COCAIN MODULATES PATHWAYS FOR PHOTIC AND NON-PHOTIC ENTRAINMENT OF THE MAMMALIAN CIRCADIAN CLOCK

J. David Glass*, Allison Brager, Adam Stowie and Rebecca A. Prosser

aDepartment of Biological Sciences, Kent State University
Kent, OH 44242, USA

bDepartment of Biochemistry and Cellular and Molecular Biology, University of
Tennessee, Knoxville, TN 37996, USA

Author Contributions:
J.D. Glass: Principle investigator; designed in vivo experiments, prepared manuscript
A. Brager and A. Stowie: Undertook in vivo experiments, data recording and analysis
R.A. Prosser: Designed in vitro experiments and undertook data collection, analysis and manuscript preparation

* Corresponding author. Tel: 1-330-672-2934; Fax: 1-330-672-3713

E-mail address: Jglass@kent.edu

Running Title: Cocaine modulates SCN clock function
ABSTRACT

Cocaine abuse is highly disruptive to circadian physiological and behavioral rhythms. The present study was undertaken to determine if such effects are manifest through actions on critical photic and non-photic regulatory pathways in the master circadian clock of the mouse suprachiasmatic nucleus (SCN). Impairment of SCN photic signaling by systemic (i.p.) cocaine injection was evidenced by strong (60%) attenuation of light-induced phase-delay shifts of circadian locomotor activity during the early night. A non-photic action of cocaine was apparent from its induction of 1 h circadian phase-advance shifts at midday. The serotonin receptor antagonist, metergoline, blocked shifting by 80% implicating a serotonergic mechanism. Reverse microdialysis perfusion of the SCN with cocaine at midday induced 3.7 h phase-advance shifts. Control perfusions with lidocaine and artificial cerebrospinal fluid had little shifting effect. In complementary in vitro experiments, photic-like phase-delay shifts of the SCN circadian neuronal activity rhythm induced by glutamate application to the SCN were completely blocked by cocaine. Cocaine treatment of SCN slices alone at subjective midday but not the subjective night induced 3 h phase-advance shifts. Lidocaine had no shifting effect. Cocaine-induced phase shifts were completely blocked by metergoline, but not by the dopamine receptor antagonist, fluphenazine. Finally, pretreatment of SCN slices for 2 h with a low concentration of serotonin agonist (to block subsequent serotonergic phase resetting) abolished cocaine-induced phase shifts at subjective midday. These results reveal multiple effects of cocaine on adult circadian clock regulation that are registered within the SCN and involve enhanced serotonergic transmission. Keywords: circadian rhythm, suprachiasmatic nucleus, light, serotonin, glutamate, dopamine
INTRODUCTION

Cocaine abuse is highly disruptive to rhythmic physiological and behavioral functions, including endocrine, autonomic and immune processes, as well as sleep and feeding (16, 29, 32, 53, 90). The interaction between cocaine and circadian rhythms is bi-directional: cocaine dramatically affects a variety of circadian clock-regulated processes and conversely, the circadian system regulates the timing and extent of cocaine utilization. Cocaine's interaction with the circadian system is evidenced by the daily pattern of cocaine self-administration (13, 68). Notably, there is an anticipatory response to such administration, suggesting that this drug may act as a circadian entraining agent, possibly influencing its own consumption (82). Marked time-of-day effects on cocaine sensitization and place preference have also been demonstrated (1, 13). Cocaine also alters the expression of circadian clock genes (including Clock, Per1 and Per2) throughout the CNS (3, 6, 39, 83, 88), which in turn modulates the activities of multiple neurotransmitter pathways, including those involved in drug reward, behavioral sensitization and place preference (1, 44, 54, 75, 89).

In mammals, the master clock that generates and maintains physiological and behavioral circadian rhythms is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (35, 51, 70). These rhythms are synchronized to the external light-dark (LD) cycle via glutamate release from retinohypothalamic terminals in the SCN (34, 52, 58). The clock is also regulated by non-photic neuropeptide Y (NPY) and serotonin (5-HT) projections arising from the intergeniculate leaflet (IGL) and the midbrain raphe nuclear complex, respectively (4, 14, 30, 47). These photic and non-
photic signals are integrated by the SCN to regulate multiple clock genes and their products that together generate circadian pacemaker activity and signaling output (5, 33).

Despite being so disruptive to biological rhythms (16, 29, 32, 53, 90), the mechanisms through which cocaine modulates circadian timing are not known. To bridge this gap in our understanding of cocaine's actions we used a combination of in vivo and in vitro experimental approaches to explore behavioral and neurochemical effects of this drug on photic and non-photic entrainment of the circadian system. Such information should provide insight into the neurophysiological basis of cocaine's pathological effects, and earmark the SCN clock as a potential target for these actions.
METHODS

Animals. Adult ~8 week-old male C57 albino (B6(Cg)-Tyrc-2J/J) and C57 black (C57BL/6-Tyrp-Brd/J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and Harlan Laboratories (Indianapolis, IN, USA), respectively. The C57 albino mice were used in the in vivo experiments, and C57BL/6J mice were used in the in vitro trials. These strains exhibit a similar degree of circadian response to i.p. administration of cocaine (Glass and Bragger, unpublished observations). All animals were housed in polycarbonate cages under a 12:12 LD photoperiod (lights-on at 0800 h) in a temperature-controlled vivarium (23°C) with food and water provided ad libitum. The experiments followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Kent State and University of Tennessee Institutional Animal Care and Use Committees.

Circadian activity measurements. General circadian locomotor activity was measured using overhead passive infrared motion detectors interfaced with a computerized data acquisition system (Clocklab: Coulbourn Instruments, Whitehall, PA). The data were collected in 1 min bins, and activity onset associated with lights-off (designated as zeitgeber time [ZT] 12) was defined by the initial 6 min period that 1) coincided with an intensity of activity that exceeded 10% of the maximum rate for the day; 2) was preceded by at least 4 hr of activity quiescence; and 3) was followed by at least 60 min of sustained activity. Under DD, activity onset is designated as circadian time (CT) 12 and is the phase reference point for the onset of the subjective night.
Phase shifts were calculated as the difference between the projected times of activity onset of baseline entrainment and days following the cocaine/photic treatment as determined by 1) back extrapolation of the least-squares line through activity onsets on days 3-7 after cocaine-photic treatment and 2) extrapolation of the least-squares line calculated from activity onset data collected the last 5 days of baseline entrainment. Assessments of changes in activity (duration and intensity) after cocaine or saline injection were undertaken using data exported from the Clocklab data acquisition system. An activity count represented an individual event registered by an overhead infrared sensor. An activity bout was defined as the sum of activity counts collected in a 1 min bin. Activity duration represented the length of increased activity bouts (relative to pre-treatment level) immediately following cocaine or saline treatment at midday. Activity intensity was the number of bouts integrated over the duration of response.

**SCN reverse-microdialysis.** The reverse-microdialysis procedures are similar to those described in our previous studies on SCN neurotransmitter release (21). Concentrically designed microdialysis probes were constructed from a 26-gauge stainless-steel outer cannula into which was inserted a beveled 32-gauge fused silica tube (polymicro Technologies, Phoenix, AZ, USA). Hemicellulose dialysis tubing (12 kDa MW cutoff; 230 um OD; Spectra-por; Fisher Scientific, Pittsburgh, PA, USA) was inserted 1.0 mm to a length of 1.0 mm, and the tip sealed with epoxy. Animals received a probe implant with the tip aimed at the lateral margin of the SCN (coordinates: AP: +0.46 mm from bregma, L: +0.02 from midline, H: -0.55 from dura; head level). Following 48 hr of recovery, animals were connected to the inflow and outflow tubings. Artificial
cerebrospinal fluid (ACSF) with or without cocaine was perfused through the probe at a rate of 1.0 µl/min using a calibrated syringe pump (CMA/100; Bioanalytical Systems Inc. West Lafayette, IN, USA). Probe tip placement was verified histologically at the end of the experiment by staining 20 µm SCN cryosections with cresyl violet. To obtain an in vitro estimate of probe efficiency of cocaine delivery via our probes, a labeled structural analog of cocaine (\(^{125}\text{I}\)-RTI-55; PerkinElmer Inc., North Billerica, MA, USA) was measured in dialysate samples collected from probes (n=3) submerged in a known \(^{125}\text{I}\)-RTI-55 standard at a perfusion flow rate of 1 µl/min at 37°C. Under these conditions, probe efficiency was calculated as 20%, and this value was used to estimate cocaine dosage delivered from the probes in the reverse-dialysis trials. It should be noted, however, that this estimate may overestimate release of cocaine from the probe in vivo, owing to tissue elements affecting drug diffusion into the brain. In this regard, the in vivo release of another lipophylic compound, ethanol, is ~20% less than that occurring in vitro (Glass and Brager, unpublished observations).

**Brain slice preparation and single unit recording.** Coronal brain slices (500 µm) containing the SCN were prepared during the daytime from adult mice (2-5 months old), housed in 12:12 LD conditions, as reported previously (60, 61, 63). Slices were prepared between ZT 0–4. Slices were maintained at the interface of a Hatton-style brain slice chamber (Hatton et al.,1980), where they were perfused continuously with warm (37°C), oxygenated (95% O2/5%CO2), glucose/bicarbonate-supplemented Earle’s Balanced Salt Solution (EBSS; MP Biomedicals, Solon, OH, USA), pH 7.4–7.5. Gentamicin (0.05%) was also added to the perfusion medium. All drugs were prepared
in warm, oxygenated EBSS and were bath-applied to the brain slices. At the onset of the drug treatments, perfusion of the standard medium was stopped, the medium was completely removed from the chamber, and fresh medium containing the drugs was applied. Previous experiments have demonstrated that changing the perfusion medium by itself does not affect the phase of the circadian clock. The procedure for neuronal recordings has been described previously (60, 63). Briefly, the spontaneous activity of single SCN neurons was recorded extracellularly using glass capillary microelectrodes filled with 3M NaCl. Each neuron was recorded for 5 min, and the data stored for later determination of firing rate using a DataWave system (Berthoud, CO). Typically, 4–7 cells were recorded during each hour. These individual firing rates were then used to calculate 2 h running averages, lagged by 1 h (± SEM), to obtain a measure of population neuronal activity. As in previous studies (56, 60), the time of peak neuronal activity was assessed visually by estimating, to the nearest quarter hour, the time of symmetrically highest activity. For example, if the two highest 2 h means are equal, then the time of peak is estimated to be halfway between them. Phase shifts were calculated as the difference in time-of-peak of untreated slices vs. drug treated slices. Using these methods, the consistency of the results obtained for each experimental manipulation is such that differences in phase of as little as one hour are often statistically significant with few (n=2 to 3) replicates (e.g., 60, 64).

**Drugs.** Cocaine hydrochloride, metergoline (5-HT$_{1A,2,5,7}$ receptor antagonist), fluphenazine dihydrochloride (dopamine D1/D2 receptor antagonist), (+) 8-hydroxy-2-
(di-n-propylamino) tetralin (DPAT; 5-HT_{1A,5,7} agonist), glutamate and lidocaine hydrochloride were obtained from Sigma-Aldrich Corp St. Louis, MO, USA.

Experimental Protocols

Effects of acute systemic administration of cocaine on photic phase-resetting. This experiment was undertaken to determine if acute cocaine perturbs photic phase-resetting in vivo. Mice under LD were individually caged and their daily activity rhythms measured for a 2 wk period prior to experimentation. On the day of experimentation, mice received an i.p. injection of cocaine (20 mg/kg; n=7) dissolved in physiological saline or saline alone (n=7) 15 min preceding a 30 min phase-delaying light pulse (25 lux) delivered from ZT 16-16.5. Immediately following the light pulse, the animals were released into constant darkness (DD) for 2 wks to assess the extent of phase-delaying using an Aschoff Type II procedure (12). Release of animals into DD is used to reveal the extent to which a phase-resetting treatment shifts clock time in the absence of an entraining photocycle that would otherwise mask a phase-resetting effect. An additional control, where mice received the same cocaine treatment, but no light pulse (n=4) was undertaken to determine if cocaine alone has a phase-resetting response at this time.

Behavioral phase-resetting effects of acute systemic cocaine administration. The phase-resetting effect of cocaine was explored to determine if systemically administered cocaine can act as a non-photic shifting stimulus. Mice under LD were caged individually, and their circadian locomotor activity rhythms measured over a two week period prior to experimentation. On the day of experimentation, animals received an
injection of cocaine (20.0 mg/kg i.p., n=7) dissolved in physiological saline or saline alone (n=7) at ZT 6, coinciding with the phase-advancing portion of the non-photic phase-response curve. Immediately following drug injection, the animals were released into constant darkness for 2 wks to assess phase-advancing responses according to the Aschoff Type II procedure. In a separate trial, mice received an injection of the 5-HT receptor antagonist, metergoline, (10 mg/kg i.p.) 15 min prior to cocaine (n=9) or saline (n=6) injection to explore a serotonergic pathway for cocaine phase-shifting actions on circadian timing.

*Phase resetting through microdialysis administration of cocaine into the SCN.* In this experiment, cocaine was administered directly to the SCN region using reverse microdialysis perfusion to determine if the SCN is a direct target of cocaine's non-photic phase-resetting effect. Mice under LD were singly caged, and their circadian activity rhythms were measured over a 2 wk period prior to experimentation. Two days prior to experimentation, the animals were surgically outfitted with a microdialysis probe stereotaxically aimed at the lateral margin of the SCN. On the day of experimentation, microdialysis probes were perfused with ACSF alone (n=4) or ACSF containing cocaine (250 μM or 500 μM; n=4/dose) from a syringe pump. Based on *in vitro* probe efficiency of ~20% this provided theoretical cocaine tissue concentrations of 50 μM or 100 μM outside the probe. These dosages were based on those from our *in vitro* trials which had a phase-resetting action. Continuous 80 min perfusion of ACSF alone or ACSF+cocaine began at ZT 6. The animals were released into DD at the onset of perfusion to assess phase-shifting responses according to the Aschoff Type II
procedure. Following experimentation, probe placement was verified histologically from fixed frozen sections stained with cresyl violet. An additional of group mice (n=4) received SCN reverse dialysis of lidocaine (50 μM) to control for a possible anesthetic action of cocaine on clock phase.

In vitro phase-resetting experiments using the SCN brain slice preparation. Drugs were bath-applied for 10 min to SCN slices at either ZT 6 or ZT 16 during the initial day in culture. In blocking experiments, metergoline (10 μM) or fluphenazine (50 μM) was applied alone for 5 min prior to, and continued 5 min after the 10 min co-application with cocaine at ZT 6. As a control for possible anesthetic effects of cocaine, slices were treated with lidocaine (50 μM) at ZT 6. In the desensitization experiments 0.01 μM DPAT was applied for 2 h starting at ZT 4. At ZT 6 this was replaced with 50 μM cocaine for 10 min. Previous experiments have demonstrated that this concentration of DPAT does not induce phase shifts by itself (64). In the glutamate experiments, cocaine was applied 5 min prior to and extended 5 min after its 10 min co-application with glutamate (1 μM) at ZT 16. Extracellular single-unit activity recordings commenced near the beginning of the second day in vitro.

Statistics. The in vivo effects of cocaine on photic and non-photic phase-resetting and locomotor activity were assessed by two-way ANOVA. A Student Neuman-Keuls post-hoc comparison test was utilized when the analysis of variance revealed significant treatment effects. The in vitro results were assessed using ANOVA. In all cases, the level of significance was set at p<0.05.
RESULTS

Cocaine attenuates circadian photic phase-resetting in vivo. There was a significant attenuating effect of cocaine on the phase-delaying response to photic stimulation at ZT 16. Vehicle controls receiving i.p. saline injection had light-induced phase-delay shifts averaging 1.50±0.10 hr. Mice pretreated with 20 mg/kg cocaine had significantly smaller phase-delay shifts that averaged only 0.60±0.20 hr (F _{1,6} =15.9; p<0.01; Fig. 1). Cocaine in the absence of a light pulse had no phase-shifting effect (0.00±0.00 hr; p=1.00). Representative actograms of these results are shown in Fig. 2.

Cocaine induces circadian in vivo phase-advance shifts at midday.
Acute i.p. injection of cocaine at midday (ZT 6) induced phase-advance shifts averaging 1.0±0.3 hr, vs. 0.3±0.1 hr and 0.1±0.1 hr for saline and uninjected controls, respectively (F _{2,7} = 9.8; p<0.01; Fig. 3). Pretreatment with the 5-HT _{1A,2,7} receptor antagonist, metergoline (10 mg/kg, i.p.), significantly attenuated the phase-advancing action of cocaine, while metergoline alone had no shifting effect vs. saline controls (0.2±0.3 hr and 0.2±0.1 hr, respectively; F _{3,9}=1.2; p>0.05). Representative actograms of the different treatment groups are presented in Fig. 4.

Cocaine and locomotor activity. Quantitative assessments of the duration and intensity of locomotor response immediately following acute cocaine and control treatments were undertaken, because behavioral arousal per se could be causally associated with the circadian phase-resetting responses. These analyses revealed that
activity duration following cocaine was equivalent to saline, but 1.4 times greater than uninjected controls ($F_{2,12}=45.7; p<0.05$). Activity intensity was ~2 times greater following cocaine compared to saline or to no injection ($F_{2,12}=6.1; p<0.05$).

The SCN is a target for the phase-shifting effects of cocaine. Localized reverse-dialysis perfusion of the SCN region with cocaine at midday in C57 albino mice significantly advanced circadian phase. Perfusion of the SCN with ACSF caused small phase-advance shifts that averaged 0.3+0.2 hr. In contrast, perfusions of the SCN with 50 µM and 100 µM cocaine in ACSF (estimated tissue concentrations) induced larger phase-advance shifts of 2.2±0.7 h and 3.7±0.9 h respectively ($F_{1,12}=16.4; p<0.01$ vs. ACSF; Fig. 5). Control perfusion with 50 µM lidocaine had no phase-resetting effect (0.05±0.17 h). Representative actograms of the different treatment groups are shown in Fig. 6.

In complimentary in vitro experiments, treatment of SCN-containing brain slices with cocaine (1-100 µM) at ZT 6 dose-dependently phase-advanced the circadian rhythm of spontaneous single-unit neuronal activity. Maximal cocaine shifting effect (3.07±0.2 h; $p<0.01$ vs. untreated controls) was attained at 50 µM (Fig 7). Co-application of metergoline completely blocked the phase-resetting action of 50 µM cocaine (0.3±0.2 h phase advance; $p<0.01$ vs. cocaine), while metergoline alone had little effect (0.2±0.2 h phase shift). Co-application of the dopamine antagonist, fluphenazine had no attenuating effect on cocaine shifting (mean phase shift = 3.15±0.4 hr). The phase-resetting action of cocaine was not replicated by application of 50 µM lidocaine to SCN brain slices at ZT 6 (mean phase shift = -0.47±0.18 h).
An additional set of experiments further explored the involvement of 5-HT receptors in cocaine’s phase resetting actions. Previous research has demonstrated that *in vitro* pretreatments that generate a low level of serotonergic signaling in the SCN brain slice block subsequent phase shifts induced by 5HT agonists applied at ZT 6 (64), consistent with a down-regulation of serotonin receptors. We tested whether cocaine-induced phase shifts show a similar sensitivity. SCN-containing brain slices were initially treated for 2 h with 0.01 µM DPAT starting at ZT 4, then treated for 10 min with 50 µM cocaine at ZT 6. As shown in Fig 8, cocaine treatment under these conditions failed to phase-advance the rhythm in neuronal activity (mean phase shift = -0.27±0.14 h, n=3).

Consistent with its *in vivo* effects on photic phase resetting, cocaine also attenuated *in vitro* phase-delays induced by glutamate application. As shown in Fig. 9, treating SCN slices with 1 mM glutamate at ZT 16 induced a 2.26±0.2 h phase delay that was completely blocked by co-application of 50 µM cocaine (0.02±0.27 h phase delay; p<0.01 vs. glutamate alone). Cocaine treatment alone at ZT 16 had no effect (0.0±0 h phase shift). This effect of cocaine was also dose-dependent, with 10 µM cocaine having little inhibitory effect on glutamate-induced phase delays.
DISCUSSION

Cocaine administration and withdrawal produce major disturbances of the daily patterns of circadian-timed homeostatic functions, including endocrine, autonomic, and immune processes, as well as sleep and feeding (16, 28, 29, 32, 53, 90). These actions indicate that cocaine may directly and/or indirectly influence the integrity of circadian clock timekeeping, which could increase susceptibility to drug abuse and addiction. At present, however, relatively little is known concerning what direct effects, if any, cocaine has on the adult circadian timing system. Here we confirm that cocaine's effects include direct actions on the SCN clock itself. These drug effects are pervasive, as they extend to functions critical to photic as well as non-photic regulation of clock timing. Notably, acute cocaine markedly attenuates the circadian phase-resetting response to photic input, the primary entrainment stimulus for timing the 24 hr sleep-activity cycle. Moreover, cocaine acts in a phase-dependent manner to produce serotonin-like circadian phase shifts directly within the SCN clock at midday, but not at night (characteristic of non-photic phase response curves). These results indicate that the circadian clock is vulnerable to cocaine's actions, which could underlie the pathological effects of cocaine abuse on behavioral, physiological and endocrine functions associated with drug abuse and addiction.

Cocaine Modulates Circadian SCN Clock Activity.

Literature on the effects of cocaine on the adult circadian system is relatively sparse, and in humans limited primarily to clinical and anecdotal reports suggestive of a link between cocaine abuse and circadian clock disruption. For example, cocaine
dampens circadian fluctuations in immune and autonomic functions, and can impair sleep (36). In more numerous laboratory animal studies, cocaine has been shown to affect the daily timing and/or pattern of multiple rhythmic functions. In rats, cocaine self-administration blunts diurnal corticosterone and prolactin rhythms up to several days post-administration (40). Also, cocaine self-binging dampens diurnal fluctuations in body temperature and heart rate (82). With respect to behavioral interactions, repeated systemic administration (s.c.) of cocaine over several days disrupts the normal 24 hr pattern of feeding behavior, with food intake increased during the light phase and decreased during the dark phase of the LD cycle (29). Chronic systemic cocaine treatment (i.p.) also disrupts the diurnal rhythm of wheel-running by increasing running during the light phase, even on days without cocaine treatment (11).

Although these results are suggestive of cocaine-mediated effects on circadian timing, a direct action of cocaine on clock functioning has never been explored. It is possible that the blunting effects of cocaine on hormonal and physiological rhythms are due to effects on central and/or peripheral systems modulating these functions, instead of effects on circadian timing per se. Also, the daily anticipatory reactions to cocaine that persist after withdrawal could reflect involvement of an extra-SCN oscillator system, similar to that proposed for methamphetamine (the methamphetamine-sensitive circadian oscillator; 50), rather than SCN circadian clock entrainment.

The present results reveal that cocaine exerts effects directly within the SCN to affect circadian phase regulation. Notably, cocaine’s daytime phase-resetting action could cause inappropriate adjustments in rhythms of sleep, feeding and other related behaviors. Interestingly this effect of cocaine is phase-dependent, in that cocaine did
not induce phase shifts when administered alone at ZT 16. The daytime phase-shifting effect of cocaine is likely manifest through enhanced SCN serotonergic activity, because SCN treatment with the 5-HT antagonist, metergoline, completely blocked this effect of cocaine. Likewise, in vitro phase shifts induced by cocaine were blocked by cotreatment with metergoline as well as by a serotonin desensitizing pretreatment with DPAT. The basis of this action could be cocaine's impairment of the serotonin transporter, resulting in increased extracellular levels of 5-HT in the SCN. Within the hypothalamus the ability of cocaine to bind to serotonin transporters is evidenced by the total displacement of the cocaine analog ($^{125}$I-RTI-55) by the selective 5-HT transport blocker, citalopram (79). Moreover, microdialysis measurements have shown that systemic administration of cocaine causes large (300-500%) increases in extracellular levels of 5-HT in various brain regions, including the nucleus accumbens (18, 27, 56), and localized perfusion of cocaine into this structure causes a striking (1,300%) elevation in 5-HT levels (9,18). The less robust effect of systemic cocaine administration on 5-HT is thought to reflect a limiting effect of raphe 5-HT$_{1A}$ autoreceptor stimulation that suppresses neuronal discharge and 5-HT release in terminal fields (10, 19, 20, 26).

A consideration basic to our data supporting serotonergic mediation of cocaine's effects in the SCN relates to the patency of serotonergic terminals in the slice preparation subsequent to deafferentation. In particular, the blocking action of metergoline on cocaine phase-resetting assumes continued release of 5-HT in the slice. Evidence for such release comes from previous research demonstrating serotonergic phase resetting in response to 5-HT reuptake (fluoxetine) and/or precursor (L-tryptophan) treatments to SCN brain slices. These observations support continued
serotonin synthesis and output from nerve terminals under these \textit{in vitro} conditions (64, 76, 77).

It must also be noted that cocaine enhances extracellular dopamine (DA) levels by inhibiting DA transporters, and this could participate in its circadian phase-resetting action. However, although DA is important for fetal SCN clock entrainment, it is most likely not involved in the intra-SCN cocaine phase-resetting demonstrated here, as the adult SCN does not respond to DA agonist stimulation (85). Moreover, our \textit{in vitro} treatment of the SCN with the dopamine antagonist, fluphenazine, had no attenuating effect on cocaine phase-resetting. Nevertheless, a DA-mediated phase-resetting action of cocaine on extra-SCN (reward) pathways is possible in view of our preliminary results showing that \textit{in vivo} application of cocaine to the ventral tegmental reward area induces phase-advance shifts at midday that are independent of induced locomotor activity (Glass and Brager, unpublished observations).

The putative 5-HT-mediated effect of cocaine in the SCN is highly relevant to the proposed role of 5-HT in regulating SCN non-photic phase-resetting. The SCN contains one of the highest concentrations of 5-HT in the forebrain, and 5-HT can act in the SCN to mediate non-photic circadian phase-resetting. Agonists of 5-HT reset the SCN clock \textit{in vitro} (46, 60, 63, 73) and \textit{in vivo} (17, 24, 25, 57, 81). Also, non-photic phase-resetting stimuli (wheel-running and sleep deprivation) increase 5-HT release in the SCN (21, 31), while depleting central 5-HT inhibits non-photic phase-resetting evoked by wheel-running (42, 80). The ability of DPAT to induce phase shifts (37, 49), and the blockade of these shifts by intra-SCN application of 5-HT$_{2,7}$ and 5-HT$_{7}$ receptor antagonists, ritanserin and DR4004, respectively (25, 37), strongly implicate SCN 5-HT$_{7}$ receptors in
this response. Conversely, the ability of DPAT to induce phase shifts in SCN brain slices prepared from 5-HT7 knockout mice (77) and the inability of the 5-HT7 antagonist, mesulergine, to prevent 5-HT-induced phase shifts in vitro (78) suggest that 5-HT5 receptors may also participate in intra-SCN serotonergic phase resetting.

The second major modulatory effect of cocaine on circadian timing is its marked attenuation (~60%) of phase-resetting by light. This is significant, as the SCN circadian clock synchronizes to the external environment largely through photic information conveyed from the retina to the clock. The principal pathway is a monosynaptic projection from retinal ganglion cells to the SCN, the retinohypothalamic tract (RHT), which is necessary for entrainment of the SCN pacemaker (34, 52, 58). Impairment of this entrainment mechanism by cocaine use could cause marked disturbances in the timing of behavioral and other circadian clock-regulated functions, and such desynchrony could be exacerbated by cocaine's non-photic shifting effect outlined above. The mechanism underlying the attenuating effect of cocaine on photic phase-resetting may again be due to increased 5-HT signaling, since 5-HT is a potent negative modulator of RHT-mediated signaling in the SCN. This is based on findings that 5-HT receptor agonists attenuate various light-activated SCN responses, including increased neuronal activity (48, 66, 87), immediate-early gene (c-fos) gene expression, and behavioral phase-resetting (66, 67, 72). Importantly, our present in vitro results demonstrating cocaine-induced inhibition of glutamate-induced phase-shifts in SCN-containing brain slices indicates that cocaine acts postsynaptically rather than by modulating presynaptic glutamate release. For example, it could be mediated by
serotonergic activation of 5-HT$_{5A}$ or 5-HT$_7$ receptors expressed on retinorecipient SCN cells (87).

**Short-Term Effects of Cocaine on Locomotor Activity.**

Acute treatment with cocaine produces psychomotor stimulation for extended periods following acute injection in mice and rats. Our analyses showed that the intensity, but not duration of cocaine-induced locomotor activity was greater (~2-fold) than that induced by saline injection. In a previous study cocaine-induced activity in mice (measured over a shorter [30 min] post-injection interval) was ~5-fold greater than saline (1), and in rats, cocaine elevates ambulatory activity for 4 hr (11). From a circadian perspective, the activity induced by cocaine could be significant, as locomotor activity/arousal can induce non-photic phase-advance shifts at midday in hamsters (15). Thus, the increased activity rather than a direct chronotypic action of the drug per se could be responsible for the phase shifts observed here. Two lines of evidence argue against this possibility, however. First, as opposed to hamsters and rats, mice do not exhibit the same degree of circadian shifting response to acute activity pulses as do other animal models such as hamsters (42). Second, cocaine’s phase-shifting action in the isolated SCN brain slice preparation confirms that cocaine can induce phase shifts independent of an increase in locomotor behavior.

**The SCN is a Direct Target for Cocaine.**

The present demonstration of a strong phase-shifting response to direct administration of cocaine to the SCN (both in vivo and in vitro) is the first evidence
showing that the adult SCN is responsive to cocaine. Our results also demonstrate that cocaine can act directly in the SCN to block photic phase-resetting. It is unlikely that these localized effects of cocaine are related to its anesthetic properties, as our in vivo and in vitro control applications of lidocaine to the SCN had no chronotypic effect. Cocaine's actions in the SCN strongly suggest that it could impair the synchrony of multiple physiological and behavioral rhythms controlled by the SCN master circadian clock. These results thus have significant health implications concerning the disruptive effects of cocaine on daily rhythmic activities throughout the brain and periphery, and place cocaine on the list of abused drugs with direct action in the SCN, including fentanyl (84), cannabinoids (2, 71) and ethanol (45, 62, 69). It is important to note that the present results do not rule out possible phase-resetting actions of cocaine mediated by regulatory sites projecting to the SCN, such as the midbrain raphe nuclei whose neuronal activities (Fos expression) are activated by cocaine (55), or the IGL that expresses 5-HT transporters (6) that could be targeted by cocaine. Both these areas also are target sites where 5-HT has been shown to reset the clock (17, 22, 23).

In the context of circadian substrate(s) for cocaine action, it also must be noted that a reciprocal linkage between cocaine and the circadian timing system exists (41). In addition to the present demonstration of cocaine's direct interaction with the circadian clock, this drug dramatically affects a variety of other circadian-related processes. For example, cocaine significantly alters the expression of circadian clock genes (including Clock, Per1 and Per2) throughout the CNS (3, 38, 39, 77, 86, 88), which in turn influences the activities of multiple neurotransmitter pathways involved in drug abuse (1, 44, 54, 75, 88). Such effects of cocaine on clock gene activity and on the SCN clock
itself provides a basis for a bi-directional interaction between cocaine and the circadian timing system that could regulate drug effects. Such an interaction is borne out by observations that the SCN is critical for time-of-day conditioned place preference (CPP) for cocaine during CPP extinction (74), and that clock genes have a powerful regulatory influence over behaviors associated with cocaine intake (1, 7, 8, 43, 44) and action (1).

Perspectives and Significance.

The present study is the first to confirm that acute cocaine exposure directly affects adult SCN circadian clock phase regulation. Notably, cocaine alters two of the fundamental regulatory systems that maintain proper circadian phase: namely, the photic and non-photic entrainment pathways. Such effects could impair the ability of the clock to maintain normal synchrony with the outside environment, and to maintain harmony among multiple internal daily rhythmic processes. We also provide evidence that cocaine's effects in the SCN are mediated by elevated serotonergic tonus, possibly produced by suppressed 5-HT transporter activity. It will be important in future studies to address the effects of chronic and binge cocaine applications on the circadian system.
REFERENCES


23. Duncan MJ, Congleton MR. Neural mechanisms mediating circadian phase resetting by activation of 5-HT7 receptors in the dorsal raphe: Roles of


37. Lovenberg TW, Bruce M. Baron BM, de Lecea L, Miller JD, Prosser RA, Rea MA, Foye PE, Racke M, Slone AL, Siegel BW, Danielson PE, Sutcliffe JG, Erlander MG. A novel adenylyl cyclase-activating serotonin receptor (5-


43. McClung CA, Nestler EJ. Regulation of gene expression and cocaine reward by CREB and ΔFosB. *Nature Neurosci* 6: 1208-1215.


46. **Medanic M, Gillette MU.** Serotonin regulates the phase of the rat suprachiasmatic circadian pacemaker in vitro during the subjective day. *J Physiol (Lond)* 450: 629-642, 1992.


76. Sprouse J, Braselton J, Reynolds L. Fluoxetine Modulates the Circadian Biological Clock via Phase Advances of Suprachiasmatic Nucleus Neuronal Firing. *Biol Psychiatry* 60: 896-899


86. Wei YM, Li SX, Shi HS, Ding ZB, Luo YX, Xue YX, Lu L, Yu CX. Protracted cocaine withdrawal produces circadian rhythmic alterations of phosphorylated GSK-3β in reward-related brain areas of rats. *Behav Brain Res* 218: 228-233.


FIGURE LEGENDS

**Figure 1.** Acute systemic cocaine attenuates phase-delay responses to a light pulse delivered at ZT 16. Bars with different letters are significantly different (p<0.05). Bars represent means + SEM. N = 7/group.

**Figure 2.** Representative double-plotted actograms of general locomotor activity showing cocaine inhibition of phase-delay responses to a light pulse delivered at ZT 16 (asterisk designates time of injection and light pulse). The light and dark phases of the LD cycle are represented above the actograms by the open and filled horizontal spaces, respectively. Shaded area represents exposure to DD.

**Figure 3.** Acute systemic cocaine administration at midday (ZT 6) phase-advances the daily locomotor activity rhythm. This effect is blocked by metergoline. Bars with different letters are significantly different (p<0.05). Bars represent means + SEM. N = 7/group.

**Figure 4.** Double-plotted actograms of general locomotor activity showing cocaine-induced phase-advance shifts at midday (ZT 6), and their inhibition by metergoline. Asterisks denote time of treatments. There was an acute behavioral response to metergoline in some mice receiving this treatment (seen here), but no phase-resetting effect. The light and dark phases of the LD cycle are represented above the actograms by the open and filled horizontal spaces, respectively. Shaded area represents exposure to DD.
**Figure 5.** Direct reverse-microdialysis perfusion of the SCN with cocaine at midday (ZT 6) phase-advances the daily locomotor activity rhythm. This effect was significantly different from controls perfused with artificial cerebrospinal fluid (ACSF) alone or with lidocaine. Bars with different letters are significantly different (p<0.05). Bars represent means ± SEM. N = 4/group.

**Figure 6.** Representative double-plotted actograms of general locomotor activity showing the phase-advancing effect of direct reverse microdialysis perfusion of the SCN with cocaine (50 µm) or artificial cerebrospinal fluid (ACSF) at midday (ZT 6). The blank days on the actograms represent the post-surgical recuperative period where activity was not measured. Asterisks designate time of perfusion and release into constant darkness. Shaded area represents exposure to DD.

**Figure 7.** Cocaine phase-advances the SCN clock at midday (ZT 6) *in vitro*. A. Shown are the 2 h means ± SEM of SCN neuronal activity in individual experiments. Top: Neuronal activity peaks near ZT 6 on the second day *in vitro* in a control (no drug) experiment. Middle: Neuronal activity peaks approximately 3 h earlier after treatment with cocaine (50 µM) applied alone at ZT 6, indicating the SCN clock had been phase-advanced by 3 h. Bottom: Co-application of the 5-HT antagonist, metergolone (Meterg; 10 µM) with cocaine completely abolishes the phase-resetting effect of cocaine. Co-application of the dopamine antagonist fluphenazine (Fluphen; 50 µM) with cocaine had no effect on cocaine phase-resetting. Lidocaine alone (50 µM) had no phase-resetting
Figure 8. Cocaine phase advances are blocked by desensitizing serotonergic pretreatment. A. Shown are the 2 h means ± SEM of SCN neuronal activity in individual experiments. Top: Neuronal activity peaks near ZT 6 on the second day in vitro after 2 h pretreatment with a low concentration of DPAT. Bottom: DPAT pretreatment prevents cocaine-induced phase advance. See Fig 7 for details. B. Histogram plot of mean phase shifts induced by cocaine alone (data repeated from Figure 7), cocaine + 0.01 µM DPAT, or DPAT alone. Bars with different letters are significantly different (p<0.05). Bars represent means ± SEM. N=3 slices (mice) for each group.

Figure 9. Glutamate-induced phase delays are inhibited by cocaine in vitro. A. Shown are the 2 h means ± SEM of SCN neuronal activity in individual experiments. Top: Treatment with glutamate (1mM) at ZT 16 delays the time of peak neuronal activity by approximately 3 h, indicating the SCN clock had been phase-delayed by 3 h. Middle: Application of cocaine (50 µM) alone to SCN brain slices at ZT 16 has no effect. Bottom: Co-application of cocaine with glutamate completely blocks the glutamate-induced phase delay. See Fig 7 for details. B. Dose response curve for cocaine
inhibition of glutamate-induced phase delays at ZT 16. Closed circles (representing means \( +\text{SEM} \)) with different letters are significantly different \((p<0.05)\). N=2 slices (mice) for each group