12:12-h light/dark cycle (LD).

Entrainment. At the start of the experiment all animals were relocated individually to the cages equipped with running wheels and had free access to food and water. Rats were then entrained to one of two lighting cycles. Half the animals were entrained to a 24-h T cycle (T24) while the other half were entrained to a 26-h T cycle (T26). Rats were housed in these cages for the remainder of the study. Food and water were regularly monitored and replenished as needed. Bedding in the waste trays was changed every week.

Pretreatment. Pretreatment began once the animals were entrained to their respective light cycle. Prior to being individually placed into the locomotor cages rats were weighed and subsequently injected with either cocaine (10 mg/kg, i.p.) or 0.9% saline at the same local time every day. Thus, the T24 group was pretreated at a consistent circadian time (CT) daily, more specifically at CT5, whereas the T26 group was pretreated at varying CTs, two hours earlier each day. At the end of each pretreatment session rats were returned to the running wheels until the subsequent session. Following the five-day pretreatment phase rats were left undisturbed until the test day.

Test for sensitization. To examine the behavioral expression of sensitization, two weeks after pretreatment, all animals were transported to the same activity boxes in which they were pretreated. Here, animals were injected with a challenge dose of cocaine (5 mg/kg, i.p.). The test injection was administered at CT5 for both the T24 and T26 groups. Locomotor activity was

recorded for 30 min parsed into successive 15-min time bins. One count of locomotor activity was defined as a consecutive interruption of each photocell.

Statistics

Behavioral sensitization to cocaine in each of the two groups was quantified as the difference in locomotor activation between cocaine- and saline-pretreated animals in response to a challenge dose of the drug. For each group, a separate mixed two-factor within-subjects design analysis of variance (ANOVA) was done on the locomotor activity counts in response to the cocaine challenge injection, with Pretreatment (drug and saline) as the between group variable and Time (15 and 30 min) as the within-subjects variable. In the presence of a significant Pretreatment × Time interaction, the effects of Pretreatment at each of the two time intervals were analyzed by two-tailed independent samples *t*-tests. All statistics were conducted using GB-STAT™ (6.5) for Macintosh. The significance value was set at $p < .05$. A Bonferroni correction was applied by dividing the alpha level (set at .05) by the number of *t*-tests conducted (two *t*-tests). The Bonferroni adjusted alpha level thus obtained was .025.

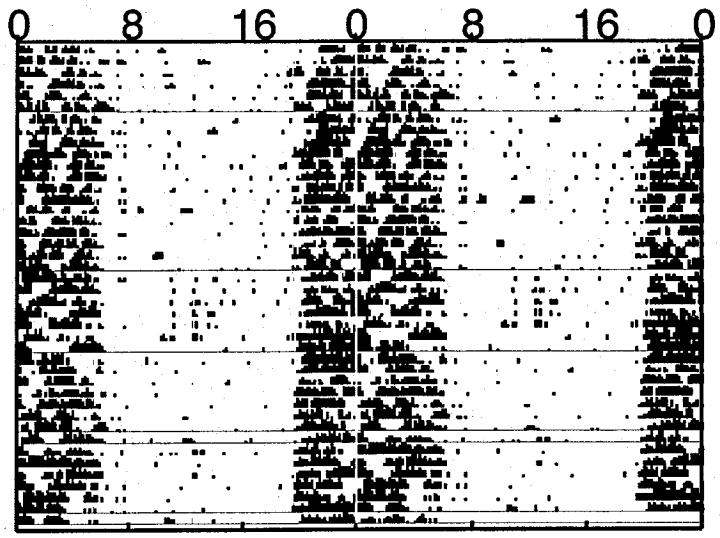
Results

Representative actograms showing the wheel running activity of subjects entrained to either a 24-h T cycle or a 26-h T cycle are shown in Figure 1. Figure 2 illustrates the mean locomotor activity counts obtained on the test for

sensitization in cocaine- and saline-pretreated animals from each of the two groups (T24 and T26). It can be seen that cocaine-treated animals entrained to a 24-h T cycle showed higher activity levels than their saline-pretreated counterparts, suggesting that this group expressed sensitization. By contrast, cocaine- and saline-pretreated animals entrained to a 26-h T cycle showed similar activity levels, suggesting that sensitization was blocked in this group. A mixed design ANOVA indicated a main effect of Pretreatment and Time for the T24 group, $F(1, 10) = 7.39$, $p = .02$, and $F(1, 10) = 35.35$, $p < .01$. No Pretreatment × Time interaction was obtained. In the T26 group, there was a significant interaction between Pretreatment and Time, $F(1, 10) = 7.94$, $p = .02$, but the results of t tests indicated that there were no significant differences in activity between cocaine- and saline-pretreated animals at neither the 15-min nor the 30-min time bins, $t(10) = 1.20$, $p = .26$, and $t(10) = -0.65$, $p = .53$, respectively.

Figure 1. Double-plotted actograms showing the daily wheel running activity of a subject entrained to a 24-h T cycle (top) and another subject entrained to a 26-h T cycle (bottom).

T24



T26

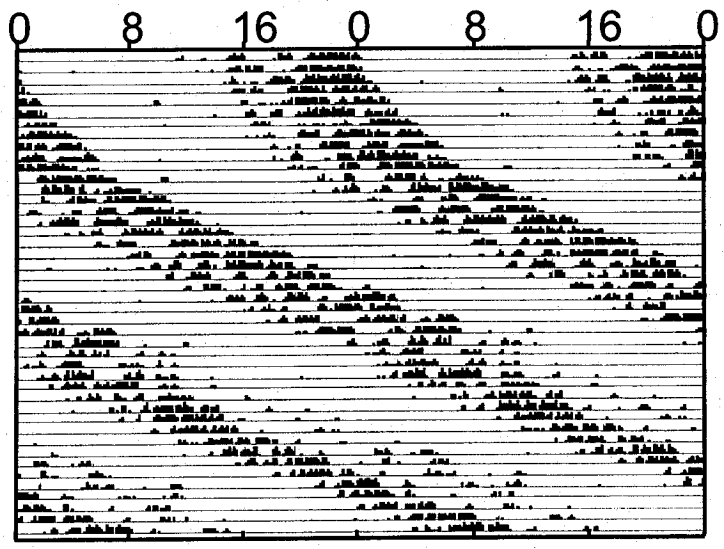
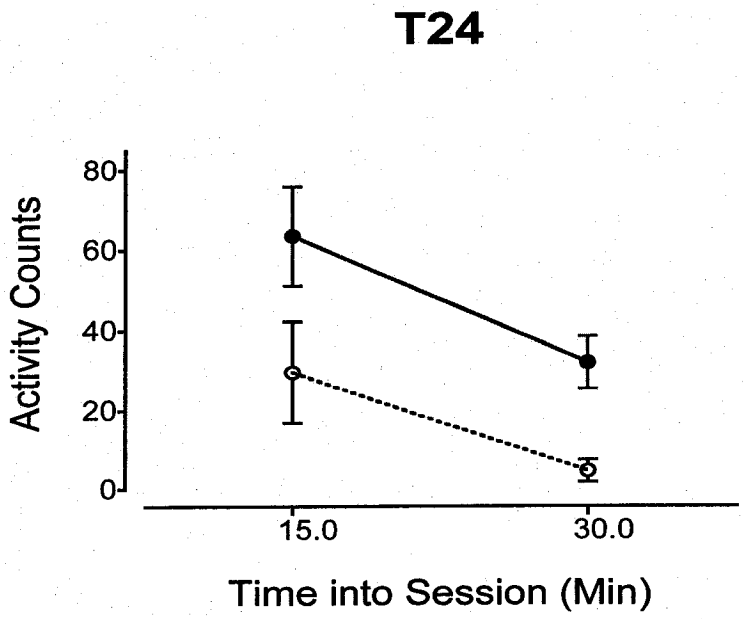
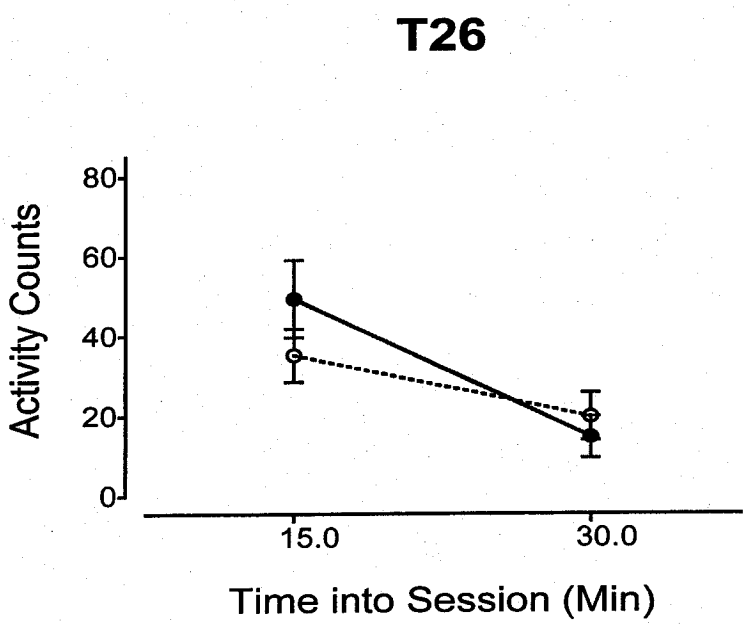


Figure 2. Mean locomotor activity counts for groups T24 and T26 on the test for sensitization. Filled and open symbols represent the cocaine- and saline-pretreated animals, respectively. Error bars represent SEM.





Discussion

The present data suggest that an inconsistent schedule of cocaine pre-treatment blocks the expression of behavioral sensitization. Sensitization was seen only in the group entrained to a 24-h T cycle. Animals in this group received their pretreatment at the same CT daily. In contrast, cocaine-pretreated animals in the group that was entrained to a 26-h T cycle and, thus, received the drug at different CTs daily, showed a similar behavioral response to that of drug naive animals in the test for sensitization. These results are in accord with the data reported by White et al. (2000) using a 33-h interval between cocaine injections. What the two studies have in common is that drug injections given at inconsistent CTs prevent the expression of behavioral sensitization. Taken together, these studies support the notion that an inconsistent drug pretreatment schedule prevents the expression of behavioral sensitization. The present study suggests that drug pretreatment at time intervals lying beyond the circadian range of entrainment was not the reason for the abolition of behavioral sensitization in the White et al. (2000) study. It would appear, instead, that the issue of whether the timing of cocaine delivery during the pretreatment phase is steady or variable is key to the observed modulation of the expression of behavioral sensitization.

However, this is not the only interpretation that can account for the results of Experiment 1. In addition to the difference in the consistency of the drug injections between the T24 and T26 groups, the two groups also differed with

respect to the length of the daily cycle. The rats that received inconsistent drug injections were entrained to a 26-h T cycle, a non-natural, experimentally simulated periodicity that differs by two hours from the 24-h periodicity seen in the natural environment. Whether or not entrainment to such an artificial daily cycle is somehow responsible for the failure to observe behavioral sensitization remains unclear. I conducted a second experiment to investigate directly this possibility.

Experiment 2

Results from a previous study indicated that contrary to animals pretreated with cocaine at the same time daily, rats pre-exposed to the drug at different times fail to express behavioral sensitization (White, Feldon, Heidbreder, & White, 2000). Experiment 1 revealed that following a challenge dose of cocaine, animals in the T26 group that were pretreated with the drug at different times each day, do not differ behaviorally from saline pre-exposed rats. However, the available data do not allow us to unambiguously assign the failure to observe sensitization to the inconsistent timing of drug delivery. Another possibility needs to be considered. Perhaps sensitization in the T26 group was blocked as a result of the longer period of the daily cycle to which animals in this group were entrained. Behavioral sensitization may be susceptible to daily cycles that diverge substantially from the internal rhythm. Therefore, the second experiment was designed to differentiate between the two possibilities that could potentially account for the results obtained in Experiment 1.

To assess whether the inconsistent timing of drug delivery or the extended cycle used to implement this inconsistency was responsible for the disruption of sensitization, I replicated the first experiment but with one exception. In Experiment 2, the schedule of cocaine injections during pretreatment was held constant in the T26 group rather than the T24 group. More specifically, instead of pre-treating group T24 at the same CT and group T26 at varying CTs, in Experiment 2, the latter group received drug injections at the same CT and the

former at varying CTs. If, indeed, inconsistent timing of drug delivery blocks the expression of behavioral sensitization, only the T26 rats should express behavioral sensitization following the challenge injection. Conversely, if the results indicate that only the T24 animals exhibit behavioral sensitization, the lack of sensitization in the T26 animals would be linked explicitly to entrainment to an unnaturally long daily cycle.

Method

Subjects and Apparatus

Twenty-four male Wistar rats, weighing between 450 and 525 g at the start of the experiment, were used as subjects. The current study used the same apparatus as Experiment 1.

Procedure

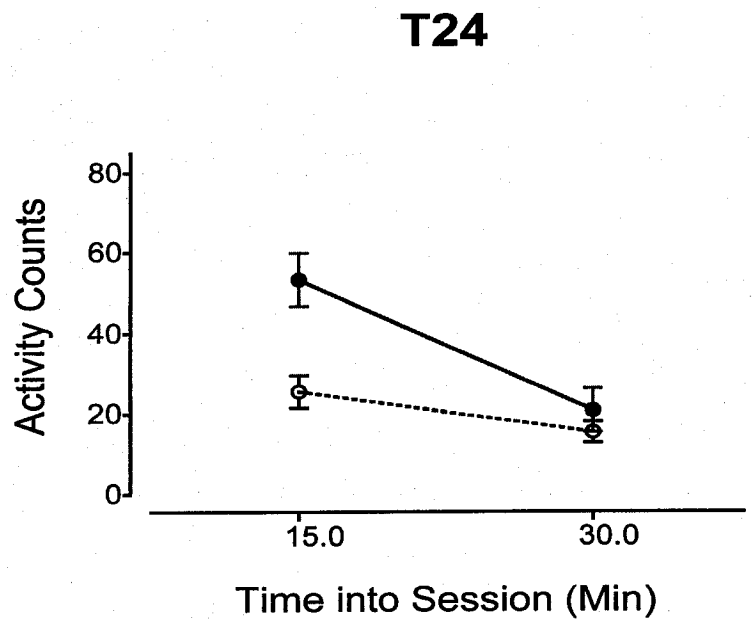
The procedures used for this experiment were similar to those described in Experiment 1. Briefly, rats were again entrained to a 24 –h T cycle or a 26-h T cycle. Once entrained animals were pretreated with cocaine (10 mg/kg, i.p.) or 0.9% saline for five consecutive days. In the current experiment, however, injections were delayed each day by two hours (based on local time). Thus, the T26 group was pretreated at a consistent CT daily whereas the T24 group was pretreated at varying CTs, two hours later each day. On the test session, two

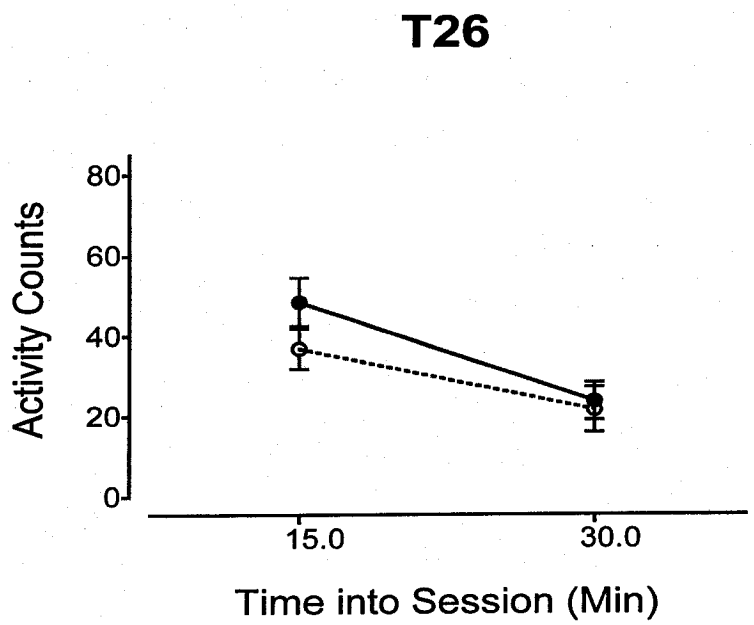
weeks following pretreatment, all animals were given a challenge dose of cocaine (5 mg/kg, i.p.).

Results

The mean locomotor activity counts obtained on the test for sensitization in cocaine- and saline-pretreated animals from each of the two groups (T24 and T26) are given in Figure 3. In the case of animals entrained to a 24-h T cycle, a mixed design ANOVA indicated a significant interaction between Pretreatment and Time, $F(1, 10) = 20.77$, $p < .01$. An independent t test revealed that the activity induced by the challenge cocaine injection in cocaine-pretreated animals was significantly different than the activity of saline-pretreated animals during the first 15 min of testing, $t(10) = 3.63$, $p = .01$. Thus, cocaine-pretreated animals entrained to a 24-h T cycle expressed sensitization. No differences in activity between cocaine- and saline-pretreated animals were found at the second 15-min interval, $t(10) = 0.91$, $p = .39$. For animals entrained to a 26-h T cycle, the mixed design ANOVA indicated that there was neither a main effect of Pretreatment, $F(1, 10) = .98$, $p = .35$, nor a significant Pretreatment × Time interaction, $F(1, 10) = 2.47$, $p = .15$. Thus, sensitization was blocked in animals entrained to a 26-h T cycle.

Figure 3. Mean locomotor activity counts for groups T24 and T26 on the test for sensitization. Filled and open symbols represent the cocaine- and saline-pretreated animals, respectively. Error bars represent SEM.





Discussion

The main conclusion that can be drawn from the results of the current study is that it is the length of the entrainment cycle that modulates behavioral sensitization, and not the inconsistent times of cocaine delivery. Only the T24 drug group expressed behavioral sensitization following the challenge-dose of cocaine. Animals in group T24 received drug pretreatment at inconsistent times but, nonetheless, expressed behavioral sensitization on the test day. By contrast, even though cocaine pre-exposure occurred at the same CTs in group T26, animals in this group failed to express behavioral sensitization. These findings suggest that the failure of group T26 to exhibit behavioral sensitization in Experiment 1 cannot be accounted for by the inconsistent timing of drug pretreatment. Instead, the expression of behavioral sensitization appears to be regulated by the length of the daily entrainment cycle.

There is substantial evidence that repeated exposure to stimulant drugs alters the neural systems that mediate their behavioral activating effects. Such treatment produces persistent presynaptic and postsynaptic changes in dopamine and glutamate neurotransmission in the NAcc and striatum (Robinson & Becker, 1986; Vanderschuren & Kalivas, 2000) as well as persistent changes in the morphology of neurons in the NAcc and prefrontal cortex (Li, Acerbo, & Robinson, 2004; Robinson & Kolb, 1997). Sensitization-related neuroplasticity is manifested as behavioral sensitization upon re-exposure to the drug. However, neural sensitization can be powerfully modulated by the circumstances

surrounding the drug challenge to the extent of preventing its behavioral expression. For example, contextual and time cues in the environment have been found to gain control over the ability of the sensitized neural circuitry to influence the drug-induced behavior (Arvanitogiannis, Sullivan, & Amir, 2000; Robinson, Browman, Crombag, & Badiani, 1998). Differentiating between the induction of neural sensitization and its behavioral expression following a drug challenge, offers up two possibilities to account for the failure of group T26 to exhibit sensitization. First, light cycles longer than the normal 24-h light cycle may prevent the development of neural sensitization. Alternatively, in spite of a sensitized neural substrate, extended light cycles may control the manifestation of behavioral sensitization. To further understand the manner in which an extended daily cycle blocks behavioral sensitization to cocaine, I conducted a third experiment.

Experiment 3

The circumstances surrounding drug intake are known to have the capacity to regulate both the development and the expression of sensitization (Anagnostaras & Robinson, 1996; Badiani, Anagnostaras, & Robinson, 1995; Badiani, Camp, & Robinson, 1997; Jodogne, Marinelli, Le Moal, & Piazza, 1994). With regard to the development of sensitization, Badiani and his colleagues have shown that drug treatment in the normal living environment (home) reduces sensitivity and subsequent sensitization to the effects of the drug as compared to giving the drug in a test environment other than home. With regard to the expression of sensitization, it has been shown that the sensitized locomotor response to a challenge injection of a drug is greater if it is given in the environment or at a time previously associated with drug pretreatment than if given in a different environmental context or at a different time (Anagnostaras & Robinson, 1996; Arvanitogiannis, Sullivan, & Amir, 2000; Jodogne, Marinelli, Le Moal, & Piazza, 1994; Vezina & Stewart, 1984).

Although the VTA and NAcc are known to be critical in the process of sensitization, these brain structures appear to be differentially implicated in the development and expression of sensitization. Several studies have suggested that while the VTA is responsible for the development of sensitization, it is the NAcc that is key in its expression (Cador, Bjijou, & Stinus, 1995; Kalivas & Weber, 1988; Vezina, 2004; Vezina & Stewart, 1990). For instance, directly infusing amphetamine into the NAcc is known to generate behavioral activation,

however, such infusions do not result in sensitization of the drug's behavioral effects (Cador, Bjijou, & Stinus, 1995; Kalivas & Weber, 1988; Vezina & Stewart, 1990). On the other hand, though infusions of amphetamine into the VTA fail to increase locomotor activity, intra-VTA drug pretreated rats exhibit behavioral sensitization to subsequent systemic or intra-NAcc amphetamine (Cador, Bjijou, & Stinus, 1995; Kalivas & Weber, 1988; Vezina & Stewart, 1990). Likewise, stimulant injections directly into the NAcc cause DA release, however, sensitized extracellular DA concentrations in the NAcc is contingent on previous psychostimulant injections into the VTA (Vezina, 1993). Moreover, both locomotor and NAcc DA sensitization are prevented when D1 DA receptors in the VTA are blocked (Vezina, 1996). Such findings have led to the idea that the induction of behavioral and neurochemical sensitization is predominantly linked to the VTA, whereas the NAcc appears to be critical in the expression of sensitization (Vezina, 2004). The distinct role of the VTA and NAcc in sensitization can be attributed to their distinct afferent and efferent connections with particular brain structures (Albanese & Minciacchi, 1983; Kalivas, 2004; Kirouac & Ganguly, 1995).

Taken together, the data from experiment 1 and 2 revealed that entrainment to an extended daily cycle (T26) blocks behavioral sensitization to cocaine. These results may be due to processes that interfere with either the development or the expression of sensitization. Revealing the stage at which sensitization is modulated by an extended daily cycle may provide insights into the neural mechanisms through which such modulation occurs.

Accordingly the goal of the Experiment 3 was to find out which stage of sensitization, induction or expression, is interrupted by entrainment to an extended daily cycle. To address this issue, two groups of animals were formed. Both groups were first entrained to a 26-h T cycle and pretreated with cocaine or saline while entrained to this cycle. After pretreatment, one group was switched to a 24-h T cycle (group T26→T24), while the other group was maintained on the 26-h T cycle (group T26). If the long entrainment cycle interferes with the development of sensitization, neither group T26→T24 nor group T26 should exhibit behavioral sensitization after drug challenge. Conversely, if entrainment to an extended daily cycle blocks the expression of sensitization, then group T26→T24 would be expected to exhibit behavioral sensitization on the test day.

Method

Subjects and Apparatus

Twenty-four male Wistar rats, weighing between 375-500 g at the beginning of the experiment, served as subjects. The rats were cared for as described in Experiment 1. The same apparatus as in Experiment 1 was used.

Procedure

In the current experiment, all animals were initially placed on a 26-h T cycle. Once entrained, rats were pretreated with cocaine (10 mg/kg, i.p.) or 0.9%

saline every 24 hours for five consecutive days. As a result, the pretreatment injections were delivered at the same local time but at different CTs daily. Following the last day of pre-exposure half of the animals were switched to a 24-h T cycle (T26→T24), while the other remained on a 26-h T cycle (T26). The test for sensitization was conducted five weeks later so as to ensure that re-entrainment to the 24-h light cycle was complete. During the test cocaine- and saline-pretreated animals from both groups were injected with a challenge dose of cocaine (5 mg/kg, i.p.). The data from one rat in the T26 drug group was lost due to malfunctioning of the photocells in the activity box. Further details about the procedure can be found in the Method section of Experiment 1.

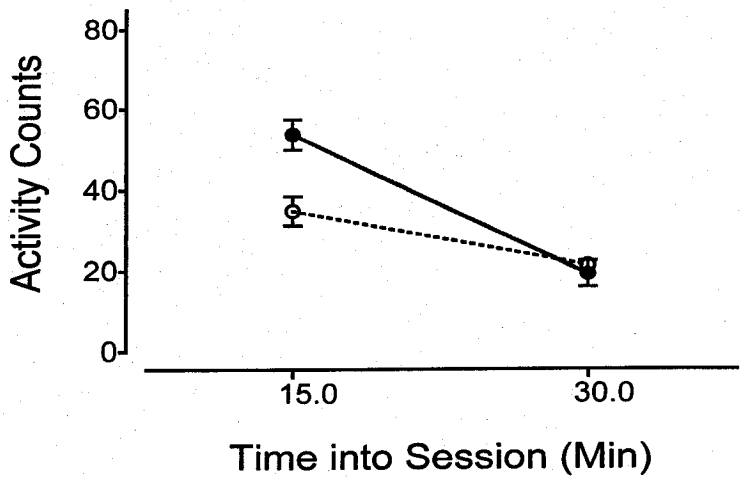
Results

Figure 4 illustrates the mean locomotor activity counts obtained on the test for sensitization in cocaine- and saline-pretreated animals from each of the two groups. In accord with the previous experiments, locomotor activity during the test for sensitization did not differ between cocaine- and saline-pretreated animals that were entrained to a 26-h T cycle both during the pretreatment and the testing phases of the experiment. A mixed way ANOVA revealed that in the T26 group there was no significant effect of pretreatment, $F(1, 9) = .01$, $p = .92$, and no significant interaction, $F(1, 9) = .09$, $p = .77$. However, in the case of the animals that received their pretreatment while entrained to a 26-h T cycle and were tested for sensitization after having been switched to a 24-h T cycle, there

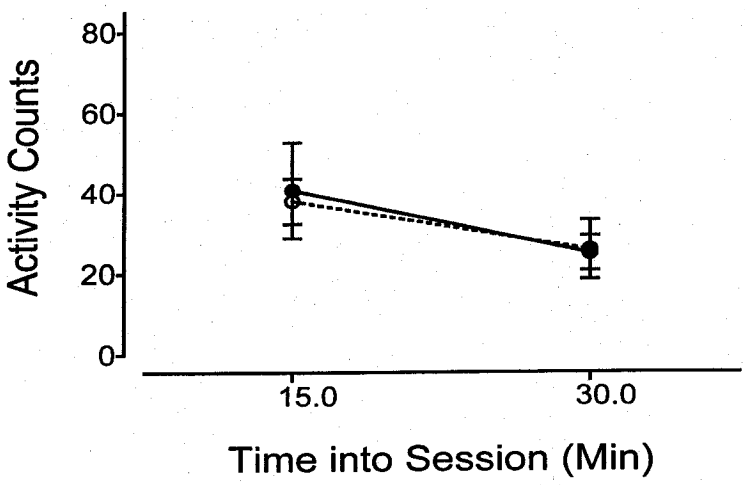
was a significant difference in cocaine-induced locomotor activity during testing. The mixed ANOVA indicated a significant pretreatment effect, $F(1, 10) = 6.32$, $p = .03$, a significant time effect, $F(1, 10) = 66.65$, $p < 0.01$, and a significant Pretreatment × Time interaction, $F(1, 10) = 12.76$, $p < 0.01$. During the first 15 min of the test for sensitization, cocaine- pretreated animals were significantly more active than their saline counterparts, $t(10) = 3.60$, $p < 0.01$.

Figure 4. Mean locomotor activity counts for groups T26→T24 and T26 on the test for sensitization. Filled and open symbols represent the cocaine- and saline-pretreated animals, respectively. Error bars represent SEM.

T26 → T24



T26



Discussion

This study provides further evidence for the idea that entrainment to a long daily cycle interferes with sensitization to cocaine. As in the previous studies, animals in group T26 failed to exhibit sensitization. However, animals pretreated with cocaine while entrained to T26 but that were subsequently re-entrained and tested for the expression of sensitization under a T24 cycle, expressed sensitization. These results suggest that extending the daily cycle modulates the expression of behavioral sensitization and does not interfere with its development. The neurobiological basis of such modulation is presently unclear.

Stimulant drugs share the property of increasing extracellular DA levels in the NAcc by acting directly on the presynaptic terminals of DA neurons. Cocaine binds to the DA transporter and inhibits DA uptake, leading to marked increases in extracellular DA concentrations (Vezina, 2004). Evidence indicates that behavioral sensitization is accompanied by increased responsiveness of the midbrain DA system (Kalivas & Duffy, 1990; Vezina, 2004; Wise & Bozarth, 1987). Acute systemic injections of stimulants increase extracellular DA in striatal terminal regions and this effect becomes sensitized following their repeated exposure (Cador, Bjijou, & Stinus, 1995; Kalivas & Weber, 1988; Vezina, 2004; Vezina & Stewart, 1990). Therefore, lengthening the light cycle may alter the ability of cocaine to increase extracellular DA levels in striatal regions. Changes in DA levels can be measured in vivo using approaches such as intra-accumbens

placement of a microdialysis probe (Vezina, 1993). Thus, it would be instructive to use in vivo microdialysis to assess possible variations in the extracellular concentrations of DA in the NAcc, elicited by cocaine challenge, between animals entrained to a normal 24-h light cycle and others entrained to a long 26-h cycle.

To further investigate differences between the groups (T26 and T24), we can look at differences in brain activation. Cocaine administration induces the activation of immediate early genes (IEGs) such as *c-fos* and *zif268* (Bhat & Baraban, 1993; Moratalla, Robertson, & Graybiel, 1992; Moratalla, Vickers, Robertson, Cochran, & Graybiel, 1993). It would be interesting to use Fos and Zif268 as markers of cell activity to determine whether the neural circuitry engaged by the cocaine challenge varies in animals entrained to the two different T-cycles. Fos has a low basal expression level, making its up-regulation readily detectable. Zif268 has a high basal level of expression and although it can be up-regulated, its down-regulation can also be studied (Herdegen & Leah, 1998).

As previously stated, the expression of behavioral sensitization has been shown to be primarily under the control of the NAcc (Cador, Bjijou, & Stinus, 1995; Kalivas & Weber, 1988; Vezina, 2004; Vezina & Stewart, 1990). Thus, from a functional perspective, the extended daily cycle may interfere with drug-induced neuronal adaptations in this brain region. The manner in which lengthening the daily cycle modulates the function of the NAcc is yet unknown. There are, however, important connections between the circadian and reward pathways that need to be addressed. One candidate structure thought to relay information from the circadian to the reward system is the paraventricular thalamic nucleus

(PVT). In addition to having efferent and afferent connections with the master clock, the PVT projects directly to the NAcc (Leak & Moore, 2001; Moga, Weis, & Moore, 1995). The projections from the PVT to the NAcc are thought to be glutamatergic (Christie, Summers, Stephenson, Cook, & Beart, 1987). Interestingly, glutamate transmission seems to play a key role in behavioral sensitization (Tzschentke & Schmidt, 2003; Vanderschuren & Kalivas, 2000). Thus, the glutamatergic connections from the PVT to the NAcc may have the capacity to modulate the expression of behavioral sensitization. According to this schema, an extended daily cycle may alter the state of the PVT through SCN signaling and, in turn, changes in the PVT may interfere with the glutamatergic transmission to the NAcc and thereby interfere with the expression of behavioral sensitization. Microdialysis could be used to examine the effects of a challenge dose of cocaine on the levels of glutamate release in the NAcc of animals entrained to two distinct light cycles.

General Discussion

Taken together, the three experiments reported in this thesis clearly show that the expression of behavioral sensitization to cocaine is suppressed by a manipulation as simple as lengthening the period of the daily light cycle by a mere two hours. Although research on how this manipulation modulates the long term neurobehavioral consequences of stimulant drugs is still in its infancy, the findings presented in this thesis point to an important interaction between the circadian system and the systems that are involved in drug addiction. Before closing this thesis, I will address some issues relevant to conceptualizing this interaction.

The molecular clockwork and its role in sensitization

The endogenous rhythm of the SCN as well as its ability to assimilate and consequently entrain to external cues arises from underlying intracellular processes. The master clock is composed of hundreds of neurons referred to as "clock cells" (Reppert & Weaver, 2002). In mammals, the most important circadian genes responsible for circadian rhythmicity in each clock cell are *Clock*, *Bmal1*, *Period* (*Per1*, *Per2*, and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*). Circadian rhythmicity within the clock cells is generated by a negative transcriptional feedback loop in which the expression of these clock genes is suppressed periodically by their protein products (Dunlap, 1999; Reppert & Weaver, 2002). It is the amalgamation of the rhythm generated within each clock cell that gives

rise to the integrated oscillatory capacity of the master clock (Herzog & Schwartz, 2002). Thus, circadian rhythms are contingent on the functioning of individual circadian genes.

Several studies have shown that knocking out one of the clock genes may alter or altogether abolish the free-running circadian period (Cermakian, Monaco, Pando, Dierich, & Sassone-Corsi, 2001; Vitaterna et al., 1994; Zheng et al., 1999). For instance, mice mutant for the *Clock* gene exhibit a longer daily period (Vitaterna et al., 1994). Conversely, the daily cycle of *Per1* and *Per2* mutant mice is shorter than the period of their wild-type littermates (Cermakian, Monaco, Pando, Dierich, & Sassone-Corsi, 2001; Zheng et al., 1999). Remarkably, the same clock genes in charge of circadian rhythmicity have, in recent years, been shown to regulate behaviors that deviate from the classical notion of timekeeping. Through the use of knockout animal models, clock genes have been found to play a pivotal role in behavioral and rewarding responses to cocaine, including sensitization (Abarca, Albrecht, & Spanagel, 2002; Andretic, Chaney, & Hirsh, 1999; McClung et al., 2005).

Behavioral sensitization to cocaine is eliminated in fruit flies mutant for PERIOD, CLOCK, CYCLE or DOUBLETIME, the mammalian homologs of PERIOD, CLOCK, BMAL1 and Casein Kinase (CSNK) respectively (Andretic, Chaney, & Hirsh, 1999). *Per1* knockout mice also fail to express behavioral sensitization to cocaine and show a decreased responsiveness to the rewarding effects of cocaine as assessed by place conditioning (Abarca, Albrecht, & Spanagel, 2002). Conversely, mice mutant for PER2 or CLOCK exhibit

heightened locomotor sensitization as well as greater sensitivity to the rewarding effects of cocaine (Abarca, Albrecht, & Spanagel, 2002; McClung et al., 2005). Taken together, these findings provide evidence that circadian clock genes have the capacity to modulate behavioral sensitization.

How do clock genes modulate behavioral sensitization?

Although the SCN is essential for circadian rhythmicity, the molecular components underlying circadian rhythms are not restricted to the SCN. They also seem to operate in other neurons in the brain as well as cells in peripheral tissues such as the liver (Amir, Lamont, Robinson, & Stewart, 2004; Dunlap, 1999; Reppert & Weaver, 2002; Yamazaki et al., 2000). The ubiquitous presence of clock genes throughout central and peripheral tissues makes it difficult to determine the mechanisms by which circadian genes regulate behaviors other than classical timekeeping. Thus, for the time being, the manner in which clock genes regulate sensitization remains unclear.

It has long been known that circadian genes (e.g., *Clock*) regulate the transcription of other clock genes (e.g., *Per*, *Cry*). Interestingly, recent studies have revealed that circadian genes may also act as transcription factors for a number of non-clock genes throughout the brain and body (Akhtar et al., 2002; Panda et al., 2002). Accordingly, it has been suggested that clock genes may be involved directly in the regulation of genes that are important for the function of neurons in drug-relevant neurocircuitry (McClung et al., 2005). For instance, the CLOCK protein may regulate DA transmission by regulating the levels of

tyrosine hydroxylase, the rate-limiting enzyme in DA biosynthesis (Andretic, Chaney, & Hirsh, 1999). In addition, PER2 has been shown to influence the glutamatergic system through regulation of the glutamate transporter (Spanagel et al., 2005).

Such findings have been obtained from experiments using mutant mice. Although clock genes may regulate sensitization through direct intracellular alterations in brain regions affected by the drug, one cannot deny the fact that circadian genes are present in various brain regions. Thus, knocking out particular clock genes must have repercussions not only on drug-relevant pathways in the brain, but also in other brain structures. If so, the above mentioned changes in DA or glutamate transmission may ensue, not from the ability that circadian genes have to regulate gene expression in these neural systems, but from indirect influences that clock genes have in other brain areas which in turn project to drug-related brain regions. Therefore, mutant animal models make it difficult to identify with certainty the way in which circadian genes modulate sensitization.

The circadian system under an extended daily cycle

As shown in the present experiments, simply lengthening the daily cycle of typical, genetically intact animals blocks behavioral sensitization to cocaine. Seemingly, the expression of behavioral sensitization is disrupted as a result of the daily 2-h phase delay the circadian system must undergo to adjust to the 26-h period of the imposed light cycle. As the central pacemaker, the SCN is believed

to be responsible for synchronicity within the circadian system (Reppert & Weaver, 2002). The period of the rat's endogenous circadian rhythm, as that of humans, is approximately 24.5 h. The period of this rhythm is readjusted daily to equal 24 h, the environmental day. Light cycles are the dominant entraining agent for circadian rhythms. The daily cycle of light and dark synchronizes the master clock in the SCN that generates the rhythm and changes its period to match the period of the environmental cycle (Reppert & Weaver, 2002). In the experiments reported in this thesis, the rats did in fact behaviorally entrain to the extended 26-h light-dark cycle. However, under certain circumstances, the rhythm of PER2 expression in specific subordinate oscillators has been shown to uncouple from that of the SCN (Amir, Lamont, Robinson, & Stewart, 2004; Damiola et al., 2000). Specifically, although PER2 expression in the central amygdala (CEA) and bed nucleus of the stria terminalis (BNST-OV) is usually consistent with PER2 rhythm in the SCN, entrainment to a 26-h T cycle abolishes this synchronicity (Harbour, Renteria Diaz, Robinson, Arvanitogiannis, & Amir, 2005).

Of note, neurons in both the BNST-OV and the CEA project to the NAcc, the key structure in the expression of behavioral sensitization (Dong, Petrovich, Watts, & Swanson, 2001; Kalivas, 2004). The projections of the BNST-OV and the CEA to the NAcc indicate that these brain regions may have the capacity to influence NAcc activity (Dong, Petrovich, Watts, & Swanson, 2001; Kalivas, 2004). As a result, the disruption of PER2 rhythm in the BNST-OV and CEA may

modify the functional activity in these regions, thereby affecting the function of the NAcc and, thus, the expression of behavioral sensitization to cocaine.

Conclusions

This thesis reports that lengthening the period of the daily light cycle can have a profound impact on the expression of behavioral sensitization to cocaine. These results underscore the role of the circadian system in the modulation of the behavioral effects of cocaine and open up a previously under-appreciated perspective on the influence that temporal features of the environment have on such effects. This highlights the need for further research to delineate the processes that are involved in the interaction between the neurobehavioral mechanisms of drugs and those of circadian timing.

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