COCAIN MODULATION OF CIRCADIAN TIMING: A PUTATIVE MECHANISM FOR DRUG DEPENDENCE

A dissertation submitted to Kent State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

by

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<table>
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<tbody>
<tr>
<td>5-HT</td>
<td>serotonin</td>
</tr>
<tr>
<td>Alpha</td>
<td>daily activity period</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BMAL1</td>
<td>brain and muscle arnt-like 1; clock gene</td>
</tr>
<tr>
<td>CLOCK</td>
<td>circadian locomotor output cycle kaput; clock gene</td>
</tr>
<tr>
<td>CPP</td>
<td>conditioned place preference</td>
</tr>
<tr>
<td>CRY</td>
<td>cryptochrome; clock gene</td>
</tr>
<tr>
<td>D1</td>
<td>dopamine receptor subtype 1</td>
</tr>
<tr>
<td>DAB</td>
<td>3’3-diaminobenzidine</td>
</tr>
<tr>
<td>DD</td>
<td>constant darkness</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EBOX</td>
<td>enhancer box</td>
</tr>
<tr>
<td>GHT</td>
<td>geniculohypothalamic tract</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IGL</td>
<td>intergeniculate leaftlet</td>
</tr>
<tr>
<td>I.P.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>I.V.</td>
<td>intravenous</td>
</tr>
<tr>
<td>LD</td>
<td>light/dark cycle</td>
</tr>
<tr>
<td>MUA</td>
<td>multi-unit neural array</td>
</tr>
<tr>
<td>NAc</td>
<td>nucleus accumbens</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>PER</td>
<td>period; clock gene</td>
</tr>
<tr>
<td>PPC</td>
<td>peak plasma concentration</td>
</tr>
<tr>
<td>PRC</td>
<td>phase response curve</td>
</tr>
<tr>
<td>RHT</td>
<td>retinohypothalamic tract</td>
</tr>
<tr>
<td>SC</td>
<td>sweetened cocaine solution</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SS</td>
<td>sweetened solution</td>
</tr>
<tr>
<td>TAU</td>
<td>free-running period</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
<tr>
<td>VIP</td>
<td>vasoactive intestinal peptide</td>
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<tr>
<td>VP</td>
<td>vasopressin</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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<tr>
<td>ZT</td>
<td>zeitgeber time</td>
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Chapter 1

Introduction

Biological Rhythms

The significance of biological rhythms to life processes is substantiated by the fact that such a large variety of organisms, from single celled bacteria to multi-cellular humans possess them. Such rhythms confer the benefit of confining costly physiological processes to the time when they will be of maximum benefit to an organism, while temporally separating antagonistic processes (Sharma, 2003). More than that, biological rhythms allow an organism to align physiological and behavioral processes with the environment in order to anticipate predictable events, such as the presence of resources, mates, or threats, and be prepared to exploit or avoid them for maximum survival and reproductive advantage (Dibner et al., 2010; Pittendrigh, 1961; Moore-Ede, 1986). There can be little doubt as to the adaptive value of biological rhythms. In experiments on fruit flies (Drosophila melanogaster) raised in constant conditions (no environmental variation in light, temperature, food availability) for over 600 generations, all generations exhibited precise daily rhythmic timing, while non-adaptive phenotypes, such as traits associated with desiccation (preferential storage of non-carbohydrates or decreased blood volume) disappeared within 25-100 generations (Rose et al., 1996). This strongly indicates a powerful selection advantage of retaining biological rhythmicity. In addition, other studies have indicated that survival outcomes are increased by rhythmic regulation of eclosion timing in midges so that it occurs near the daily occurrence of optimum temperature (Kureck, 1979) and nest departure behavior in
seabirds, which greatly reduced predation (Daan and Tinbergen, 1980). Further, electrolytic lesion of the master pacemaker in hamsters resulted in an approximately 7 month (~47%) reduction in lifespan (Hurd and Ralph, 1998). As this internal timing system is the principle regulator of physiological and behavioral rhythmicity (Pittendrigh, 1961), knowledge of the mechanisms underlying biological rhythmicity is necessary for understanding basic aspects of physiology and behavior (Rusak and Zucker, 1975).

While biological rhythms operate on a number of different time scales (tidal, daily, lunar, annual), the most studied of these are rhythms that repeat approximately once per day, or circadian rhythms (derived from \textit{circa diem}, meaning approximately a day). These rhythms are not driven by external stimuli, but rather are generated within the individual (endogenously) and persist in the absence of environmental stimuli with a period approaching 24 hours, called the free-running period (tau; Aschoff, 1981; Sharma, 2003). The sleep/wake rhythm provides an excellent example of this. In humans living under experimental conditions in which environmental timing cues have been removed, the sleep/wake rhythm repeats, or has a tau of, ~24.80 hours; longer than a solar day (Mills et al., 1964). This contrasts with the endogenous period of mice, for example, which is shorter than the solar day, at approximately 23.5 hours (Edgar et al, 1991).

Because the free-running period of most, if not all, organisms is not 24 hours, it is essential that the internal clock possess a mechanism for synchronizing with the environment (Pittendrigh 1958; Pittendrigh and Bruce, 1957; Bruce, 1960; De Coursey, 1959). The 24 hour light-dark cycle (LD) represents the most stable and predictable environmental variable, thus making light an ideal cue to utilize for this purpose (Pittendrigh 1961; Aschoff, 1981; reviewed in Gillette and Abbot, 2006). Indeed, photic
stimulation is the most potent environmental input which synchronizes the biological clock timing with the environment (entrainment) by resetting the internal clock each day (reviewed in Pittendrigh, 1961). This process involves a non-parametric (non-linear) phase-shifting response of the circadian clock to light across the 24 hour day. Namely, the clock is not sensitive to light during the day, but has differential sensitivity during the night. During the early night, light phase-delays the clock, while exposure to light during the late night phase-advances the clock (Pittendrigh, 1961). Such differential response to light (the phase-response curve; PRC) confers the ability for an organism to entrain its clock to a constantly changing 24 hour cycle throughout the year (or in humans to re-entrain to artificial shifts in the LD cycle imposed by jetlag or shift work). In addition to light, circadian rhythms can also be entrained to variety of non-photic stimuli such as food (Reviewed in Mistlberger, 2009), access to receptive mates (Honrado and Mrosovsky, 1989), exercise (Reebs and Mrosovsky, 1989), social cues (Aschoff et al., 1971; Goel and Lee, 1995), as well as a wide variety of pharmacological agents (reviewed in Mistlberger et al., 2000) and drugs (Scheving, 1968; Ruby et al., 2009; Brager et al., 2009; Glass et al 2012; Stowie et al 2015).

Circadian Physiology

In mammals, the master circadian clock is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. This bilateral nucleus, composed of approximately 20,000 neurons, is located just dorsal to the optic chiasm. Several lines of evidence support the SCN as being the master circadian pacemaker. First, total electrolytic lesion results in complete arrhythmicity in drinking, locomotion, and endocrine function in rats (Moore and Eichler, 1972; Stephan and Zuker, 1972). Notably, in hamsters with SCN
lesions, rhythmicity is restored by transplanted grafts of fetal SCN cells placed in the third ventricle (Lehman et al., 1987). Similar grafts from Tau mutant hamsters which have an abnormally short period length impose this short period in WT recipients with SCN lesions (Ralph et al., 1990). Electrophysiological recordings from ex vivo SCN slices also maintain circadian oscillation, with peak output occurring during the light phase (Inouye and Kawamura, 1979; Green and Gillette, 1982; Shibata and Moore, 1988) without input from the rest of the brain or the environment. Interestingly, while SCN transplants surrounded by a semi-permeable membrane are capable of restoring some rhythms such as locomotor activity, others (e.g. reproductive behavior, endocrine function) were not restored (Silver et al., 1996). This suggests that the SCN imparts time information to extra-SCN brain regions as well as to the periphery via both paracrine and direct neural signaling. A large subpopulation of SCN neurons manufacture and secrete vasopressin (VP), and evidence that blockade of VP receptors does not affect sleep/wake rhythms suggests VP acts as a diffusible signal from the master pacemaker (Kruisbrink et al., 1987). Intact neural connections are also critical for the SCN to communicate timing information throughout the body. A large portion of the SCN projections terminate nearby in the hypothalamic supraventricular zone (Morin et al., 1994), as well as contralateral projections strongly linking both SCN nuclei (Pickard, 1982; Buijs et al., 1994). Other targets within the hypothalamus include the medial preoptic area (Watts and Swanson, 1987), the bed nucleus of the stria terminals (Watts and Swanson, 1987), the lateral septum (Watts and Swanson, 1987), the dorsomedial hypothalamus (Watts and Swanson, 1987; Dai et al., 1998), and the arcuate nucleus (Watts and Swanson, 1987). The SCN also projects to areas which lie within the
thalamus, namely the paraventricular nucleus (Moore, 1996; Dai et al., 1998), and the intergeniculate leaflet (IGL; Card and Moore, 1989). Altogether, these projections, especially those modulating the neuroendocrine (Lehman et al., 1987, Meyer-Bernstein and Morin, 1999; reviewed in Kalsbeek et al., 2006) and autonomic nervous systems (la Fleur et al., 2000; Buijs et al., 2001; reviewed in Kalsbeek et al., 2006), enable circadian control of the daily timing of physiological processes from cellular metabolism to behavior.

There are three primary afferents to the SCN. First, the SCN receives direct input from photosensitive retinal ganglion cells in the eye via the retinohypothalamic tract (RHT; Moore and Lenn, 1972; Moore, 1973). Transection of this tract to the SCN completely abolishes entrainment to a light-dark (LD) cycle, demonstrating both the importance of this connection with the eyes and the role of the SCN in maintaining normal daily circadian rhythms (Johnson et al., 1988). The RHT terminates mainly within a ventral SCN subpopulation of vasoactive intestinal peptide (VIP) immunoreactive neurons (Hendrickson et al., 1972; Tanaka et al., 1993), conveying photic information via glutamatergic (Shirakawa and Moore, 1994; Ding et al., 1994) and peptidergic signaling through substance P (Hamada et al., 1999) and pituitary adenylate cyclase-activating peptide (Chen et al., 1999). Photic phase-resetting within the SCN is induced via glutamatergic and peptidergic activation of calcium/modulin kinase/mitogen-activated protein kinase/cAMP response element-mediated transcriptional activation (Obrietan et al., 1998; Obrietan et al., 1999; Butcher et al., 2002) as well as cGMP-dependent protein kinase II (Oster et al., 2003). These signaling cascades result in downstream chromatin remodeling (Crosio et al., 2000) as well as the induction of immediate early
and clock genes (Albrecht et al., 1997; Shearman et al., 1997; Morris et al., 1998) which are critical to photic phase-resetting of the molecular circadian clock. The second major afferent is a non-photic pathway to the SCN via serotonergic input from the dorsal and median raphe nuclei (Meyer-Bernstein and Morin, 1996; Leander et al., 1998; Dudley et al., 1998; Dudley et al., 1999; Glass et al., 2000, Glass et al., 2003), which terminates on the same VIP-immunoreactive region of the SCN as the RHT (Kiss et al., 1984).

Similar to the differential effects of light on entrainment, non-photic stimulation of the SCN elicits phase-advances of behavioral rhythms during the subjective day and small phase-delays during the late subjective night (reviewed in Mistlberger et al, 2000). A critical role for serotonin signaling in mediating non-photic circadian phase-resetting has been well documented. Serotonin applied to the SCN slice in vitro phase-shifts rhythmic electrical output (Prosser, 2003) and antagonism of serotonin receptors attenuates phase-advances elicited by novel wheel running at midday in hamsters (Glass et al., 2003), as well as affecting phase and rhythm stability under both LD and in constant darkness (DD; reviewed in van Esseveldt et al., 2000). Serotonergic signaling has also been shown to modulate photic phase-resetting by influencing the bioavailability of glutamate within the SCN (Selim et al., 1993; Srkalovic et al., 1994). The third major input pathway to the SCN, the geniculohypothalamic tract (GHT) originating from the IGL, integrates photic (retinal) and non-photic (raphe) input to the SCN via NPY and GABAergic transmission (Grossman et al., 2004; Glass et al., 2010). Pharmacological activation of GABA<sub>A</sub> receptors within the SCN disrupt photic and non-photic phase-resetting (Novak and Albers, 2004) as well as attenuating serotonin release from the raphe (Glass et al., 2003), while blockade of GABA receptors modulate photic phase-
resetting in the SCN (Ehlen and Paul, 2009; Freeman et al., 2004). NPY administration to the SCN, both in vivo (Albers and Ferris, 1984; Huhman and Albers, 1994) and in vitro (Medanic and Gillette, 1993) produce phase shifts, likely linked to NPY mediated reduction of Per1 and Per2 expression in the SCN (Fukuhara et al., 2001). Additionally, phase-resetting in the SCN to light is attenuated by pretreatment with NPY (Weber and Rea, 1997), and mice deficient in NPY have disrupted responses to light (Kim and Harrington, 2008). These mechanisms reset the master circadian pacemaker and facilitate the synchronization of the organism with the environment, as well as alignment of the internal milieu.

**Molecular mechanisms of circadian physiology**

Circadian rhythmicity is generated at the molecular level by interlocking transcriptional and translational positive and negative feedback loops operating in a cell-autonomous fashion (Welsh et al, 1995). This mechanism is highly conserved in all known organisms from cyanobacteria (reviewed by Iwaskai and Kondo, 2000), flies (reviewed by Williams and Sehgal, 2001), and mammals (reviewed Reppert and Weaver, 2001) to plants and fungi (reviewed by Loros and Dunlap, 2001; Alabadi et al., 2001). In mammals, the rhythmic Brain and Muscle Arnt-like 1 (BMAL1; Honma et al., 1998; Oishi et al., 1998), and the non-oscillating Circadian Locomotor Output Cycles Kaput (CLOCK; Shearman et al., 1999; Tei et al., 1997) proteins form a heterodimer and bind the enhancer box (EBOX) promoter region driving the transcription of the Period (Per 1, 2, and 3) and Cryptochrome (Cry 1 and 2) genes (Reviewed in Reppert and Weaver, 2001; Gekakis et al., 1998; Hogenesch et al., 1998; Takahata et al., 1998). Peak expression and nuclear translocation of these proteins occurs around midday resulting in interference
with CLOCK-BMAL1 mediated transcriptional activation of \textit{mCry} and \textit{mPer} as well as an increase in \textit{mBMAL1} transcription (Kume et al., 1999; Shearman et al. 2000). The transcriptional/translational loops of these core “clock genes” repeat with a period approaching 24 hours and comprise the molecular basis for the mammalian circadian clock.

The role of these genes in driving the daily timing of behavioral and physiological processes is supported by many lines of evidence. BMAL1 is necessary for the generation and maintenance of circadian rhythms, as daily rhythms of \textit{Per} transcripts are abolished in \textit{Bmal1} knockout mice, which are also entirely arrhythmic in constant darkness (Bunger et al., 2000). Homozygous mutant \textit{Clock} mice manifest a lengthened locomotor period compared with wild type (WT) littermates, while heterogeneous expression of the mutation results in altered individual cell rhythms periods (Herzog et al., 1999) and eventual loss of rhythm cohesion under constant lighting conditions (Vitaterna et al., 1994). \textit{Clock} mutant mice also have significantly reduced expression of \textit{mPer} and \textit{mCry} transcripts, further supporting an important positive induction role for \textit{Clock} (Jin et al., 1999). The critical involvement of the \textit{Cry} genes is demonstrated by arrhythmic wheel running behavior in constant darkness resulting from targeted deletion of both \textit{Cry} homologs (van der Horst et al., 1999, Vitaterna et al., 1999). Interestingly, deletion of only the \textit{Cry2} gene results in a lengthening of circadian period (van der Horst et al., 1999), while loss of the \textit{Cry1} gene shortens it (van der Horst et al., 1999; Vitaterna et al., 1999). This evidence suggests a complex regulatory organization that is both partially redundant and partially antagonistic. Like CRY, PER proteins have been shown to weakly inhibit CLOCK-BMAL1 mediated transcription of both \textit{mPer} and \textit{mCry}
Importantly, $mPer1$ (Albrecht et al., 1997, Shigeyohsi et al., 1997) and PER1 (Field et al., 2000) expression are both rapidly induced by acute light exposure during the dark phase, and pretreatment with an antisense oligonucleotide against $mPer1$ disrupts photic phase-resetting (Akiyama et al., 1999). $mPer2$/PER2 expression is also induced by acute light exposure, but only during the early dark phase (Shearman et al., 1997; Zylka et al., 1998), and mice with an $mPer2$ gene with a selective deletion of a critical protein-protein interaction domain show weak behavioral rhythms under constant lighting conditions (Zheng et al., 1999). In addition, non-photic phase-resetting taking place during the light phase disrupts PER levels within the SCN of hamsters (Maywood et al., 1999, Horikawa et al., 2000). Taken together, this evidence strongly indicates a critical role for $Per$ genes in daily resetting of the mammalian clock. These interconnected transcriptional and translational feedback loops drive the time keeping signal within the SCN as well as in extra-SCN and peripheral oscillators throughout the brain and body.

**Mesolimbic Reward Pathway**

Behavioral processes critical to the maintenance and propagation of life (e.g. eating, reproduction) are promoted through neurochemical association of relevant stimuli and a subjective feeling of reward. Animals will perform work, sacrificing energy or opportunities to engage in other activities, to gain access to a rewarding activity/substance. There is significant evidence that the reinforcing physiological and behavioral effects of many drugs of abuse, including cocaine, are regulated via dopaminergic and glutamatergic neurons in the mesolimbic reward pathway. Knockout of a functional dopamine receptor subtype 1 (D1) completely abolishes cocaine self-
administration in mice (El-Ghundi et al., 1998), implicating dopamine as a critical mediator of reward-related signaling within the central nervous system. The dopaminergic neurons of the ventral tegmental area (VTA) are necessary for the development of behavioral sensitization; a process by which initial drug administration elicits exaggerated behavioral responses to subsequent drug exposure (Kalivas and Alesdatter, 1993; Wolf, 1998, Wolf et al., 2004), conditioned place preference (CPP); a behavioral measure of drug reward (Ungless et al., 2001; Harris et al., 2004; Borgland et al., 2006), and reinstatement following cocaine withdrawal (Tang et al., 2004; Lu et al., 2003). Additionally, electrical stimulation of the dopaminergic cells of the VTA and substantia nigra greatly increases cocaine self-administration (Corbett and Wise 1980; Wise, 1981). Dopamine released from the VTA targets many areas, but activation of the nucleus accumbens (NAc) has been shown to be critical for the generation of motivated behaviors in response to stimuli (Reviewed in Wise, 1998; Nestler, 2005). Lesions of the NAc abolish reward mediated responses to cocaine and amphetamine (Roberts and Zito, 1987), and performance on CPP tasks following ablation of the accumbens appears to be proportional to the amount of dopamine which remains (Roberts et al., 1980). Glutamatergic neurons in the accumbens have also been shown to be important for cocaine reinstatement (reviewed in Kalivas and O’Brien, 2006), relapse (Cornish and Kalivas, 2000) and potentially for maintenance of self-administration (Conrad et al., 2007). Furthermore, rats and monkeys will work to gain access to cocaine injections to the prefrontal cortex (Phillips et al., 1981; Goeders and Smith 1983), demonstrating the involvement of higher processing areas in mediating reward signaling. Lesions to the dorsal portion of the hippocampus significantly reduce CPP responding to cocaine
and electrical stimulation to the glutamatergic, but not dopaminergic, neurons of the hippocampus induced cocaine seeking in rats trained to self-administer (Vorel et al., 2001). Interestingly, the opposite effect is observed within the amygdala, where pharmacological blockade of dopaminergic, but not glutamatergic receptors, attenuate CPP in rats (See, 2001). Together via a highly complex interaction, these brain regions imbue environmental stimuli such as food or drugs with significance and increase the behavioral drive to gain access to these activities or substances.

Drugs of Abuse and Circadian Disruption

Disruption of circadian functioning has been documented for several drugs of abuse. Acute ethanol administration in the early subjective night attenuates glutamate induced phase-delays within the mouse SCN slice \textit{in vitro} (Prosser et al., 2008) but not during the late subjective dark phase (Brager et al., 2011). \textit{In vivo}, acute ethanol inhibits photic phase-resetting in a species dependent way, occurring during the late subjective night in hamsters (Ruby et al., 2009) and the early subjective night for mice (Brager et al., 2011). Alone, ethanol did not affect circadian phase \textit{in vitro} (Prosser et al., 2008). Chronically, ethanol also attenuates photic phase-resetting in hamsters (Ruby et al., 2009) and mice (Brager et al., 2009) and disrupted entrainment to light under a dim skeleton photoperiod (Brager et al., 2010). Methamphetamine administered in drinking water under DD for 44 days increases period length, alpha, and overall activity (wheel-running) in rats (Honma et al., 1986) as well as mice (Tataroglu et al., 2006), though these disruptive effects disappear within a few cycles following withdrawal. Interestingly, methamphetamine is also able to induce the molecular machinery of the clock when the SCN is destroyed (Honma et al., 1987), suggesting a separate oscillator system exists
for this drug. Tetrahydrocannabinol has been shown to alter the phase of hormone secretion in humans, particularly melatonin (reviewed in Lissoni et al., 1986), disrupt sleep/activity rhythms in mice (Lewis and Brett, 2010), as well as disrupt the circadian transcription of a number of transcription factors, notably CREB (Butovsky et al., 2005). Collectively, this evidence suggests that the daily timing pathway is highly susceptible to manipulation by commonly abused pharmacological agents.

**Cocaine**

Within the central nervous system, cocaine binds to and inactivates the reuptake channels for dopamine, norepinephrine, and serotonin increasing the bioavailability of these signaling molecules within the synapse. The most common form of this drug is cocaine hydrochloride salt, though it also occurs less commonly as a paste unbound to the hydrochloride ion (“free base”) as well as in crystalline hydrochloride form. These forms and various routes of drug administration combine to provide a variety of peak plasma concentration (PPC) levels and speed of peak concentration. Cocaine hydrochloride and crystalline hydrochloride have PPCs of 40-90 and 35 minutes respectively when administered intranasally in human volunteers (Javaid et al., 1978; Wilkinson et al. 1980; Bromley and Hayward 1988) which is comparable to orally administration routes (50-90 mins; Van Dyke et al., 1978). Peak plasma concentrations are much faster via smoking of either “free-base” or cocaine hydrochloride (~3 minutes; Paly et al., 1982; Cook et al., 1985) as well as via intravenous (i.v.) injection (1-5 minutes; Javaid et al., 1978) than via oral cocaine administration. Systemic and neural bioavailability of cocaine is relatively consistent across administration methods (60-80%), though orally consumed cocaine results in a greater subjective “high” than other
administration routes (Van Dyke et al., 1978). At neutral pH in solution (7.0), cocaine rapidly degrades into several metabolites, but mostly into benzoylecgonine via nonenzymatic hydrolysis (Stewart et al., 1979; Garrett and Seyda, 1983), as well as minor oxidative elimination within the liver. At room temperature, this process begins within 15 minutes and is mostly complete by approximately 45 minutes (Das Gupta, 1982).

**Circadian Modulation of Physiological and Behavioral Cocaine Response**

The significance of circadian influence on the physiological and behavioral processes associated with cocaine abuse has only recently come to light. Cocaine seeking behavior exhibits circadian rhythmicity, as evidenced by the strong influence of time of day in the acquisition of cocaine self-administration (Ozburn et al., 2012), as well as the confinement of cocaine self-administration to the dark portion of the daily light/dark cycle in both rats and mice (Roberts et al., 1997; Roberts et al., 2002; Stowie et al., 2015). Additionally, self-administration of cocaine exhibits a pronounced free-running rhythm under constant lighting conditions (Falk et al., 1996; Bass et al., 2010). The primary behavioral response to cocaine, the psychomotor increase in arousal and ambulation, as well as reinforcement undergo daily oscillations in cocaine responsiveness (Abarca et al., 2002; Uz et al., 2002) as do processes associated with cocaine dependence such as behavioral sensitization (Cornish and Kalivas, 2001; Abarca et al., 2002, Uz et al, 2002), extinction (Gabriele et al., 2009) and reinstatement of drug seeking (Sorg et al., 2011). Circadian modulation of cocaine is likely mediated through indirect projections from the SCN through the medial preoptic area which have
been shown to modulate the rhythmic activity of some dopaminergic neurons within the VTA (Luo and Aston-Jones, 2009).

At the genetic level, circadian clock genes are also critical for the regulation of physiological processes associated with cocaine abuse. In mutant *Drosophila melanogaster* which have core clock genes knocked out, sensitization to cocaine is completely abolished (Andretic et al., 1999). Mice possessing a dominant negative *Clock* mutation acquire CPP much more quickly than WT litter mates (McClung et al., 2005), and these mice display a loss of circadian oscillation in acquisition and maintenance of cocaine self-administration (Ozburn et al., 2012). In PER1 knockout mice, there is a marked decrease in behavioral sensitization as well as in CPP to cocaine indicating that in addition to behavioral responses, cocaine reinforcement is also under direct circadian regulation (Abarca et al., 2002). Interestingly, PER2 knockout mice display exaggerated cocaine sensitization and CPP which suggests that these two proteins have antagonistic functions in the regulation of physiological and behavioral responses to the drug (Abarca et al., 2002). This evidence strongly implicates the circadian timekeeping system in modulating many aspects of cocaine abuse, from the level of the gene to behaviors which support the initiation and maintenance of cocaine abuse.

**Specific Aims and Hypotheses**

The following specific aims were designed to investigate:

1) **Acute Cocaine Action within the Circadian Pathway for Photic and Non-photic Entrainment**
a). Investigate the effects of acute cocaine on photic phase resetting (Experiment 1).

**Hypothesis:** Cocaine pretreatment will attenuate the delayed onset of circadian activity rhythms in response to light exposure during the early dark phase.

b). Assess independent phase resetting effects of acute cocaine (Experiment 2).

**Hypothesis:** Acute cocaine administration during the middle of the light period will advance the phase of the circadian locomotor rhythm.

2) Chronic Cocaine Actions in the Circadian System

a). Validate orally consumed cocaine for use in long-term circadian measurement of chronic cocaine effects (Experiments 3, 4).

**Hypothesis:** Cocaine dissolved in drinking water possesses the stability in solution to act as a non-photonic circadian stimulus over long administration intervals.

b). Characterize chronic cocaine effects on expression of endogenous circadian free-running rhythm (Experiment 4).

**Hypothesis:** Under constant conditions, chronic cocaine self-administration will result in a lengthening of endogenous free-running period.

c). Investigate masking of chronic circadian effects by light (Experiments 3, 8).

**Hypothesis:** Lengthening of free-running period and abnormal entrainment to light will be masked under a normal light/dark cycle in both locomotor activity and extra-SCN oscillators.

3) Neurochemical Mechanisms for Cocaine Circadian Actions

a). Acute cocaine effects within the circadian pathways are mediated by serotonergic
signaling (Experiment 5).

**Hypothesis:** Antagonism of serotonin receptors will abolish phase advancement to light period cocaine administration as well as prevent disruption of phase-resetting to light during the early dark phase.

b). Circadian disruption by chronic cocaine is mediated through serotonergic signaling. (Experiments 6, 7).

**Hypothesis:** Mice insensitive to the serotonergic effects of cocaine will display phase advances in locomotor activity rhythms in response to acute cocaine injections at midday, and no disruption of photic phase-resetting during the early dark phase will occur.
CHAPTER 2

Materials and Methods

Animals

Adult male C57BL/6J (*Mus musculus*) were used in experiments 1, 2, 3, 4, and 7 (The Jackson Laboratory, Bar Harbor, ME). In experiments 5 and 6, transgenic mice with a cocaine-insensitive serotonin (5-HT) transporter resulting from a single amino-acid substitution in the I172M allele (Thompson et al 2011), back-crossed to a C57BL/6J background were used. All mice were between 6-10 weeks old at the start of experimentation. Animals were singly housed in polycarbonate cages under a 12:12 LD photoperiod (LD; light on at 0700) in a temperature controlled vivarium (23°C) with food and water provided *ad libitum*. All experiments adhered to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Kent State University Animal Care Use Committee.

Circadian Activity Measurements

Circadian locomotor activity was measured with infrared motion detectors interfaced with a computerized data acquisition system (ClockLab; Coulbourn Instruments,
Whitehall, PA). The timing of drinking activity was measured with a photocell sensor (H20-93; Coulbourn Instruments, Whitehall, PA) mounted in front of the animal’s water bottle and interfaced with the computerized data acquisition system. The circadian parameters analyzed included onset and offset, tau, length of daily activity (alpha), and phase angle of entrainment. Data were collected in 5-minute bins, and activity onset was characterized by an initial period of activity that: (i) exceeded 10% of the maximum rate for the day; (ii) was preceded by at least 4 hours of activity quiescence; and (iii) was followed by at least 60 minutes of sustained activity. Activity offset was defined as a final period of activity that: (i) was immediately preceded by at least 60 minutes of activity; and (ii) was followed by at least 4 hours of inactivity. Tau was calculated using a least-squares regression line through a minimum of 7 daily activity onsets. Under constant darkness (DD), activity onset was the reference point for the beginning of the subjective night (designated as circadian time 12). Phase-shifts were calculated according to a modified Aschoff type II procedure (Ashoff, 1981) as determined by: (i) back extrapolation of the least-squares line through activity onsets on days 1 to 10 after treatment; and (ii) extrapolation of the least-squares line calculated from activity onset data collected for a minimum of 7 days prior to treatment. Phase angles were calculated as the average weekly onset time subtracted from the time of lights off.

*Bout Analysis*
Qualitative assessments of locomotor and drinking activity episodes were undertaken using data exported from the ClockLab data acquisition system and were expressed as counts. For locomotor activity, counts were defined as activity episodes of any duration which exceeded 1 episode/min proceeded and followed by a minimum of 5 minutes of quiescence. Due to the relatively low number of drinking events compared to locomotor activity, drinking counts represented the total number of episodes integrated for the light and dark phases of the 24 h day.

**Drinking Analysis**

*Fluid Intake*

Daily intake of 0.5 mg/ml, 0.1 mg/ml cocaine solution, or water alone was measured during the light phase using 50 ml or 15 ml tubes (Fisher Scientific, Pittsburgh, PA). Daily fluid intake was estimated to the nearest 0.25 ml, and expressed either as volume consumed (ml) or dosage (mg drug/kg of body weight).

*Cocaine Preference*

Cocaine preference was expressed as a percentage representing the amount of daily cocaine intake (ml) over total daily fluid intake (ml).
Multi-unit Neural Activity

Implantation

Experiment 7 was conducted using Multi-unit Neural Activity (MUA) measurement. Bipolar electrodes composed of two recording Teflon-coated stainless steel wires (inside diameter 75 µm; tip distance ~120 µm; A-M Systems, WA, USA) and one uncoated platinum-iridium wire (inside diameter 75 µm; A-M Systems, WA, USA) which acted as a signal ground in the cortex. Mice were anesthetized with sodium pentobarbital (Ovation Pharmaceuticals, Deerfield, IL) and electrodes were implanted in the ventral tegmental area (-460 µm from Bregma, +75 µm lateral, -475 µm vertical) with three stainless steel surgical screws placed in the skull to act as anchors. Hygon-tray material (Coltene, Cuyahoga Falls, OH) was built up in a conical shape from the skull to secure the electrode in place.

Recording

Mice recovered for one week prior to connection to a computerized monitoring system via communtator (Dragonfly R & D, Ridgeley, WV) to a head stage buffer amplifier (TL082; Texas Instruments, TX, USA). Electrical output was processed by differential input integration amplifiers (INA 101 AM; Burr-Brown, AZ, gain x10) and subsequently amplified (AC amplifier; band-pass 500 Hz-5 kHz; gain x10,000). Spiking was
discriminated by amplitude and counted in 1 min bins via computer-based window discrimination (KPCI-1801HC; Keithley Instruments, OH, USA).

**Electrode Placement Verification**

At the conclusion of recording, mice were euthanized by overdose of sodium pentobarbital (Virbac, Fort Worth, TX) and a current of 3µA was passed through each electrode input for 60 seconds. Brains were extracted and post-fixed in a phosphate-buffered solution containing 4% paraformaldehyde and 2% potassium ferrocyanide (Sigma-Aldrich, St. Louis, MO) for 48 hours, then transferred into a 30% sucrose cryoprotectant solution in preparation for sectioning. Brains were frozen and cut into 20 µM sections on a cryostat (ThermoFisher Scientific, Waltham, MA). Free floating sections were stained for tyrosine hydroxylase (TH) via immunocytochemistry. Placement of electrode was verified via bright-field microscopy and determined to be in the VTA if the ventral most portion of the potassium ferrocyanide staining was within the bounds identified by the tyrosine-hydroxylase staining.

**Immunocytochemistry**

Free-floating adjacent sections underwent 3 rinses (15 minutes) in Tris buffered saline containing 0.1% bovine serum albumin, 0.1% triton x-100, and 0.01% merthiolate before
the first and following all subsequent incubations. Initial incubation was done in 0.01% H$_2$O$_2$ for 30 minutes to reduce nonspecific binding of endogenous peroxidases. Primary incubation was done with rabbit polyclonal immunoglobulin (IgG) antibody against TH (Santa Cruz Biotechnologies, Dallas, TX) for 24 hours. Next, sections were incubated for 90 minutes in an anti-rabbit IgG biotinylated antibody (Vector Laboratories, Burlingame, CA), and then for a further 60 minutes in Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA). The final incubation was 3,3'-Diaminobenzidine (DAB)/hydrogen peroxide (DAB peroxidase substrate kit; Vector Laboratories, Burlingame, CA) solution with nickel added to aid in the visualization of the stain.

**Drugs**

Cocaine hydrochloride (Sigma-Aldrich; St. Louis, MO) was used in all experiments. Cocaine stored at room temperature in a solution at a pH of 5.5 or higher decomposes to non-biologically active metabolites within approximately 13 days (Murray and Al-Shora, 1978; das Gupta, 1982). For this reason, cocaine solutions for intraperitoneal (i.p.) injection were prepared immediately prior to use. Solutions utilized for chronic drinking experiments were prepared at a pH of 3, as cocaine stored at a pH of 4 or below does not undergo significant degradation for up to 45 days (Murray and Al-Shora, 1982) and stored at 4°C. To further ensure accuracy of cocaine dosage, solutions presented to the mice at room temperature were discarded and replaced with a fresh
solution in 5 day intervals. Metergoline (5-HT$_{1A,2,5,7}$ receptor antagonist) was obtained from Sigma Aldrich (St. Louis, MO).

**Euthanasia**

At the end of experimentation, all non-MUA animals were euthanized via carbon dioxide asphyxiatio (primary) followed by cervical dislocation (secondary) as per the Animal Use Regulations for Kent State University. For multi-unit neural array, animals were euthanized by overdose of pentobarbital sodium (~20 mg/kg; Euthasol; Virbac Animal Health, Fort Worth) followed by decapitation as secondary euthanasia.

**Statistical Analysis**

A univariate or two-way analysis of variance (ANOVA) with subsequent Student Newman–Keuls post hoc testing was used to assess treatment differences of mean rhythm period, solution consumption, electrical output, and activity bouts and was reported as a mean ± S.E.M. The level of significance, in all cases, was set at $p < 0.05$

**Experimental Protocols**

**Acute Cocaine Action within the Circadian Pathway for Photic and Non-photic Entrainment**

*Experiment 1 – Modulation of photic shifting by acute cocaine presentation during dark phase*
Possible modulation of photic phase-resetting by cocaine was assessed in this experiment. Mice were individually housed and daily locomotor activity rhythms were recorded for a 2 week period in order to establish baseline rhythm expression. On the day of experimentation, mice received i.p. injections of either saline or cocaine dissolved in physiological saline (20 mg/kg; n=7 for all groups) at zeitgeber time (ZT) 16.25, 15 minutes prior to a 30 minute light pulse beginning at ZT 16.5. The timing of the light pulse was selected to occur within the most responsive phase-delaying region of the mouse photic PRC, and the cocaine administration was done 15 minutes prior in order to allow the drug to diffuse across the blood-brain barrier and take effect. Following the light pulse, mice were immediately released into constant darkness for two weeks and phase shifts were calculated to assess cocaine effects on photic phase-shifting. Additionally, a group of mice (n=4) received a cocaine injection but were not subjected to the light pulse in order to determine whether cocaine alone has a phase-resetting response at this time.
Experiment 2 – Non-photic phase modulation by acute cocaine administration at midday

This experiment was undertaken to determine if systemically administered acute cocaine could act alone as a non-photic stimulus. Mice were individually housed and maintained under an LD cycle for two weeks to acquire baseline data. On the day of experimentation, mice received i.p. injections of either saline or cocaine dissolved in saline (20 mg/kg; n=7 per group) at ZT 6. Cocaine administration was done at this time to coincide with the most responsive portion of the mouse non-photic PRC in order to maximize potential effects elicited by the injection. Following cocaine administration animals were immediately released into constant darkness for two weeks and phase-shifts were calculated to characterize the non-photic phase resetting properties of acute cocaine administration using an Aschoff Type II procedure (Ashoff 1980).

Chronic Cocaine Actions in the Circadian System

Experiment 3 – Characterization of free-choice oral cocaine consumption

Characterization of oral cocaine self-administration was undertaken in this experiment. Mice (n=9) were subjected to a free-choice drinking regimen consisting of two 50ml bottles, one containing tap water (pH 3) and the other a cocaine solution (0.1 mg/ml, pH 3). Fluid consumption was measured daily to the nearest 0.25 ml and the position of the bottles was switched to eliminate possible left/right location preference. On day 22, the
bottle containing the cocaine solution was replaced with a second tap water bottle, initiating a state of withdrawal. On day 37, the cocaine solution was reintroduced, marking the beginning of the reinstatement period. Assessment of drinking behavior was stopped on day 65.

**Experiment 4 – Chronic cocaine effects on free-running period**

This experiment investigated the effect of temporally limited daily cocaine access on circadian behavioral activity and rhythm period. Initially, mice were individually housed under LD to establish baseline circadian behavioral activity measurements. After 7 days, mice were randomly assigned to a “forced” consumption regimen in which they had free access to a 50ml bottle containing tap water 23 hours a day from ZT 5.5-6.5 which was replaced with a 15ml bottle containing a sweetened solution (SS; 0.16% sodium saccharine, 3% glucose) or a sweetened cocaine solution (SC; .5 mg/ml) at midday from ZT 5.5-6.5. This time was selected to occur 30 minutes prior to and following ZT 6, the time of maximum phase-advancement to non-photic stimulation in the mouse PRC. A sweetened solution was utilized as an incentive to promote drinking during a time during which mice typically do not engage in this behavior. Fluid consumption was estimated to the nearest 0.25 ml. Both SS and SC solutions were withdrawn from days 54-60 to observe any disruptions in the temporal organization of locomotor activity associated with activity onset. In order to assess the effects of
cocaine on free-running period, the mice were released into DD on day 66, with the daily presentation of cocaine or sweetened solution occurring at the same external time as previously under LD. Withdrawal of both cocaine and control solutions occurred under DD on day 144 in order to examine possible residual effects of the cocaine treatment on circadian activity. Locomotor activity bout, entrainment, and length of daily activity (alpha) analysis were undertaken prior to and during cocaine administration to examine drug effects on these parameters. An activity ratio, calculated as the locomotor activity taking place between ZT 4.5-5.5 divided by the total daily activity count, was performed for the last week of baseline and cocaine treatment to quantify possible anticipatory activity to cocaine presentation.

**Neurochemical Mechanisms for Cocaine Circadian Actions**

**Experiment 5 – Serotonergic mediation of cocaine’s phase-advancing effects at midday**

Mice were singly housed under LD for a two week baseline acquisition period prior to assessment of non-photic phase resetting. On the day of the experiment, mice received i.p. injections of either saline or cocaine (20 mg/kg) following i.p. pretreatment with either DMSO or the broad serotonin receptor antagonist metergoline (10 mg/kg; n=6 and 7 respectively). Pretreatment occurred 15 minutes prior to saline/cocaine administration at ZT 11.75 and 12 respectively. Following saline/cocaine injections, animals were released into constant darkness for one week and phase-shifts were
calculated according to an Aschoff Type II procedure to assess the role of 5-HT signaling pathways in mediating cocaine induced phase-resetting in the circadian system.

**Experiment 6 – Cocaine effects on photic phase-resetting in M172 mice**

The role of serotonergic signaling pathways in mediating attenuation of light induced photic-phase resetting by acute systemic cocaine was assessed in this experiment. M172 and WT mice were singly housed and maintained under LD for two weeks to acquire baseline locomotor activity rhythms. On the experimental day, as in Experiment 1, mice were given either an i.p. injection of saline or cocaine dissolved in physiological saline (20 mg/kg) 15 minutes prior to a 30 minute light pulse at ZT 16.5. This time point was chosen to coincide with the previous experiment in which cocaine attenuated the photic resetting effects of light at this time (Experiment 1). Immediately following the light pulse, mice were placed into DD for two weeks and phase shifts were calculated following an Aschoff Type II procedure.

**Experiment 7 – Cocaine effects on non-photic phase-resetting in M172 mice**

In this experiment, the role of serotonergic signaling in mediating the non-photic phase resetting effects of acute systemic cocaine was examined. M172 and WT mice were
individually housed under LD for two weeks to establish baseline recording. On the day of the experiment, as in Experiment 2, mice were injected with either an i.p. injection of saline or cocaine dissolved in physiological saline (20 mg/kg) at midday (ZT 6). Injections took place at this time to coincide with the previous experiment in which cocaine administration at this time caused phase-advances (Experiment 2). Immediately following i.p. injections, mice were placed into DD for two weeks and phase shifts were calculated following an Aschoff Type II procedure.

Chronic Cocaine Actions on Core Circadian Clock Output

Experiment 8 – Cocaine effects on neuronal multi-unit activity in the ventral tegmental area

In this experiment, the effects of chronic oral cocaine consumption on the signaling output of neurons in the VTA were assessed. Following implantation of the recording electrode and recovery period, the summed electrical output of a region of the VTA in mice was recorded for 7-10 days in order to establish a stabilized baseline output. Subsequently, the drinking solution available to the mice was replaced with a cocaine solution (0.5 mg/ml; pH 3) for one week at which time animals were euthanized and their brains were extracted to be processed for electrode placement verification. In
addition to regional electrical output, drinking events and general locomotor activity were also measured.
CHAPTER III

Results

Acute Cocaine Action within the Circadian Pathway for Photic and Non-photic Entrainment

Experiment 1 - Modulation of photic shifting by acute cocaine presentation during dark phase

Acute, systemic cocaine administration has significant attenuating effects on the phase-delaying response to photic stimulation at ZT 16.5 (Figure 1). Mice that received the vehicle i.p. injections displayed large phase-delay shifts (1.50±0.10 h) which were much smaller in mice which had been pretreated with 20 mg/kg cocaine injection (0.60±0.20 h; $F_{1,6}=15.9; p<0.01$). Intraperitoneal injections of cocaine at ZT 16.25 without a subsequent light pulse did not result in any independent phase-resetting (0.00±0.00 h; $p=1.00$).
Figure 1: Left: Acute systemic cocaine attenuates phase-delay responses to a light pulse delivered at zeitgeber time (ZT) 16. Bars with different letters show significant difference (P < 0.05). Values are expressed as means ± S.E.M. Right: 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 light-dark (LD) cycle are represented above the actograms by the open and filled horizontal spaces, respectively. Shaded area represents exposure to constant darkness (DD).
Experiment 2 - Non-photic phase modulation by acute cocaine administration at midday

Cocaine alone induces phase-shifting when applied during the light phase. Intraperitoneal cocaine injections at ZT 6 induced significant phase-advancement compared to vehicle injections of saline (Figure 2; 1.0±0.3 h vs. 0.3±0.1 h respectively; F_{2,7}=9.8; p<0.01).
Figure 2: Left: Acute systemic cocaine administration at midday (ZT 6) phase-advances the daily locomotor activity rhythm. Bars with different letters show significant difference (P < 0.05). Values are expressed as means ± S.E.M. Right: Double-plotted actograms of general locomotor activity showing cocaine-induced phase-advance shifts at midday (ZT 6). Asterisks denote time of treatments. 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.
Chronic Cocaine Actions in the Circadian System

Experiment 3 - Characterization of free-choice oral cocaine consumption

Quantification of the preference for consuming a cocaine solution (0.1 mg/ml) over water revealed an initial preference for drinking cocaine (~66%; average daily intake was 15.1±.05 mg/kg) which decreased until there was no significant preference between cocaine and water bottles at around day 10 (Figure 3). This preference increased during cocaine withdrawal (54±.03 vs. 46±.04%; F\textsubscript{2,20}=5.108; p=0.049) and again during reinstatement compared to both baseline and withdrawal (65±.04% vs. 46±.04% vs. 54±.03%, respectively; F\textsubscript{2,20}=6.587; p=0.007) strongly suggesting that the oral cocaine regiment is sufficient to induce reinforcement. Daily timing of oral cocaine consumption occurred with a significant preference for the dark portion of the LD cycle (Figure 4; 167±13.75 counts vs. 36±44.58 counts respectively; F\textsubscript{1,5}=7.89; p=0.04), which was indistinguishable for light/dark preference prior to cocaine exposure (data not shown). The number of total daily drinking events was also unaffected by the introduction of cocaine (234.67±39.54 counts vs. 248.33±56.20 counts; F\textsubscript{1,5}=0.04; p=0.85).
Figure 3: *Left:* Plot showing average daily cocaine and water consumption as means ± S.E.M. Notice the significant increase in preference following a period of withdrawal (shaded region). *Right:* Bar graphs showing reference (left) and dosage (right) for mice pre- and post-withdrawal.
Figure 4: Representative double plotted actogram (top) and drinkogram (bottom) illustrating that oral cocaine self-administration takes place primarily during the dark (active) phase of the light dark cycle. Horizontal filled bars represent the dark phase of the 24-h LD cycle. “C” indicates when cocaine treatment was initiated.
**Experiment 4 - Chronic cocaine effects on free-running period**

Exposure to sweetened water or sweetened cocaine solution (0.5 mg/ml) under LD had little effect on the phase angle of entrainment (-0.42±0.04 h vs. -0.50±0.023 h respectively; F_{1,69}=2.28; p=0.14) or alpha (11.77±0.06 h vs. 11.75±0.09 h respectively; F_{1,13}=0.03; p=0.87). Additionally, under LD, withdrawal of cocaine did not affect period (24.02±0.01 h vs. 24.02±0.02 h respectively; F_{1,8}=1.85; p=0.21) or alpha (11.75±.09 h vs. 11.67±0.10 h respectively; F_{1,13}=0.33; p=0.57). Under constant darkness, however, free-running period (tau) was significantly longer compared to controls (Figure 5; 24.06±0.01 h vs. 23.96±0.01 h, respectively; F_{1,9}=67.55; p<0.01). It is notable that this lengthening of tau persisted for 32 days after the withdrawal of the cocaine solution until the end of the experiment. Activity count analysis revealed that cocaine exposure caused no change in locomotor activity compared with pre-cocaine (days 59-65; 112.50±19.99 counts vs. 90.92±19.38 counts, respectively; F_{1,9}=0.59; p=0.46).

Evaluation of possible anticipatory activity to the presentation of cocaine via anticipation ratio revealed that the mice showed no increased activity prior to cocaine presentation compared to the same time prior to the start of the cocaine regimen (0.01±.003 % vs. 0.02±.003 %, respectively; F_{1,69}=1.15; p=0.29).
**Figure 5:** *Left:* Representative double-plotted actograms of general locomotor activity for mice receiving a daily 1-h pulse of sweetened water (top actogram) or sweetened cocaine solution (0.5 mg/ml; bottom actogram) at midday. The shaded area represents constant darkness. C, cocaine onset; SW, sweetened water onset; W, withdrawal. Horizontal filled bars represent the dark phase of the 24-h LD cycle. Note the difference in free-running period between the control and drug treated mice before and 3 weeks following withdrawal. Exposure to either fluid evoked behavioral arousal evident in the actograms. *Right:* Bar graph depicting the mean ± S.E.M. free-running period between control and cocaine mice. Group *n*’s are shown as numbers appearing within the bar for each group. Asterisk indicates significant differences between means.
Neurochemical Mechanisms for Cocaine Circadian Actions

Experiment 5 – Serotonergic mediation of cocaine’s phase-advancing effects at midday

Pretreatment with the 5-HT\textsubscript{1A,2,7} receptor antagonist metergoline (10 mg/kg i.p.) significantly attenuated the cocaine (20 mg/kg i.p.) mediated phase-advance at midday compared with controls as seen in Experiment 2 (Figure 6; -0.11±0.13 vs. 0.98±0.17 h respectively; F\textsubscript{1,17}=10.61; p>0.01). Note that no phase shift is induced by metergoline pretreatment followed by saline injection.
Figure 6: Left: Acute systemic cocaine administration at midday (ZT 6) phase-advances the daily locomotor activity rhythm (as seen in Experiment 2). This effect is blocked by metergoline. Bars with different letters show significant difference ($P < 0.05$). Values are expressed as means ± S.E.M. Right: 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.
Experiment 6 – Cocaine effects on photic phase-resetting in M172 mice

Consistent with the Experiment 1, i.p. cocaine pre-treatment prior to a light pulse during the early night significantly decreased the phase-resetting actions of light in WT mice compared to saline injected controls (Figure 7; 0.84±0.17 h vs. 1.60±0.07 h, respectively; p<0.01). SERT Met172 mice were indistinguishable from WT mice receiving a i.p. saline injection, however the diminished photic phase-resetting caused by systemic administration of cocaine was absent in these mice resulting in shifts similar to those seen in WT saline injected mice (1.38±0.24h vs. 1.49±0.45 h, respectively; p>0.84).
Figure 7: In vivo photic phase resetting of the SCN circadian clock. (A)
Representative double-plotted actograms of general locomotor activity showing
acute cocaine (20-mg/kg i.p.) attenuation of photic phase-delay responses to
light pulses delivered at ZT 16.5 in WT mice (lower left panel) but not
in SERT Met172 mutant mice (lower right panel) relative to saline controls (top
panels). (B) Histogram representation (mean ± S.E.M.) of behavioral rhythm data
showing inhibition of the phase-resetting action of acute cocaine (20-mg/kg i.p.)
in WT mice but not in SERT Me172 mutant mice relative to saline controls. Bars
with different letters are significantly different ($p < 0.05$). Numbers in bars are N's
per group.
**Experiment 7 – Cocaine effects on non-photic phase-resetting in M172 mice**

Consistent with **Experiment 2**, acute systemic administration of cocaine via i.p. injection resulted in a phase-advance at midday in WT mice compared with saline controls (**Figure 8**; 0.65±0.10 h vs. 0.28±0.13 h, respectively; p<0.04). This treatment did not elicit any phase-resetting in SERT Met172 mice, which were comparable to saline injected controls, indicating the successful abolishment of non-photic cocaine-induced clock-resetting in these mutants (-0.16±0.25 h vs. -0.02±0.01 h, respectively; p>0.4).
Figure 8: *In vivo* non-photic phase resetting of the SCN circadian clock. (A) Representative double-plotted actograms of general locomotor activity showing phase-advance shifting in response to acute cocaine (20-mg/kg i.p.) in WT mice (bottom left panel) that was not expressed in SERT Met172 mutant mice (bottom right panel) or in saline-injected controls (top panels). (B) Histogram representation (mean ± S.E.M) of behavioral rhythm data showing the phase-resetting action of acute cocaine (20-mg/kg i.p.) in WT mice but not in SERT Met172 mutant mice. Saline injections had little phase-resetting effect. Bars with different letters are significantly different (p < 0.05).
Chronic Cocaine Actions on Core Circadian Clock Output

Experiment 8 – Cocaine effects on neuronal multi-unit activity in the ventral tegmental area

Two-way ANOVA revealed a significant interaction between treatment and lighting conditions ($F_{1,20159}=18.164; p<0.001$), as well as significant main effects of cocaine treatment ($F_{1,20159}=5427.69; p<0.001$) and illumination ($F_{1,20159}=9053.28; p<0.001$). Post-hoc comparison of VTA output during the light and dark phase during baseline recording reveals a strong circadian fluctuation in electrical activity (Figure 9; 24402.68±3.70 spikes/minute vs. 24066.61±3.69 spikes/minute, respectively; $q=90.89; p<0.001$). This output rhythm was significantly altered by introduction to cocaine dissolved in drinking water due to increased expression both during the light (24402.68±3.70 spikes/minute vs. 24690.85±2.615 spikes/minute, respectively; $q=77.93; p<0.001$) and dark phase (24066.61±3.69 spikes/minute vs. 24323.26±2.68 spikes/minute, respectively; $q=69.41; p<0.001$).
**Figure 9:** A: Representative double-plotted actograms showing the circadian nature of spontaneous electrical output (MUA) from the VTA (top) and daily episodic drinking behavior (bottom). Note that the VTA rhythm is anti-phase to the behavioral rhythm. Horizontal filled bars represent the dark phase of the 24-h light-dark cycle. “C” indicates initiation of forced cocaine regimen. 

B: Photomicrograph of coronal brain section stained for tyrosine hydroxylase showing location of the recording electrode tip (PT) placed in the VTA (left portion of section). Following the end of the experiment, current was passed through the electrode to visualize electrode tip placement, resulting in the lesion evident in the right portion of section. 

C: Line plot illustrating mean hourly spikes/minute values during the light and dark phases (grey shading) of the light-dark cycle before and during cocaine self-administration.
Chapter IV

Discussion

The role of the circadian timing system in regulating physiological and behavioral responses to cocaine has only just begun to be understood. Reinforcement to cocaine (Ozburn et al., 2012), drug seeking (Sorg et al., 2011), psychomotor response (Abarca, 2002), behavioral sensitization (Uz et al., 2002), and drug-associated neurological remodeling (McClung et al., 2005) are under circadian control, suggesting that circadian involvement in the development and maintenance of drug abuse is more pervasive than previously thought. Reciprocally, cocaine administration has been shown to disrupt circadian modulation of daily feeding, endocrine and immune function, as well as clock gene expression outside of the SCN (Giorgetti and Zhdanova, 2000; Broadbear et al., 1999; Fox et al., 2008; Zhou et al., 1999; Irwin et al., 2007; Uz et al., 2005; Lynch et al., 2008). To date, however, very little is known about cocaine’s actions on circadian function per se or of the mechanism(s) which might underlie them. As the circadian system and the physiological response to cocaine potentially form a reciprocal regulation loop, it is critical to understand how cocaine disrupts the mammalian clock, which may produce psychological and physiological stress and contribute to a devolving pattern of increased cocaine abuse, withdrawal, and relapse. The focus of the present research project, therefore, was to elucidate cocaine effects on circadian function on
acute and chronic scales, as well as to investigate the location and mechanism of cocaine's actions.

**Acute Cocaine Effects**

The present work is the first to show that acute cocaine treatment directly affects the circadian system by altering the phase of the daily locomotor activity rhythm and disrupting the phase-resetting response to light. Specifically, acute systemic cocaine administered in the middle of the subjective day phase-advances the locomotor circadian rhythm approximately one hour. Importantly, cocaine directly targets the master pacemaker itself, as perfusion of cocaine directly into the SCN via microdialysis or *in vitro* on explanted SCN slices also results in an advanced daily rhythm peak (Glass et al., 2012). Additionally, systemic cocaine administered just prior to a light pulse during the early subjective night greatly reduces normal phase-resetting of behavioral rest-activity rhythms in response to light. The disruptive effects on photic phase-resetting also occur within the master pacemaker, as phase-delays to glutamate administration are completely abolished by cocaine pretreatment in SCN explants *in vitro* (Glass et al., 2012). Interestingly, recent evidence suggests that cocaine actions within the SCN may be mediated by the core clock gene PER2. Systemic administration
of cocaine at midday elicited phase-advances approximately 3.5 times greater than in WT mice, and the inhibition of photic phase-resetting during the early dark phase was also significantly stronger (Brager et al., 2013), indicating an inhibitory role of PER2 in cocaine-mediated circadian disruption. These results strongly suggest that cocaine is capable of altering circadian functioning and implicates the SCN as the direct target of cocaine’s actions.

**Chronic Cocaine Effects**

Substance abuse is a chronic behavior, taking place over months to years, making understanding long-term cocaine exposure effects on the functioning of the circadian time keeping system critical. To date, the number of circadian studies on chronic cocaine are few, and the most common methods of administration (intravenous infusion, catheterization, i.p. injection) are not suitable for long term circadian study due to their hindrance of circadian behavioral rhythms. The present work is the first to utilize cocaine dissolved in drinking water for assessing the disruptive effects of chronic cocaine in the circadian pathway; a method which provided a non-invasive, long-term, and self-administered regimen of drug application. Importantly, orally administered cocaine has been shown to have similar reinforcement properties and produce a PPC comparable with other methods of administration, though with a longer time course and greater subjective “high” (Post et al., 1974; van Dyke et al., 1978; Tang and Falk, 1987, 1990; Falk et al., 1990, 1991; Meisch et al., 1990, 1993; George et al., 1991; Lau et al., 1991; Marcucella et al., 1992; Seidman et al., 1992; Jentsch et al., 1998; Ma et al.,
has been previously shown that cocaine has disruptive effects on circadian regulation of corticosterone and prolactin rhythms (Mantsche et al., 2000), body temperature and heart rate (Tornatzky and Miczek, 1999), daily feeding patterns (Asami et al, 1996), and immune function (Irwin et al., 2007), but the present results reveal for the first time that these cocaine-mediated alterations in circadian expression may reflect disrupted function within the circadian system itself. Under LD circadian rhythm parameters (phase-angle of entrainment, alpha, frequency and distribution of activity), during chronic cocaine treatment were indistinguishable from water controls. However, under DD the free-running period, a critical endogenous property of the circadian clock, was observed to be significantly longer in the cocaine exposed mice. Subsequently, it was determined that this lengthening of tau presented after three, but not two, weeks of chronic cocaine self-administration. Importantly, this alteration in free-running period was persistent remaining long after access to cocaine was removed. In addition, mice on a chronic cocaine regimen displayed highly disrupted entrainment to a weak photoperiod. These mice entrained much faster to a 5-hour delayed skeleton photocycle, though 147° out of phase with water-only controls, effectively reversing the rest-activity rhythms in these mice (Stowie et al., 2015). As seen with free-running period, this entrainment effect persisted weeks after cocaine was discontinued. The persistent nature of the lengthened free-running period as well as the abnormal light entrainment suggest long lasting, possibly permanent perturbation of circadian function which may result in chronic physiological and psychological stress, contributing to increased cocaine abuse and further circadian disruption. Furthermore, the present results
demonstrate that while three weeks of chronic cocaine is sufficient to alter the endogenous period of the clock, these effects are masked under an LD cycle, theoretically desynchronizing the activity rest/activity from other internal rhythms generated by the master circadian clock.

**Location and Mechanism of Action**

Within the central nervous system, cocaine acts via blockade of reuptake transporters for dopamine, serotonin, and norepinephrine. While there are few dopamine receptors within the SCN itself, serotonin has been shown to phase-reset the clock as well as disrupt photic phase-resetting during the dark phase (Selim et al., 1993; Srkalovic et al., 1994; Glass et al., 2003; Prosser et al., 2003), consistent with cocaine effects in the present results. Acute systemic cocaine injection phase-advances the clock phase at midday, as does direct infusion of cocaine into the SCN in vivo as well as to the SCN slice in vivo (Glass et al., 2012). Photic phase-resetting is also attenuated by cocaine pretreatment preceding a light pulse during the early subjective dark phase, both in vivo and in vitro (Glass et al., 2012). Subsequently, it was demonstrated that pretreatment with the serotonergic receptor antagonist metergoline significantly attenuated non-photic phase-advances in behavioral activity rhythms by systemic cocaine injection as well as abolishing analogous shifts in SCN explants (Glass et al., 2012), strongly suggesting a serotonergic mechanism directly within the master pacemaker in mice. Further evidence for a critical role for serotonin in mediating cocaine effects within the circadian system is provided by the use of SERT MET 172 mice which are insensitive to the serotonergic effects of cocaine. The present work demonstrates that these mice do not display
disrupted photic phase-resetting in response to systemic cocaine pre-treatment, nor is the clock phase advanced by cocaine injection at midday. These effects are also absent in SCN slices exposed to cocaine in vitro, though they are still elicited by the serotonergic receptor agonist 8-OH-DPAT (Prosser et al., 2014). These mice also do not develop a long free-running period when subjected to a three week continuous access cocaine regimen (Prosser et al., 2014). Though the mechanism requires further investigation, it is possible that cocaine acts as a non-photic entrainment stimuli and disrupts phase-resetting to light by reducing the transcription/translation of Per/PER as previously associated with serotonergic signaling from the raphe (Horikawa et al., 2000). Taken together, these lines of evidence strongly implicate a cocaine-mediated serotonergic response that occurs directly in the SCN.

**Extra SCN cocaine-mediated circadian disruption**

While it has been shown that cocaine’s effects within the circadian pathway can be largely explained by its direct action within the SCN (Glass et al, 2012; Prosser et al., 2014), the large number of brain and peripheral areas which are affected by cocaine suggests that circadian disruption could also occur outside the master pacemaker. While dopaminergic signaling does not appear to be critical for mediating cocaine effects in the SCN, the mesolimbic reward pathway is heavily modulated by dopamine and possesses indirect regulatory influence on the circadian pathway through the VTA via the IGL (Card and Moore, 1989). Microinjection of cocaine into the VTA at midday elicited phase-advances in the behavioral rest-activity rhythm of mice (Brager et al., 2019).
suggesting a possible role for dopamine-mediated cocaine effects within the circadian pathway downstream of the SCN. The present work is the first to characterize a disruption of circadian neuronal firing within the VTA (part of the mesolimbic reward pathway) in response to chronic cocaine exposure. The increased amplitude of electrical output rhythm was observed under LD with no observable disruption in the behavioral rest-activity rhythm, providing further evidence for cocaine-mediated perturbation of circadian function being masked under bright light. In addition, rhythm disruption in the VTA developed after only one week of cocaine self-administration, while evidence suggests that serotonergic disruption within the SCN requires a longer exposure period (Stowie et al., 2015), indicating a possible role of dopamine signaling within the reward pathway in contributing to the development of persistent changes within the master pacemaker.

**Future Directions**

The present work has demonstrated chronic cocaine effects on core circadian function which are masked under an LD cycle. It will be critical to establish the length of time which these effects continue to act within the circadian pathway. Though very time intensive, the protocol used in Experiment 4 or the modified entrainment to light experiment (Glass et al., 2012) in which the disrupted rhythms are recorded for months after cocaine access is withdrawn would be demonstrate how long the clock is affected by persistent cocaine-mediated perturbations. It is also unknown what the potential behavioral or physiological effects of this disruption might be. Given the widespread influence which the circadian timekeeping system has throughout the body, especially via regulation of the neuroendocrine and autonomic nervous systems (Lehman et al.,
the potential for deleterious health outcomes associated with long-term cocaine-mediated circadian disruption requires further investigation. Electrophysiological recordings, like those in Experiment 8, within extra-SCN oscillators will be important for characterizing rhythm disturbance caused by exposure to a chronic cocaine regimen. Also, alteration in the sleep/wake activity cycle or longevity would be useful in assessing effects of long term cocaine-mediated circadian perturbation. Additionally, the mechanism by which persistent changes are made in the circadian system deserves additional attention. While the present study suggests that cocaine acts in an acute manner via serotonergic signaling in the SCN, how tau and the light entrainment mechanism are altered long after cessation of drug presentation is not known. Recent work has characterized cocaine induced epigenetic and transgenerational modification in both cocaine resistance and reinforcement (Vassoler et al., 2013; Pierce and Vassoler, 2014), suggesting a potential mechanism for persistent changes in circadian physiology. Understanding of how cocaine elicits long-term circadian disruption could potentially be utilized for improving health outcomes associated with cocaine abuse as well as the development of improved intervention strategies which might increase the success of cocaine abstinence and relapse-resistance.


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