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Circadian rhythms in reward and underlying neural circuits

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Graduate Program in Anatomy and Cell Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of
Philosophy
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CIRCADIAN RHYTHMS IN REWARD AND UNDERLYING NEURAL CIRCUITS

(Thesis format: Integrated-Article)

by

Ricardo M Baltazar

Graduate Program in Anatomy and Cell Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

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London, Ontario, Canada

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ABSTRACT

The circadian timing system influences many aspects of behaviour and physiology and previous evidence suggests a circadian modulation of drug-seeking behaviour and responsiveness to drugs of abuse. To further characterize daily rhythms in reward, diurnal variations in the rewarding value of systemic amphetamine and of the natural reward behaviour mating, were assessed. Diurnal rhythms were evident for both mating and amphetamine-related reward, but differed in the pattern of their timing with a peak during the mid-dark period for mating reward, and peaks during the late night and midday for amphetamine reward, with a nadir prior to the light-to-dark transition. To identify potential mechanisms for rhythmicity in reward, diurnal rhythms in expression levels of core clock proteins (Per1 and BMAL1) and tyrosine hydroxylase (TH), as well as makers of neural activation (cFos) were examined. Western blot analyses revealed rhythmic Per1 and Bmal1 protein levels in the nucleus accumbens (NAc) but not ventral tegmental area (VTA). By contrast, TH protein levels were rhythmic in both the NAc and VTA, with a peak in NAc coinciding with the peak of sex reward, while the peak in the VTA associated with the peak in amphetamine reward. Rhythmic expression of cFos immunoreactivity (IR) was observed in NAc, medial prefrontal cortex (mPFC), and VTA, with peak expression during the late night. Absence of clock gene oscillations in the VTA suggests that rhythms in mesolimbic activity are driven by timing signal originating elsewhere; and the mPFC was hypothesized to be a key mediator. The effects of mPFC lesions or inactivation on diurnal rhythms in cFOS-IR and amphetamine CPP were determined at previously observed peak and nadir times. Lesions attenuated the peak in cFos-IR in the NAc,

eliminating the diurnal rhythm, but had no effect on VTA rhythms. Moreover, daily differences in amphetamine reward were eliminated by mPFC lesions and inactivation, via an increase at the nadir. Together, these results indicate that diurnal rhythms of mesolimbic activity may be functionally relevant for rhythms in reward and that the mPFC plays a critical role in mediating such diurnal fluctuations.

Keywords: reward, circadian, sexual behaviour, psychostimulants, amphetamine, clock genes, Per1, BMAL1, dopamine, conditioned place preference, cFos, medial prefrontal cortex, excitotoxic lesions, GABA agonists, mesolimbic system,

CO-AUTHORSHIP

Chapter 1: Introduction was partially adapted from a review entitled “Bidirectional interactions between the circadian and reward systems: is restricted food access a unique zeitgeber” written by Dr. Ian Webb and Ricardo Baltazar, with inputs from Dr. Lique Coolen, and Dr. Michael Lehman. Chapter 2 is a published manuscript in the Journal for Biological Rhythms entitled “Diurnal variation in natural and drug reward, mesolimbic tyrosine hydroxylase, and clock gene expression in the male rat” written by Ricardo Baltazar and Ian Webb (shared first authors) with inputs from Dr. Lique Coolen and Dr. Michael Lehman. The experiment data included were collected and analyzed by Ricardo Baltazar and Ian Webb, with technical assistance with western blot analysis from Xu Wang. Chapter 3 entitled “Medial prefrontal cortex modulates diurnal rhythms in neural activation in the mesolimbic dopamine system”, was written by Ricardo Baltazar, with inputs from Dr. Lique Coolen and Dr. Michael Lehman. Studies were designed by Ricardo Baltazar, Dr. Lique Coolen, and Dr. Michael Lehman, conducted by Ricardo Baltazar with help from Dr. Ian Webb, and analyzed by Ricardo Baltazar. Chapter 4 entitled “Medial prefrontal cortex modulates diurnal rhythms in amphetamine reward’ was written by Ricardo Baltazar, with inputs from Dr. Lique Coolen and Dr. Michael Lehman. Studies were designed by Ricardo Baltazar, Dr. Lique Coolen, and Dr. Michael Lehman, conducted by Ricardo Baltazar with help from Dr. Ian Webb, and analyzed by Ricardo Baltazar.

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LIST OF ABBREVIATIONS

ACA, anterior cingulate area

BLA, basolateral amygdala

BNST, bed nucleus of the stria terminalis

CeA, central amygdala

CPP, conditioned place preference

CT, circadian time

DA, dopamine

DAB, diaminobenzidene

DAT, dopamine transporter

DG, dentate gyrus

DOPA, 3,4-dihydroxyphenylalanine

DOPAC, 3,4-Dihydroxyphenylacetic acid

ERK, extracellular-regulated kinases

GABA, gamma-aminobutyric acid

hr, hour

HVA, homovanillic acid

HVA, homovanillic acid

IL, infralimbic

i.p., intraperitoneal

IR, immunoreactive

IR, immunoreactivity

LD, light/dark

MAOA, monoamine oxidase A

mPFC, medial prefrontal cortex

NAc, nucleus accumbens

PB, phosphate buffer

PBS, phosphate buffered saline

PFC, prefrontal cortex

PL, prelimbic

s.c. , subcutaneous

SCN, suprachiasmatic nucleus

TH, tyrosine hydroxylase

VTA, ventral tegmental area

ZT, zeitgeber time

CHAPTER 1:Introduction¹

¹ Sections 1.1-1.4 were adapted from Webb IC, Baltazar RB, Lehman MN, Coolen LC (2009). Bidirectional interactions between the circadian and reward system: is restricted food access a unique zeitgeber? *European Journal of Neuroscience*, 30(9),1739-48.

Most organisms must select between different stimuli in their environment to maximize their chances for survival. To facilitate this selection, mammals possess specialized brain systems that allocate value to external stimuli (Tobler, Fiorillo, & Schultz, 2005). This determines whether a given stimulus is approached or avoided, and with what priority. Continued experience with the stimulus alters neural activity and structure in these circuits, and dictates the intensity and direction of future behavioural responses (Pitchers et al., 2010; Wolf, Sun, Mangiavacchi, & Chao, 2004). In general, stimuli that increase the frequency of the behaviours required to obtain them, are termed rewards (Kelley, 2004). Rewarding stimuli include resources necessary for survival, such as food and sex, but also substances with no apparent biological relevance, such as drugs of abuse. The value of a rewarding stimulus is subjective, and at any given time depends on various factors, such as the organisms internal homeostatic drive, its affective state, and its genetic background (Berridge, 2004; Murray, 2007; Nestler, 2000). Similarly, researchers have reported that the time-of-day also affects many rewards and reward-motivated behaviours. The mammalian circadian system regulates diurnal rhythms in many biological processes (Dibner, Schibler, & Albrecht, 2010), and has been reported to regulate the activity in brain areas related to reward. Likewise, the circadian system is also influenced by the timing of rewards (Kosobud et al., 2007), suggesting a bidirectional interaction between these two systems. The nature of this interaction remains poorly understood. The purpose of this work was to demonstrate the circadian regulation of reward. In particular, to provide a comprehensive characterization of daily rhythms in reward, natural and drugs of abuse, demonstrate their circadian properties, and

elucidate the underlying neural mechanisms that mediate them. The following literature review will summarize the general properties of the mammalian mesolimbic reward and circadian systems, and discuss the current evidence for circadian regulation of reward.

1.1 THE MESOLIMBIC REWARD SYSTEM

Anatomical mapping of reward-related brain areas, facilitated by the discovery that rats will learn to bar press when it is contingent upon electrical self-stimulation (Olds & Milner, 1954), has indicated that this system is comprised of a distributed series of brain structures located in both the midbrain and the basal forebrain. Central to this system is the mesolimbic dopaminergic pathway which originates in the ventral tegmental area (VTA) and projects via the medial forebrain bundle to the nucleus accumbens (NAc), amygdala, hippocampus, prefrontal cortex (mPFC) and other rostral structures (Kelley, 2004). As a whole, this system is thought to be the common substrate for reward associated with both natural stimuli and drugs of abuse as it is critical for the expression of appetitive behaviour in general (Ikemoto, 2007; Kelley & Berridge, 2002; Koob, 1992).

To date, the most thoroughly characterized reward-related neurotransmitter is dopamine and, though there exists a large body of literature, its functional role in this system remains a matter of debate (Berridge, 2007). Reward is comprised of multiple components including “liking” or the hedonic aspects associated with consumption and “wanting”, which reflects increased incentive salience (i.e., the attraction to external stimuli with appetitive properties). There is no compelling evidence implicating

dopamine in hedonic effects but a wealth of data suggests a role in reward seeking and incentive motivation (Berridge, 2007; Ikemoto & Panksepp, 1999). Recent evidence suggests that the induction of reward-related neuroplasticity is dependent upon the coordinated release of both dopamine and glutamate in the NAc (Kelley, 2004). GABA, serotonin, endocannabinoids, and opioid peptides are also involved in the regulation of the reward system (Koob, 1992) with the latter group of transmitters likely mediating hedonic effects via release in the NAc (Pecina, 2008).

1.2 THE CIRCADIAN SYSTEM

1.2.1 Circadian rhythms

Rhythmic control of essential life processes is ubiquitous throughout nature (Bell-Pedersen et al., 2005; Rapp, 1987). Nearly all organisms have developed mechanisms to drive daily rhythms in cellular processes, physiology, and behaviour (Dunlap, 1996, 1999). In mammals, increased complexity has driven the development of a multi-oscillator systems controlled hierarchically by a master time-keeping mechanism located in the brain (Ko & Takahashi, 2006; Mohawk, Green, & Takahashi, 2012; Reppert & Weaver, 2002). Precise control of the timing of biological processes has obvious advantages; it allows for the allocation of valuable resources to times of greatest benefit and maximizes biological efficiency by synchronizing complementary life processes. The role of a master clock is to organize the many behavioural and physiological rhythms to anticipate regularly occurring external events, thus conferring ecological validity upon this system. The particular environmental cycle that entrains circadian rhythms is referred to as a *zeitgeber*, or ‘time-giver’. The most consistent *zeitgeber* is the 24-hr

cycle of light and dark. As a result, clock mechanisms tend to oscillate with a near 24-hr cycle, termed *circadian* from the Latin for ‘*approximately a day*’. The circadian system is typically synchronized to the external daily light cycle, but can entrain to other zeitgebers that confer a survival advantage, such as the timing of rewarding stimuli (Angeles-Castellanos, Salgado-Delgado, Rodriguez, Buijs, & Escobar, 2008; Kosobud, et al., 2007; Mendoza, Angeles-Castellanos, & Escobar, 2005).

1.2.2 The master circadian clock

The master mammalian circadian oscillator is located in the suprachiasmatic nucleus (SCN) in the ventral portion of the hypothalamus (A. C. Liu, Lewis, & Kay, 2007; Reppert & Weaver, 2001, 2002; X. Zheng & Sehgal, 2008), a heterogeneous structure that is subdivided into shell and core regions. In general, the shell and core have two separate and unique functions. The shell region contains the endogenously self-sustaining clock mechanism, while the core is responsible for entraining the master clock to rhythms in the environment. The self-sustaining cellular clock mechanism consists of positive and negative transcriptional and translational feedback loops that drive oscillations of clock genes and their protein products (DeBruyne, Weaver, & Reppert, 2007; Ko & Takahashi, 2006; Reppert & Weaver, 2001, 2002). An entire cycle takes approximately 24-hours. The positive loop of this mechanism is made up of the transcription factors BMAL1 and Clock, which bind to E-boxes downstream of the clock genes *per(1&2)* and *cry* and regulate their expression. Once transcribed and translated, the corresponding clock proteins together make up the negative loop of the clock mechanism and inhibit *bmal1* and *clock* expression. These four clock genes make up the

central gears of the molecular clockwork mechanisms. Many additional core clock genes have been identified since, and are responsible for fine-tuning the timing of the circadian clock (Ukai & Ueda, 2010). Molecular clock gene oscillations culminate in the expression of rhythmic output signals from the SCN, including electrical, endocrine and metabolic, that synchronize subordinate extra-SCN oscillators (Albrecht, 2012; Dibner, et al., 2010).

1.3 CIRCADIAN INFLUENCES ON THE REWARD SYSTEM

1.3.1 Diurnal variation in reward and motivation

Initial support for circadian regulation of reward comes from research on addiction and drugs of abuse in humans. Long-standing anecdotal evidence of a daily rhythm in the admittance of drug overdose patients to the emergency room was formalized by studies reporting a peak in the evening (Morris, 1987; Raymond, Warren, Morris, & Leikin, 1992). Similarly, time-of-day differences in the efficacy and pharmacology of drugs-of-abuse have been known for some time (Ede, 1973; Erickson, Lee, Zautcke, & Morris, 1998; Lemmer, 1996), and nicotine self-administration is reported to be highest during the early morning and late evening (Mooney, Green, & Hatsukami, 2006). In animals, behavioural responses to many reward-related stimuli also vary by time-of-day. For instance, a diurnal rhythm in septal or hypothalamic electrical brain self-stimulation was observed in rodents that peaks during the middle-to-late dark (active) phase of the light/dark cycle, and persists under constant conditions with a near 24 hr period, suggesting it is circadian in nature. (Terman & Terman, 1970, 1975). Additionally, self-administration of addictive substances, such cocaine and alcohol, also

varies by time-of-day for monkeys and rodents (Baird & Gauvin, 2000; Deneau, Yanagita, & Seevers, 1969; Gauvin et al., 1997; Roberts & Andrews, 1997; Roberts, Brebner, Vincler, & Lynch, 2002). Time-of-day differences have been reported in several other behavioural measures of drug reward, including drug-induced conditioned place preference (CPP), sensitization, and locomotor activity (Abarca, Albrecht, & Spanagel, 2002; Akhisaroglu, Ahmed, Kurtuncu, Manev, & Uz, 2004; Gaytan, Lewis, Swann, & Dafny, 1999; Kurtuncu, Arslan, Akhisaroglu, Manev, & Uz, 2004). The general tendency amongst these findings is for increased drug consumption and reward during early-middle light-phase. Conversely, there is also evidence that drugs of abuse alter circadian rhythms and normal sleep-wake cycles (Hasler, Smith, Cousins, & Bootzin, 2012; Kosobud, et al., 2007), giving credence to bidirectional interaction between the reward and circadian systems.

1.3.2 Diurnal variation in mesolimbic dopaminergic activity and other neurotransmitters

Diurnal rhythms in different behavioural responses to rewarding stimuli suggest that circadian regulation exists at multiple levels of the mesolimbic reward system. Given the central role of dopamine within this system, researchers have initially focused on the activity of this neurotransmitter, with reports of daily rhythms in the concentrations of extracellular dopamine and markers of dopaminergic activity. Specifically, dopamine concentration in dialysate from the nucleus accumbens and striatum peak during the active phase, as does the dopamine precursor 3,4-dihydroxyphenylalanine (DOPA), and metabolites of dopamine, 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (Castaneda, de Prado,

Prieto, & Mora, 2004; Paulson & Robinson, 1996; Schade et al., 1993; Shieh, Chu, & Pan, 1997). Similarly, markers of dopamine activity, such as the concentration of tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine synthesis (Sleipness, Sorg, & Jansen, 2007), the concentration and activity of the dopamine transporter (DAT), which regulates dopamine clearance from the synapse (Sleipness, Jansen, Schenk, & Sorg, 2008; Sleipness, et al., 2007), and dopamine D2 receptor function (Tonissaar, Herm, Rincken, & Harro, 2006), are also rhythmic in PFC or NAc dialysate, with peaks during the dark phase. Concentrations of dopamine D2 and D3 receptors also vary diurnally in the striatum but instead peak during the late light phase (Akhisaroglu, Kurtuncu, Manev, & Uz, 2005).

Dopaminergic inputs to the prefrontal cortex and striatum originate in the VTA, making it a likely candidate to drive the observed rhythms in upstream brain regions, yet little is known in regards to molecular rhythms in this region. A group of researchers have recently reported diurnal rhythms in the neural impulse activity of a newly discovered population of neurons in the VTA (Luo & Aston-Jones, 2009a; Luo, Georges, & Aston-Jones, 2008). These neurons, which fire selectively during the active-phase, are non-dopaminergic, and express a vesicular glutamate transporter, suggesting that they are glutamatergic. Diurnal rhythms have also been reported in the activity of other prominent neurotransmitters in these areas, but not as extensively as those of dopamine. For instance, daily variations in glutamate, GABA, cholecystinin, and the serotonin metabolite 5-HIAA have been detected in the rat NAc (Castaneda, et al., 2004; Schade, et al., 1993). These results, taken together, suggest that diurnal rhythms in dopaminergic

and non-dopaminergic activity at multiple levels of the mesolimbic reward system may contribute to daily variations in reward. However, experimental manipulations are needed to directly evaluate this hypothesis.

1.3.3 Rhythmic clock gene expression in the mesolimbic reward system

The observed rhythms in reward and mesolimbic neurophysiology, if circadian in nature, may be mediated by intrinsic localized clock gene oscillations or, alternatively, may be conferred by neural projections or humoral output from the master clock. Self-sustaining clock gene oscillations are common to many mammalian bodily tissues (Dibner, et al., 2010; Guilding & Piggins, 2007; Yoo et al., 2004), where they regulate rhythms in cellular activity and physiology independently of the SCN while using many of the same molecular components (Dardente & Cermakian, 2007). Equally, core circadian clock genes (Reppert & Weaver, 2002) are expressed in mesolimbic structures and, in some instances, are rhythmic. Time-of-day differences in *Per(1&2)* expression have been reported for the PFC, NAc, striatum, VTA, amygdala, and hippocampus (Iijima, Nikaido, Akiyama, Moriya, & Shibata, 2002; Li, Liu, Jiang, & Lu, 2009; Uz, Akhisaroglu, Ahmed, & Manev, 2003). Peaks in clock gene expression generally occur during the mid-to-late dark phase throughout the reward system with a few exceptions during the mid light phase; such as *per1* in the NAc core, the central amygdala (CeA), and the VTA; and *per2* in the mPFC, the amygdala and hippocampus.

The recent development of a transgenic mouse model expressing a bioluminescent *per1* gene has permitted the continuous monitoring of rhythms in clock gene expression from isolated brain regions in vitro over many days, and thus facilitated

the identification of self-sustaining extra-SCN oscillators. (Yamazaki et al., 2000). Under these in vitro conditions, a rhythm in *per1* bioluminescence was not observed in the VTA and striatum (Abe et al., 2002), while the rhythm in striatal *per2* quickly dissipates after a few cycles (Natsubori, Honma, & Honma, 2012). It is unclear whether the loss of bioluminescence rhythms in these brain areas is due the dependence on an SCN entrainment signal or from being uncoupled from the reward network. In either case, the lack of clock gene rhythms suggests that daily changes in electrical (Luo et al., 2008; Luo and Aston-Jones, 2009), and dopaminergic activity (Castaneda, et al., 2004; Schade, et al., 1993; Tonissaar, et al., 2006) are driven by a signal originating elsewhere. The mPFC is a good candidate for driving rhythms in these arrhythmic reward areas as its excitatory glutamatergic efferents modulate the activity in many reward-related brain regions (Gabbott, Warner, Jays, Salway, & Busby, 2005), including the VTA and striatum, it is involved in the execution of reward behaviour (Hyman, Malenka, & Nestler, 2006; Miller & Cohen, 2001; Ridderinkhof, van den Wildenberg, Segalowitz, & Carter, 2004; Rolls, 2004), and exhibits rhythms in clock gene expression (Li, et al., 2009) and mesolimbic dopamine activity (Sleipness, et al., 2008). However, the role of the mPFC in the regulation of reward rhythms has yet to be examined.

The specific phase relationship between oscillations in clock gene expression and their protein products are critical to maintain the molecular clock mechanism (Ko & Takahashi, 2006). Though rhythms of clock gene expression have been examined in various regions of the mesolimbic system, little is known in regards to their protein products. However, diurnal rhythms in the number of cells that express

immunoreactivity (IR) for Per1 and Per2 were reported in some reward related brain regions; where the peaks in Per1-IR in the NAc shell, and in Per2-IR in the CeA was in phase with those of gene expression; while those of Per1-IR in the PFC and Per2-IR in the hippocampus had opposite phases (Amir, Harbour, & Robinson, 2006; Angeles-Castellanos, Mendoza, & Escobar, 2007; Lamont, Robinson, Stewart, & Amir, 2005). One group of researchers have reported daily differences in the concentrations of Per1 protein in the striatum that peaks in the early light phase of the light/dark cycle, and approximately 16 hours after the peak in gene expression (Akhisaroglu, et al., 2004; Uz, et al., 2003). As tissue was sampled from the entire striatum, these findings lack the spatial resolution necessary to make meaningful hypotheses regarding a functional relationship between rhythms in clock gene expression and mesolimbic activity.

In the master clock, clock proteins drive rhythms in clock gene expression by regulating the binding of the transcription factor Clock to E-boxes in their promoter regions (Ko & Takahashi, 2006; Reppert & Weaver, 2001). The promoter regions for monoamine oxidase A (*maoa*), TH, and the dopamine transporter also contain E-box elements (Hampp et al., 2008; Kawarai, Kawakami, Yamamura, & Nakamura, 1997; Yoon & Chikaraishi, 1992), which suggests that clock genes may directly regulate dopamine synthesis and metabolism. Indeed, Hampp et al. (2008) have reported that the *maoa* promoter is regulated by clock genes in vitro and that MAOA is downregulated in *per2* mutant mice. In addition, Clock has been localized to VTA dopaminergic neurons and mutations of this gene results in an overall increase in mesolimbic dopamine activity and synthesis (Coque et al., 2011; McClung, Nestler, & Zachariou, 2005; Spencer et al.,

2012). This includes increased VTA TH expression and phosphorylation, increased firing rates of VTA dopaminergic neurons, decreased soma size of VTA dopamine neurons, increased dopamine metabolites DOPAC and homovanillic acid in the dorsal striatum, and increased D1 and D2 receptor expression in the dorsal striatum. Thus, there appear to be direct links between circadian clock genes and mesolimbic dopaminergic activity.

It remains to be determined if other prominent mesolimbic neurotransmitter also express E-boxes in their promoter regions. However, there is some evidence for clock gene regulation of glutamate signalling. For instance *Per2* mutant mice exhibit altered glutamate and dopamine activity, specifically a decrease in the expression of the glutamate transporter EEAT, which increases extracellular glutamate, in the brain (Hampp, et al., 2008). Furthermore, DNase mediated cleavage of *Per1* in mice blocks morphine-induced increases in extracellular-regulated kinases (ERK) (Y. Liu et al., 2007). ERK activation is important for glutamate and dopamine signalling and for mediating the rewarding effects of cocaine (Valjent et al., 2000; Valjent et al., 2005).

1.3.4 Clock gene manipulation and reward processing

In addition to influencing dopaminergic and glutamatergic activity, studies with clock gene knock out or mutant animals have linked these genes to the regulation of reward. *per1* knockout mice do not sensitize to repeated cocaine injections nor do they show a cocaine-induced conditioned place preference response (Abarca, et al., 2002). Similarly, DNase mediated cleavage of *per1* inhibits morphine-induced conditioned

place preference when applied prior to conditioning but has no effect on recall (Y. Liu et al., 2005), suggesting that *Per1* may be important for the coding of learned reward associations. By contrast, *per2* mutants exhibit hypersensitization and exhibit a normal place preference response to cocaine (Abarca et al., 2002). Clock mutant mice also show an increased sensitivity to reward. These animals exhibit an elevated place preference to cocaine, increased cocaine-self administration, an increased preference for sucrose, and require lower currents to sustain electrical brain self stimulation (McClung et al., 2005; Ozburn, Larson, Self, & McClung, 2012; Roybal et al., 2007). At present, it is unknown if these multiple effects stem from a lack of clock gene expression in the mesolimbic dopaminergic system or elsewhere, or if they are due to a pleiotropic effect. In any case, the current body of data taken together strongly suggests that clock genes influence reward and motivation, and may do so via a direct modulation of dopaminergic activity.

1.3.5 The role of the SCN in regulating reward rhythms

Many extra-SCN oscillators are capable of self-sustained rhythmic output, however, these rhythms eventually dissipate in the absence of an entrainment signal (Yamazaki, et al., 2000). Individual cells in many rhythmic tissues are robust oscillators that sustain long-lasting circadian output in vitro (Nagoshi et al., 2004; Welsh, Yoo, Liu, Takahashi, & Kay, 2004), but the degree to which tissues are able to maintain circadian output independently of the SCN is positively correlated with the strength of coupling between the individual cells (Dibner, et al., 2010; Guilding & Piggins, 2007; Welsh, Takahashi, & Kay, 2010). Thus, the function of the SCN is to entrain rather than sustain rhythms in extra-SCN oscillators (Nagoshi, et al., 2004). While intrinsic mesolimbic

clock genes have been implicated in the regulation of reward activity, SCN lesions blunt or eliminate rhythms in Per1 and Per2 gene and protein expression in the amygdala and the bed nucleus of the stria terminalis (BNST), suggesting that the SCN also involved in the regulation of reward rhythms (Amir, Lamont, Robinson, & Stewart, 2004; Iijima, et al., 2002; Lamont, et al., 2005).

Two possible pathways have been suggested for SCN-entrainment of the mesolimbic system, however, the degree to which these pathways contribute to rhythmicity remains to be determined. The first, an indirect projection from the SCN to the VTA via the medial preoptic nucleus (Luo & Aston-Jones, 2009b). The second from the SCN to the VTA via lateral hypothalamic orexin neurons (Deurveilher & Semba, 2005; Korotkova, Sergeeva, Eriksson, Haas, & Brown, 2003). This second pathway is currently more promising as orexin neurons are known to be important for reward-induced behavioural and molecular responses (Aston-Jones, Smith, Moorman, & Richardson, 2009; Borgland, Taha, Sarti, Fields, & Bonci, 2006; Harris, Wimmer, & Aston-Jones, 2005; H. Zheng, Patterson, & Berthoud, 2007). Furthermore, there have been reports of SCN-dependent, diurnal rhythms in orexin cell activation and orexin release into the VTA (Akiyama et al., 2004; Estabrooke et al., 2001; Lee, Hassani, & Jones, 2005; Marston et al., 2008; Moorman & Aston-Jones, 2010; Zhang et al., 2004).

1.4 THESIS RATIONALE AND OBJECTIVES

1.4.1 Rationale

Time-of-day differences have been reported in behavioural responses to drugs of abuse, including self-administration (Baird & Gauvin, 2000), conditioned place preference (Kurtuncu, et al., 2004), behavioural sensitization (Akhisaroglu, et al., 2004), and drug induced locomotor activity, while diurnal changes in behavioural responses to natural reinforcers have yet to be characterized. A growing body of evidence suggests that rhythms in reward are controlled at the level of the mesolimbic reward system, as time-of-day differences have been reported for markers of dopamine, glutamate, and GABA signalling within areas of this system (Castaneda, et al., 2004; Luo, et al., 2008; Sleipness, et al., 2008). The precise mechanism that mediates rhythms in reward requires further elucidation, as does the nature of the timing signal that drives these rhythms. Tissues level quantification of gene expression has revealed that clock genes are rhythmic within the mesolimbic system (Iijima, et al., 2002; Li, et al., 2009; Uz, et al., 2003) and are important for normal reward behaviour and mesolimbic activities (McClung, Sidiropoulou, et al., 2005; Ozburn, et al., 2012). Moreover, their protein products can bind to the promoter regions of genes involved in the synthesis and metabolism of dopamine (Hampp, et al., 2008), however, daily oscillations of clock gene proteins have yet to be characterized. Clock gene rhythms are not ubiquitous to all mesolimbic areas that express oscillations in neural activity (Abe, et al., 2002), and may be driven by a timing signal originating elsewhere. The role of the master clock is to entrain extra-SCN oscillators (Nagoshi, et al., 2004; Welsh, et al., 2010), and surely two putative entrainment pathways have been identified (Deurveilher & Semba, 2005; Korotkova, et

al., 2003; Luo & Aston-Jones, 2009a), however, rhythms in reward can persist in the absence of an SCN timing signal (Iijima, et al., 2002; Sleipness, et al., 2007), which suggests that the mesolimbic system is intrinsically rhythmic. The mPFC is a potential candidate for regulating rhythms in reward, as it is important for regulation of reward (Hyman, et al., 2006; Miller & Cohen, 2001; Ridderinkhof, et al., 2004; Rolls, 2004), exhibits rhythms in clock gene (Angeles-Castellanos, et al., 2007; Li, et al., 2009) and dopamine activity (Castaneda, et al., 2004), and its excitatory glutamatergic efferents innervate other rhythmic mesolimbic areas (Gabbott, et al., 2005).

1.4.2 Hypothesis

Behavioural responses to drugs of abuse and natural rewards are dependent on the time of day. These daily differences reflect rhythms in the reward system, specifically in mesolimbic activity and clock gene expression. The prefrontal cortex plays an important role in orchestrating the timing of rhythms in reward and mesolimbic activity.

1.4.3 Objectives

1. To characterize diurnal and circadian rhythms in amphetamine and sex reward using the conditioned place preference paradigm (Chapter 2).
2. To characterize diurnal rhythms in protein markers of dopamine synthesis, and clock gene expression in the mesolimbic reward system (Chapter 2).
3. To characterize diurnal rhythms in cFos immunoreactivity in reward-related brain regions (Chapter 3).
4. To demonstrate the role of the prefrontal cortex in modulating rhythms in mesolimbic cFos immunoreactivity (Chapter 3).

5. To demonstrate the role of the prefrontal cortex in modulating rhythms in amphetamine conditioned place preference (Chapter 4).

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CHAPTER 2:

Diurnal Variations in Natural and Drug Reward, Mesolimbic Tyrosine Hydroxylase and Clock Gene Expression in the Male Rat¹

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2.1 INTRODUCTION

Convergent evidence indicates that the hypothalamic suprachiasmatic nucleus (SCN) is the locus of the master circadian pacemaker controlling daily rhythms in physiology and behaviour (Moore, 1983; Moore & Eichler, 1972; Ralph, Foster, Davis, & Menaker, 1990). This endogenous clock is primarily synchronized to the environmental light/dark cycle via photic stimulation and entrains the internal milieu to the external twenty-four hour world (Klein, Moore, & Reppert, 1991). The circadian clock mechanism is thought to be comprised of transcriptional/translational feedback loops that result in the rhythmic expression of core clock genes (e.g., *period1*, *clock*, *bmal1*) and their protein products with a periodicity of about 24h (Ko & Takahashi, 2006; Reppert & Weaver, 2002). Although clock genes are rhythmically expressed at extra-SCN brain regions and in peripheral organs, the phase relationships between these central and peripheral oscillators appear to be maintained by the SCN (Yoo et al., 2004).

The influence of the circadian timing system is ubiquitous and recent evidence suggests a diurnal or a circadian modulation of reward and motivation. Rodents have been reported to self-administer drugs of abuse in a diurnal fashion (Baird & Gauvin, 2000; Brebner, Froestl, Andrews, Phelan, & Roberts, 1999; Gauvin et al., 1997; Roberts & Andrews, 1997; Roberts, Brebner, Vincler, & Lynch, 2002) and daily rhythms have been observed in psychomotor stimulant-induced conditioned place preference, sensitization, and locomotor activation (Akhisaroglu, Ahmed, Kurtuncu, Manev, & Uz, 2004; Evans, Ghiselli, & Patton, 1973; Gaytan, Lewis, Swann, & Dafny, 1999; Gaytan, Yang, Swann, & Dafny, 2000; Kuribara & Tadokoro, 1982; Kurtuncu, Arslan,

Akhisaroglu, Manev, & Uz, 2004; Urba-Holmgren, Holmgren, & Aguiar, 1977; Uz, Akhisaroglu, Ahmed, & Manev, 2003; Uz, Javaid, & Manev, 2002). However, conflicting results have been reported and, in many cases, the diurnal variation appears dose-dependent (Baird & Gauvin, 2000; Gaytan, Swann, & Dafny, 1998a, 1998b; Roberts, et al., 2002). To build upon this body of evidence and to extend these observations to more natural rewards, here we test the hypothesis that daily rhythms exist in the rewarding value of natural stimuli (i.e., sex) and that these may differ from those related to drugs of abuse. We also examine diurnal variation in the locomotor activating effect of acute systemic amphetamine administration.

The mechanisms responsible for rhythmic drug responsiveness are not entirely clear. However, as reward processing is largely mediated by the mesolimbic dopaminergic pathway connecting the midbrain with limbic forebrain areas (Hyman, Malenka, & Nestler, 2006; Kelley, 2004; Kelley & Berridge, 2002), regulation likely occurs at the level of this system. Indeed, daily rhythmic electrical activity and *c-fos* expression have been detected in neural subpopulations within the nucleus accumbens (NAc) and the ventral tegmental area (Angeles-Castellanos, Mendoza, & Escobar, 2007; Luo, Georges, & Aston-Jones, 2008; Yamazaki, Kerbeshian, Hocker, Block, & Menaker, 1998), and virtually all indices of NAc dopaminergic activity exhibit a diurnal rhythm (Castaneda, de Prado, Prieto, & Mora, 2004; Shieh, Chu, & Pan, 1997; Sleipness, Sorg, & Jansen, 2007b; Tonissaar, Herm, Rincken, & Harro, 2006). The observed rhythms in reward and dopaminergic activity, if circadian in nature, may be mediated by intrinsic clock gene oscillations within the mesolimbic system, or, alternatively, may be conferred

by neural projections or humoral output from other rhythmic structures including the SCN. Thus, to further explore potential mechanisms underlying natural or drug reward rhythms, daily variations in tyrosine hydroxylase (TH; the rate limiting enzyme in dopamine synthesis) and core clock protein levels were examined in both the NAc and the VTA, central components of the mesolimbic reward system.

2.2 METHODS

2.2.1 Animals

Young adult male Sprague-Dawley rats (300-400g, Charles River Laboratories, QC, CAN) were singly housed in clear plexiglass cages under a 12:12 light/dark cycle (~350lux L/0 lux D) with food and water available *ad libitum*. A minimum of two weeks prior to any experimental procedures were allotted to allow for stable entrainment. To facilitate testing during the dark phase of the LD cycle, red lights (~10-15 lux) were employed during transport of the animals to the testing room and over the course of the manipulations. All experiments were approved by the University of Western Ontario Animal Care Committee and are in accordance with the guidelines set out by the Canadian Council on Animal Care.

2.2.2 Drugs

D-amphetamine sulphate (Sigma-Aldrich Ltd. ON, Canada) was dissolved in sterile saline, calculated based upon the free base, and administered in a volume of 1mL/kg body weight.

2.2.3 Conditioned Place Preference Testing

Apparatus

The conditioned place preference (CPP) apparatus (Med Associates Inc., VT, USA) consisted of two distinct plexiglass chambers (28 L x 21 W x 21 H cm) connected by a narrow hallway (12 L x 22 W x 21 H cm) via sliding doors. One chamber had black walls with a floor consisting of parallel steel bars, the other had white walls with a metal grid floor, and the connecting hallway had grey walls with a flat plexiglass grey floor. All compartments were covered with a clear ventilated ceiling that was hinged and could be opened to move animals in and out or locked to prevent escape. The time spent within each chamber was monitored by an array of infrared beams and was recorded via Med PC software (Med Associates Inc.). The chambers were cleaned thoroughly with a solution of 70% ethanol to ensure that they remained olfactory neutral following each trial. To minimize the potential for photophobic responses during the light phase of the LD cycle, the CPP apparatus was positioned to equalize light intensity across the chambers.

General Procedure

CPP testing consisted of three phases taking place over four days. During the pre-conditioning phase, performed on day 1, rats were placed in the central hallway and allowed free access to the entire apparatus for 15 minutes. The chamber in which the animal spent most time was designated the initially preferred chamber. Any animal spending more than twice as much time in one chamber as compared to the other was considered an outlier and excluded from the study (2 % of all animals). To facilitate

comparisons with previous work, a biased design was used for the sex reward experiments (Pfaus, Kippin, & Centeno, 2001; Tenk, Wilson, Zhang, Pitchers, & Coolen, 2009), while an unbiased approach was used for the drug reward studies (Tzschentke, 1998, 2007). With a biased design, conditioning assignments were organized such that the reward was paired with the initially non-preferred chamber. For the unbiased approach, half of the animals had the reward paired with the initially preferred chamber and half with the initially non-preferred chamber. To minimize any potential entrainment to the reward, conditioning took place over two days with only one reward pairing. On one of the conditioning days, the animals received the reward and were confined to one of the chambers for 30 min. On the alternate day, the animals did not receive the reward and were placed in the opposite chamber, again for 30 min. These manipulations were performed in counterbalanced order across animals. On day 4, the animals were retested for chamber preference, as in the pre-conditioning phase.

Each animal was conditioned and tested at only one of four zeitgeber times (ZT): ZT05, ZT11, ZT17 or ZT23, where, by convention, ZT12 corresponds to lights off. To rule out a change in preference due to habituation alone, control animals which did not receive the reward on either of the conditioning days, were included for each experiment and were tested at one time point.

The change in chamber preference was expressed as the time spent in the reward-paired chamber post-conditioning minus pre-conditioning. To determine the extent of reward conditioning and to identify any diurnal variation, the change in chamber

preference at each time point was compared both to the negative control and to all other time points via unpaired t-tests.

2.2.4 Open Field Apparatus

Horizontal locomotor activity was assessed via a plexiglass open field activity chamber (43.2 x 43.2 cm; Med Associates Inc.) equipped with a 16 x 16 infrared beam array to monitor the animal's movement. The chamber was wiped with 70% alcohol and lined with fresh corn cob bedding prior to each trial.

2.2.5 Experiment 1: Diurnal and circadian variation in male sexual performance and sex-related reward

Sexual Experience

To ensure adequate performance during the conditioning stage, while at the same time preventing potential entrainment to the reward, sexually naïve male rats (n = 37) were given eight bouts of sexual experience (2 sessions/wk over a 4 wk period); randomized over four different time points (for half the animals, n = 20: ZT04, 10, 14, or 23 and for the other half, n = 17: ZT02, 11, 13, or 22). During each trial, males received a sexually receptive female in the home cage. These young sexually experienced adult females were ovariectomized and implanted with subcutaneous silastic capsules (1.98 mm inner diameter, 0.5 cm length, Dow Corning Corp., MI, USA) containing 5% 17- β -estradiol benzoate (Sigma-Aldrich Ltd., ON, CAN). To induce sexual receptivity, 500 μ g progesterone (Sigma-Aldrich Ltd., ON, CAN) dissolved in 0.1 ml of sesame oil was administered s.c. 4 hours prior to the mating sessions. The males were allowed to mate

until either the first post-ejaculatory intromission or until one hour had elapsed. To assess any temporal variations in sexual performance, the percentage of animals ejaculating and the latency to ejaculation were recorded and compared across time points. Daily variation in the percentage of ejaculation and latency to ejaculations were assessed via Fisher's exact test or Kruskal-Wallis ANOVA followed by Dunn's multiple comparison tests, respectively.

Experimental Protocol

The conditioning trials were carried out using a biased design as described above. On one of the conditioning days, the males received a receptive female in the home cage and, immediately following ejaculation, were placed in the sex-paired chamber. On the alternate day, males were removed from their homecages and placed into the opposite chamber. Animals that failed to ejaculate within one hour were removed from the study leaving 6-7 animals per time point (ZT5, n = 7; ZT11, n = 6; ZT17, n = 7; ZT23, n = 6). A control group (n = 12) consisting of males that did not receive sex on either conditioning day also was included at ZT17. This time point was selected post-hoc to coincide with the observed peak in sexual reward.

To determine if the observed diurnal variation was circadian in nature, a separate group of sexually naive males was housed under constant darkness and tested at approximately circadian time (CT) 05 (n = 10) and 17 (n = 10). As circadian phase is difficult to assess from rat locomotor activity rhythms measured via telemetry and wheel-running associated reward (Lett et al. 2000; 2001) may have influenced our results, the

males were placed into darkness for only two days prior to testing under the assumption that their internal phase would not drift significantly within that time. Thus, CT was estimated in reference to the light-dark schedule prior to being placed into constant conditions.

2.2.6 Experiment 2: Diurnal variation in amphetamine-related reward

To prevent injection related anxiety during conditioning, rats received sterile saline (1 ml/kg, s.c.) on three consecutive days prior to testing. On one of the conditioning days, the animals received amphetamine (1.75 or 2.5 mg/kg, s.c.) and were confined to the drug-paired chamber. On the alternate conditioning day, the rats received saline (1 ml/kg, s.c.) and were placed in the non-paired chamber. At both the lower dose (n = 12 per time point) and the higher dose (n = 24 per time point except at ZT23 where n = 23), conditioning assignments were carried out in an unbiased fashion. A control group (n = 12) of animals receiving saline on both conditioning days was run at ZT23, a time-point chosen post-hoc to coincide with the observed peak in 2.5 mg/kg amphetamine reward.

2.2.7 Experiment 3: Diurnal variation in amphetamine-induced locomotor activity

To determine if amphetamine-induced locomotion varied by time-of-day, male rats were tested at one of four times (ZT05, ZT11, ZT17 or ZT23; n = 8 per time point). Horizontal locomotor activity was first recorded following saline injections (1 ml/kg, s.c., at the same time as the test for amphetamine) on three consecutive days. On the fourth day, each animal received amphetamine (1 mg/kg, s.c.). Immediately following each

injection, the animals were placed into the open field apparatus for 1 h. Data were analyzed by conventional or Kruskal-Wallis ANOVA followed by Bonferroni comparisons or Dunn's multiple comparison tests where appropriate.

2.2.8 Experiment 4: Diurnal variation in mesolimbic TH and clock gene protein levels

Tissue Collection and Protein Isolation

Rats were euthanized with sodium pentobarbital (125 mg/kg, i.p.) at 6 different time points (ZT02, 06, 10, 14, 18, and 22; n = 8 per time point). Brains were rapidly removed, frozen on dry ice, and stored at -80°C until processing. The VTA, the NAc, and the SCN were microdissected and homogenized mechanically in RIPA buffer (50mM Tris-HCL; 150mM NaCL; 1% Nonidet P 40; 0.1% sodium dodecyl sulphate [SDS]; 0.5% sodium deoxycholate) supplemented with a protease inhibitor cocktail tablet (Hoffman-La Roche Ltd., ON, CAN.). The homogenization mixture was centrifuged at 12,000 rpm for 20 min at 4°C and the supernatant collected. Protein concentrations were determined using a BCA assay (ThermoFisher Scientific Inc., MA, USA) and a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific Inc., MA, USA).

Western Blot Analysis

Protein samples of equal volume (15µL) and concentration (10µg/µL) were boiled at 96°C for 4 min using an AccuBlock Digital Dry Bath (Labnet International, NJ, USA) and cooled on ice prior to loading on a 10% polyacrylamide gel and separation under reducing conditions using a Mini Trans-Blot Cell system (Bio-Rad Laboratories Ltd., ON, CAN) and Tris-Glycine-SDS running buffer (25mM Tris; 192mM Glycine; 0.1%

w/v SDS, pH 8.3; Bio-Rad Laboratories Ltd.). Precision Plus Protein All Blue Standards (Bio-Rad Laboratories Ltd., ON, CAN) were used as molecular weight markers. All samples were run in quadruplicate and balanced by time point across the individual gels.

Following separation, the proteins were transferred to Millipore Immobilon-FL polyvinylidene difluoride membranes (Millipore, MA, USA) using the Mini Trans-Blot Cell wet blotting system (Bio-Rad Laboratories Ltd., ON, CAN). The membrane transfer was run in transfer buffer [10% methanol in Tris-Glycine (25mM Tris; 192mM Glycine pH 8.3), Bio-Rad Laboratories Ltd., ON, CAN] at 85V for 1 h at room temperature (RT).

Next, the membranes were blocked in a 2:3 solution of Odyssey Blocking Buffer (OBB; LI-COR Biosciences, NE, USA) and Tris-Buffered Saline (TBS; 50mM Tris and 150mM NaCl, pH 8.0) for 40 min on a shaker tray at RT. Samples were then individually incubated for 16 h on a shaker at 4°C with goat anti-Period1 (1:4,000, custom made by Bethyl Laboratories, Inc., TX, USA) or rabbit anti-Bmal1 (1:200, Affinity BioReagents, CO, USA). All blots were also simultaneously incubated with mouse anti-TH (1:200,000, Chemicon International, CA, USA) and mouse anti-GAPDH (1:200,000; Chemicon International), the latter of which was used as a loading control. All antibodies were diluted in 2:3 mix of OBB with TBS-T (TBS + 0.05% v/v Tween-20, pH 8.0). Following three 10 min washes in TBS-T, the membranes were incubated in secondary antibodies diluted in a 2:3 mix of OBB and TBS-T for 1 h at RT. The secondary antibodies used included Alexa-680 conjugated donkey anti-goat (1:5000; Invitrogen, CA, USA), Alexa-680 conjugated goat anti-rabbit (1:5000, Invitrogen), IR-

Dye 800 conjugated goat anti-mouse (1:10,000, Rockland, PA, USA), and IR Dye 800 CW conjugated goat anti-mouse (1:10,000; LI-COR Biosciences). Bands of immunoreactivity were visualized and images captured using an Odyssey 2.1 scanner (LI-COR Biosciences). Ratios of protein/GAPDH intensity levels were determined for each protein sample and averages calculated for each animal. Ratios for each animal were normalized to the overall mean for all time points and thus expressed as fold changes over the mean expression across the day. Data were analyzed by conventional or Kruskal-Wallis ANOVA followed by t-tests, Bonferroni comparisons or Dunn's multiple comparison tests where appropriate.

All antibodies resulted in single bands of immunoreactivity, except for Period1, which labelled multiple bands (see results section) similar to those described previously for the mouse (Chilov, Hofer, Bauer, Wenger, & Gassmann, 2001). Specificity of the Period1 and Bmal1 antibodies was demonstrated by a complete loss of immunoreactivity following preabsorption with the immunizing peptides (1 and 10ug/mL) overnight at 4°C prior to incubation with the membrane.

2.3 RESULTS

2.3.1 Experiment 1: Diurnal and circadian variation in sexual performance and sex-related reward

During the training period, measures of sexual performance varied with a diurnal pattern. The percentage of animals that ejaculated during the mid-night to the mid-day (ZT14 – ZT04; $p < 0.0001 - 0.022$) was significantly higher as compared to time points

surrounding the light-to-dark transition; (ZT11 - ZT13; Fig. 1A). The latency to first ejaculation also varied diurnally ($p < 0.001$) with significantly longer latencies observed near the light-to-dark transition (ZT11-ZT13) as compared to other time points (ZT02, ZT04, ZT22 and ZT23; $p < 0.001-0.05$; Fig. 1B).

The ejaculation-induced change in preference also varied by time-of-day as rats spent significantly more time in the sex-paired chamber only at ZT17 as compared to the no sex control animals ($p = 0.006$; Fig. 1C). In addition, the change in preference was significantly higher at ZT17 as compared to ZT05 ($p = 0.050$). This daily variation remained under constant conditions (CT17 vs. CT05; $p = 0.046$) suggesting that the daily rhythm in sex-related reward may be circadian in nature.

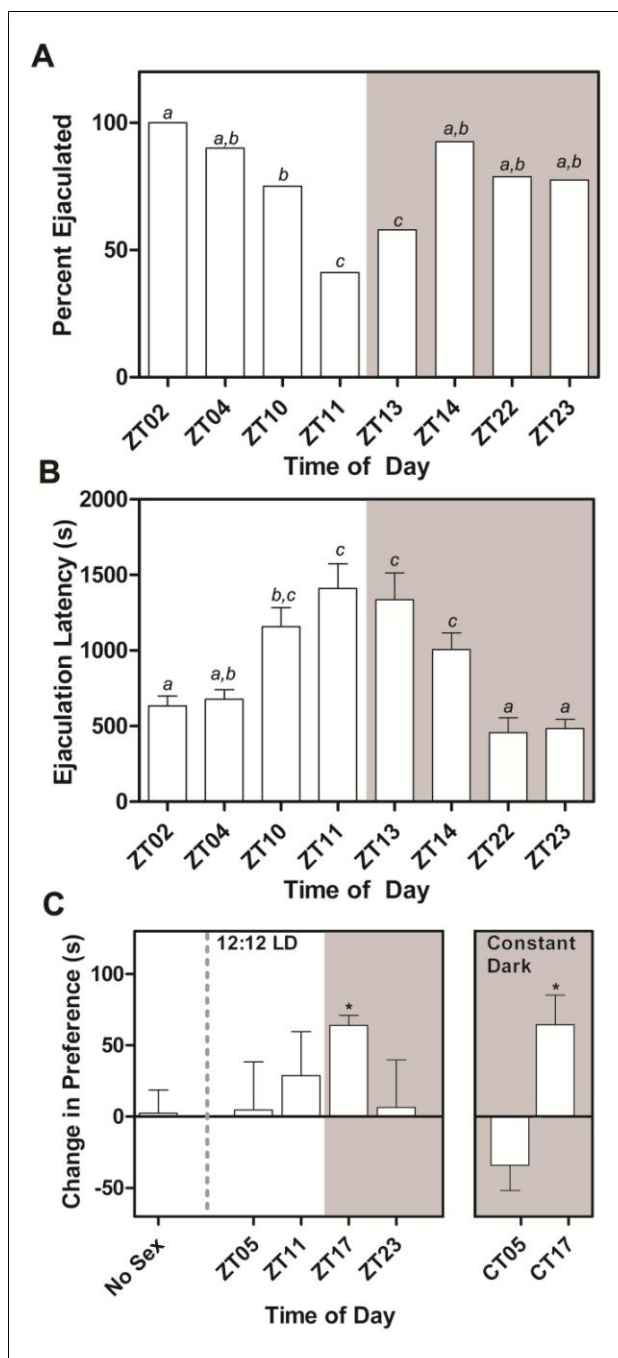


Figure 1 – Diurnal variation in sexual behaviour and sex-related reward in the male rat. A. The percentage of male rats that ejaculated during training at different time points. B. The mean latency to first ejaculation during training at different time points. C. The change in chamber preference induced by ejaculation under a 12:12 light:dark cycle

and under constant darkness. A no sex negative control was performed at ZT16. Data are expressed as mean \pm SEM. The non-shaded and shaded areas correspond light and dark, respectively. Bars with different letters are significantly different and the * denotes a significant difference between the labelled bar and the no sex control, ZT05 or CT05.

2.3.2 Experiment 2: Diurnal variation in amphetamine-induced reward

At all time points, 1.75 mg/kg amphetamine did not induce a significant change in chamber preference as compared to the saline negative control and the magnitude of the change in preference did not vary across time points (Fig. 2). However, at 2.5 mg/kg, amphetamine induced a significant change in preference at ZT05 ($p = 0.007$), ZT17 ($p = 0.026$) and ZT23 ($p = 0.011$) relative to saline control group. Comparisons across the time points revealed that the change in preference was significantly higher at ZT05 ($p < 0.001$), ZT17 ($p = 0.003$) and ZT23 ($p < 0.001$) as compared to ZT11.

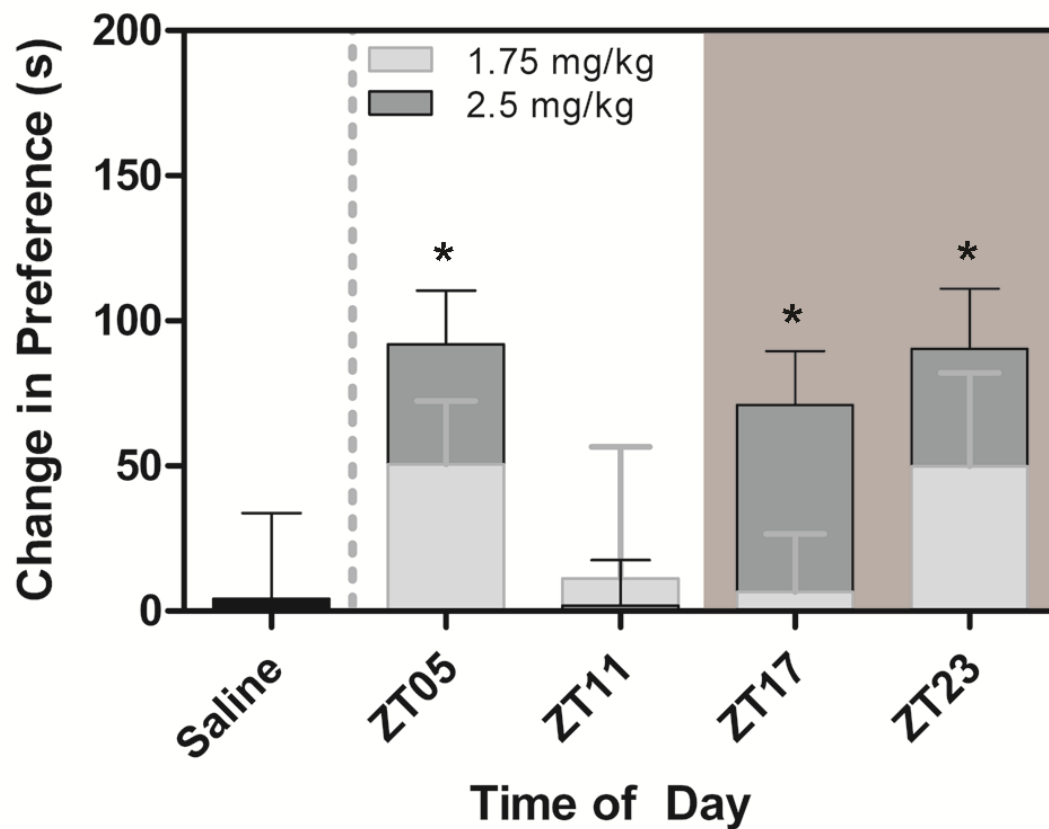


Figure 2 – Diurnal variation in the conditioned place preference induced by two systemic doses of amphetamine. Data are expressed as mean \pm SEM. * = significantly different from ZT11 and the saline control at 2.5 mg/kg dose.

2.3.3 Experiment 3: Amphetamine-induced locomotor activity varies in a diurnal fashion

As expected, baseline locomotor activity following saline injections varied diurnally ($K = 11.0$, $p = 0.015$) with significantly greater horizontal locomotion observed during the mid-dark period as compared to the late-light period (ZT17 vs. ZT11, $p = 0.015$; Fig. 3A). The locomotor activity elicited by amphetamine also varied significantly by time-of-day ($F = 6.9$, $p = 0.001$; Fig. 3B) with more ambulations recorded at ZT23 as compared to all other time points ($p < 0.050$). As compared to the saline control injections, the total horizontal distance travelled following amphetamine administration was significantly higher at ZT05 ($p = 0.003$), ZT11 ($p < 0.001$), and ZT23 ($p < 0.001$). When subtracted from the saline baseline data, locomotor activity induced by amphetamine varied significantly by time of day ($F = 0.34$, $p = 0.008$, Fig. 3C) and post-hoc analysis revealed significantly higher activity at ZT23 as compared to ZT17 ($p = 0.005$; Fig. 3C).

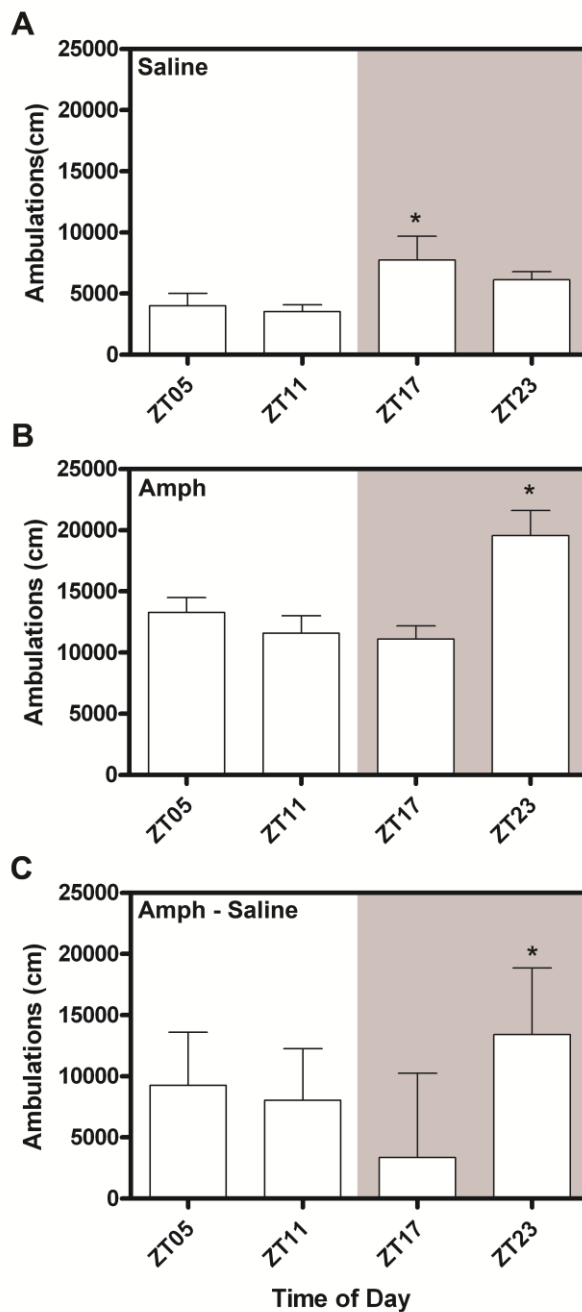


Figure 3 - Diurnal variation in the locomotor activating effect of acute amphetamine (1 mg/kg) administration. A. Daily variation in baseline horizontal locomotor activity following saline administration. B. Daily variation in horizontal locomotor activity following amphetamine administration C. Daily variation in horizontal locomotor activity

following amphetamine administration with the baseline saline data subtracted out. Data is expressed as mean \pm SEM. * = significantly different from ZT11; # = significantly different from all other time points; + = significantly different from ZT17.

2.3.4 Experiment 4: Rhythmic TH and clock gene expression in the VTA, NAc and SCN

Although the overall ANOVA was non-significant, subsequent post-hoc analysis revealed that VTA TH protein levels were significantly higher at ZT6 as compared to ZT10 ($p = 0.010$), 14 ($p = 0.005$), and 18 ($p = 0.024$; Fig. 4A). However, neither Period1 (~100 kDa; ~80 kDa; ~45 kDa; Fig. 5A-C) or Bmal1 (Fig. 5D) varied significantly across the day in this region. Within the NAc, TH protein levels also varied in a diurnal fashion ($F = 2.9$, $p = 0.023$; Fig. 4B) and were significantly higher at ZT14 as compared to ZT06 ($p = 0.011$). The NAc Period1 ~45 kDa band showed a daily rhythm as well ($F = 2.8$, $p = 0.036$) with increased levels observed at ZT22 as compared to ZT02 ($p = 0.027$, Fig. 5G). However, the ~150 kDa (Fig. 5E) and ~100 kDa (Fig. 5F) bands did not show a significant variation across the day. NAc Bmal1 protein levels also varied diurnally ($K = 24.6$, $p < 0.001$; Fig. 5H) with increased expression observed at ZT22 ($p < 0.001$) and ZT18 ($p < 0.001$) as compared to ZT02, and at ZT18 as compared to ZT06 ($p < 0.001$). As expected, rhythmicity was detected in the SCN Per1 ~100 kDa band ($F = 3.9$, $p = 0.009$; Fig. 5J) with protein levels significantly higher at ZT02 ($p = 0.035$) and ZT14 ($p = 0.011$) as compared to ZT06. By contrast, the SCN ~45 kDa Period1 band (Fig. 5K) and Bmal1 (Fig. 5L) did not vary significantly across the day.

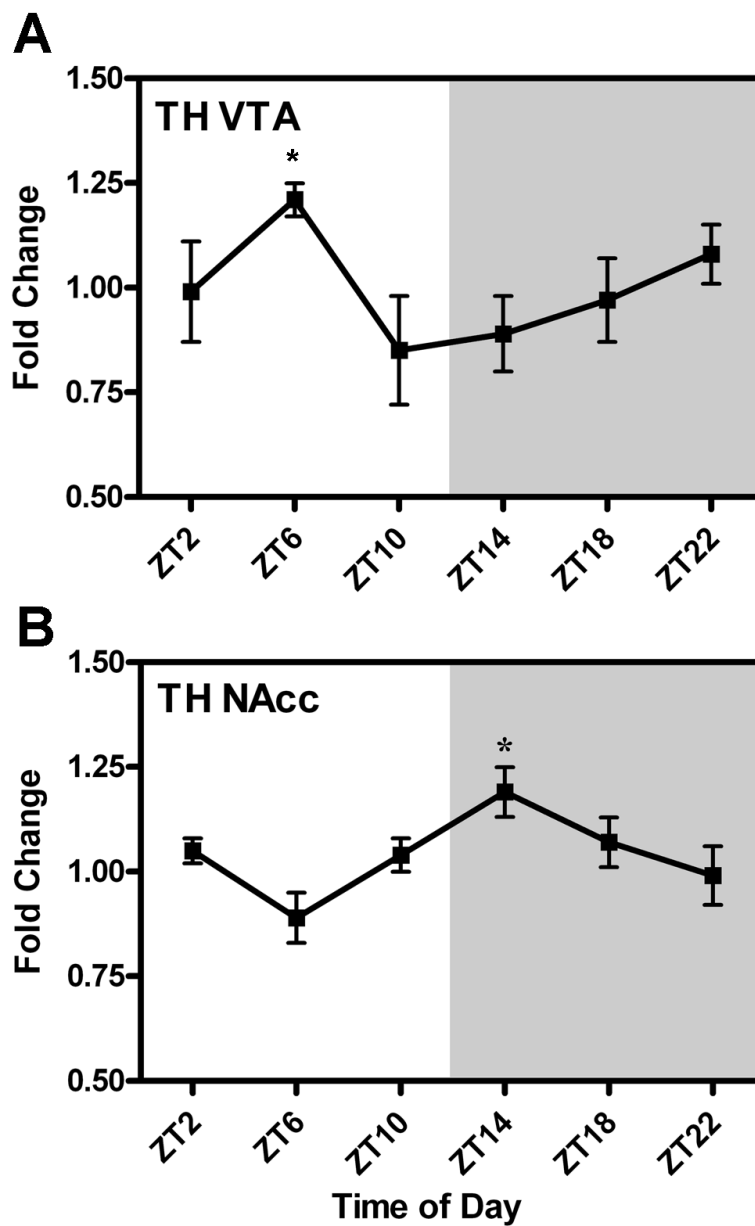


Figure 4 – Diurnal variation in tyrosine hydroxylase (TH) expression in the reward system. Shown are the fold change in the ratios of TH to GAPDH relative to the overall mean of all time points in the **A.** ventral tegmental area (VTA; * = significantly different from ZT10-18) and **B.** the nucleus accumbens (NAc; * = significantly different from ZT6). Data are expressed as mean \pm SEM..

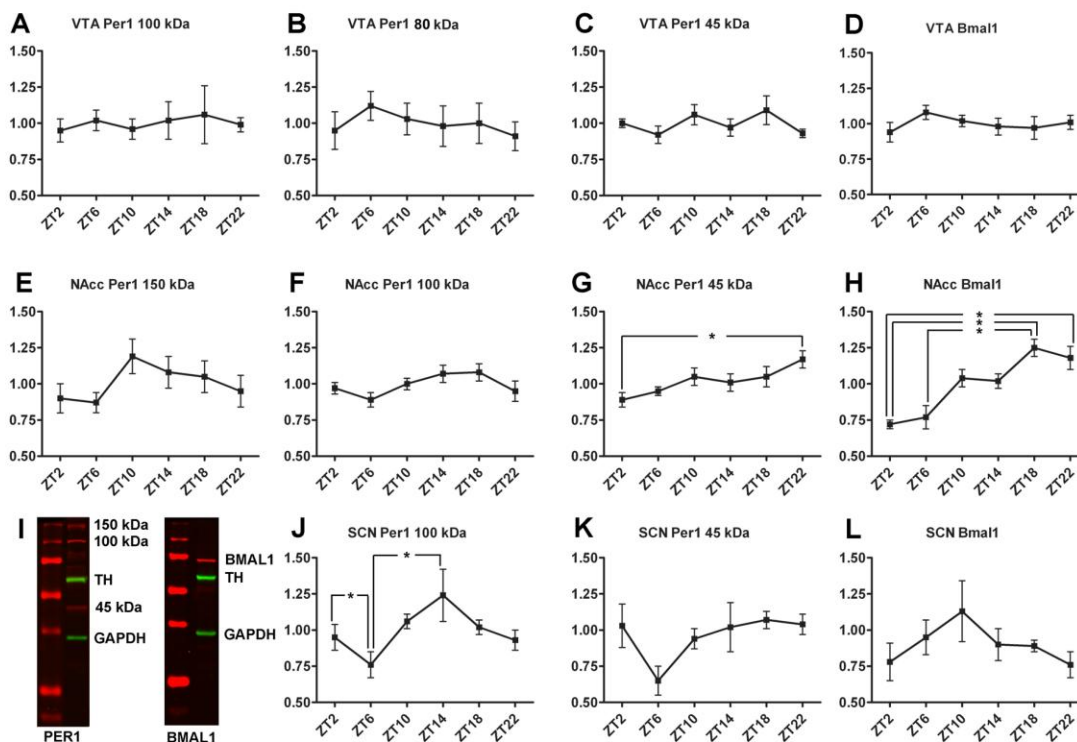


Figure 5 – Diurnal variation in clock gene expression in the reward system and in the suprachiasmatic nucleus (SCN). Shown are the fold change in ratios of Period1 and Bmal1 to GAPDH relative to the overall mean for all time points in the ventral tegmental area (VTA; 3 bands; ~100, 80, 45 kDa; A-D), the nucleus accumbens (NAcc; 3 bands; ~150, 100, 45 kDa; E-H) and the SCN (I-L). Also shown are the western blots for Period1, Bmal1, tyrosine hydroxylase (TH), and GAPDH (I). Protein standards (from top to bottom): 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37kDa, 25 kDa, 20kDa. Data is expressed as mean \pm SEM.

2.4 DISCUSSION

We show here that mating and drug-related reward as assessed by conditioned place preference vary in a diurnal fashion but exhibit a difference in timing relative to each other, with the peak in sex reward occurring in the early night (ZT17) and maximal amphetamine-related reward occurring both during the night and early day (ZT17-05) with a nadir in the late day (ZT11). The differences in timing of peaks and nadirs observed for these rewards are suggestive of modulation at multiple levels of the mesolimbic reward system. Indeed, analyses of TH, *Period1*, and *Bmal1* revealed a differential temporal expression with cyclic expression of clock genes observed in the NAc and TH rhythmicity seen in both the VTA and NAc. Interestingly, the rhythms of TH expression in these areas were out of phase with each other, with the peak in sex reward coinciding with increased NAc TH and the peak in amphetamine reward with elevated VTA TH.

We speculate that the differences between these structures in their rhythms of mesolimbic DA synthesis may contribute to the differences in timing observed between sex and amphetamine-related reward. In the NAc, daily variation in TH protein levels as well as other indices of DAergic activity (DOPA, and the DA metabolites HVA and DOPAC) has been reported previously, with peaks occurring, as we observed for TH, during the night (Castaneda, et al., 2004; O'Neill & Fillenz, 1985; Paulson & Robinson, 1996; Shieh, et al., 1997). Similar rhythms in the NAc also have been reported for DA transporter protein levels (Sleipness et al., 2007b) and DA D₂ receptor functioning (Tonissaar, et al., 2006). Taken together, these observations suggest that endogenous DA

release from dopaminergic terminals in the NAc is high during the night, corresponding to the peak time of sex-related reward (Fig. 6). Thus, it appears that the reward system is designed to maximize NAc DA release in response to natural rewards during the night, when locomotor activity levels are high and when encounters with biologically relevant stimuli (e.g., a sexually receptive female) are most likely to occur in the natural environment. Conversely, the high levels of TH protein seen during the daytime in the VTA may reflect increased somatodendritic release of DA and inhibition of VTA DA neurons via the activation of D2 receptors (Adell & Artigas, 2004). The timing of peak amphetamine-induced reward therefore occurs when inhibition of VTA dopamine neurons is high, and TH and DA in the NAc are intermediate to low. Thus, amphetamine, and perhaps other drugs of abuse, appear most rewarding at times when the propensity for evoked NAc DA release is relatively low.

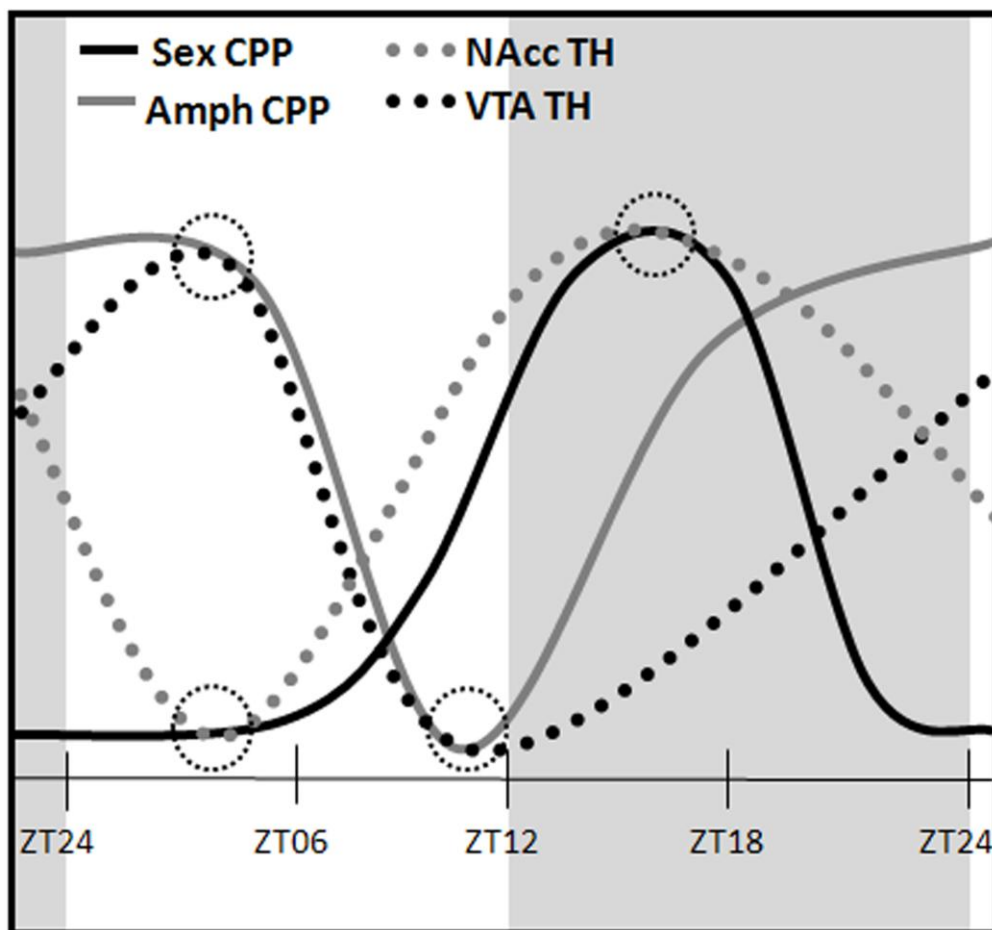


Figure 6 – A schematic overview of phase relationships between the rhythms in sex and amphetamine-related reward and tyrosine hydroxylase (TH) expression in the nucleus accumbens (NAc) and the ventral tegmental area (VTA). The circles highlight overlapping peaks and nadirs between reward rhythms and mesolimbic TH protein levels.

It is important to note, however, that TH expression may be altered by reward presentation. Indeed, there is data to suggest that drugs of abuse may induce TH expression in reward-related areas (Ferrari, Le Novere, Picciotto, Changeux, & Zoli, 2002). However, to our knowledge, there is no data regarding the influence of sexual experience or drug administration on mesolimbic TH rhythms. As well, the affinity of TH for its substrate is regulated by phosphorylation and TH protein levels may not accurately reflect the activity of this enzyme. Further study will be needed to address these issues and to evaluate the hypothesized causal links based upon the current correlational data.

Rhythmic clock gene expression in the NAc also may contribute to the diurnal variation in reward via a direct modulation of DA synthesis. Clock genes have the potential to directly regulate dopamine synthesis and metabolism as the promoter regions for monoamine oxidase A (*maoa*), TH, and the dopamine transporter all contain E-box elements (Hampp et al., 2008; Kawarai, Kawakami, Yamamura, & Nakamura, 1997; Yoon & Chikaraishi, 1992). Supporting this possibility, Hampp et al. (2008) reported that the *maoa* promoter is regulated by clock genes *in vitro* and that MAOA is downregulated in *period2* mutant mice. Other work utilizing *clock* mutant mice has indicated increased levels of TH protein in the VTA of these animals (McClung et al., 2005). Studies with clock gene knock out or mutant mice also suggest that these genes regulate reward processing, although such regulation appears complex. For instance, *period1* knockout mice do not sensitize to repeated cocaine injections or develop a cocaine-induced conditioned place preference while *period2* mutants show hypersensitization and a

normal place preference to cocaine (Abarca et al., 2002). As well, *clock* mutant mice exhibit increased firing of VTA dopaminergic neurons, increased cocaine-induced place preference, and require lower currents to sustain intracranial self stimulation (McClung, et al., 2005; Roybal et al., 2007). Thus, the current body of data support the view that clock genes can influence reward and motivation, and may do so, in part, via a direct modulation of dopaminergic activity, in particular, at the level of the NAc.

As clock genes may contribute to the expression of rhythms not only via signals from the SCN but also by intrinsic rhythmicity in extra-SCN brain areas and in peripheral tissues (Yoo et al., 2004), it may be that the observed diurnal rhythmicity in reward and TH expression stems from clock gene oscillations within structures of the reward system. Indeed, many of the core circadian clock genes have been localized to reward-related areas and have been reported to oscillate in these structures (Amir, Harbour, & Robinson, 2006; Angeles-Castellanos, et al., 2007; Feillet, Mendoza, Albrecht, Pevet, & Challet, 2008; Lamont, Robinson, Stewart, & Amir, 2005; McClung, et al., 2005; Verwey, Khoja, Stewart, & Amir, 2007). The current results confirm that *period1* and *bmal1* are expressed in both the NAc and the VTA, and, in the case of the NAc, the expression appears rhythmic in nature. Thus, the observed rhythmicity in NAc TH expression, and the previously reported circadian rhythm in NAc electrical activity (Yamazaki, et al., 1998) may be mediated in part by local rhythmic clock gene expression. By contrast, the apparent lack of clock gene rhythmicity in the VTA suggests that the diurnal variation in TH expression and the previously described rhythm in VTA electrical activity (Luo, et al., 2008) are driven by an extra-VTA signal, perhaps via an indirect projection from the

SCN (Luo & Aston-Jones, 2009). One caveat regarding this interpretation is that the VTA is a heterogeneous population comprised of both DA and non-DA cells (Fields, Hjelmstad, Margolis, & Nicola, 2007), and it may be that only specific cell types in the VTA rhythmically express clock genes. In this case, sampling from the entire VTA cell population may mask any intrinsic rhythmicity in a specific subpopulation. Cell type-specific analyses of clock gene expression in the VTA, as well as the NAc, would be desirable to address this issue.

Given that the development of a conditioned place preference is dependent upon associative learning processes, the daily variation seen in this response could potentially be due to a rhythm in the propensity for learning. If this is true then one would predict that a CPP response would vary in a similar fashion across the day regardless of the type of reward presented. The current results indicate that there are differential rhythms for sex and amphetamine related reward, thus suggesting that the diurnal variation is due to a rhythm in reward itself and not in the capacity for associative learning. It may also be argued that the development of a “time-stamp”, the phenomenon where time-of-day is encoded along with other contextual elements during memory formation (Cain, Ko, Chalmers, & Ralph, 2004; Ralph et al., 2002), may have influenced our results. In the current experiments, however, conditioning and testing occurred at the same time of day; thus, any daily variation is not attributable to differences between testing and training times. Moreover, the time stamp studies to date have utilized multiple conditioning trials and it is unclear if the formation of a time-stamp occurs with a single reward pairing.

Contrary to what one might expect, we also found interesting differences in the timing of performance vs. reward with respect to both sex and amphetamine. In agreement with previous work (Harlan, Shivers, Moss, Shryne, & Gorski, 1980), we found that male rat sexual performance varies in a diurnal fashion with a nadir occurring near the light-to-dark transition. By contrast, sex-induced reward peaks during the mid-dark period and ebbs during the mid-day. The reasons for this difference in the timing of peak sexual performance and reward are unknown but it seems reasonable to postulate that reward need not be constantly elevated to sustain a previously reinforced behaviour. Similarly, there is a difference between the timing of peak amphetamine-induced locomotor activity and reward. At the higher dose, the reward induced by systemic amphetamine administration also shows a robust rhythm with a nadir prior to dark onset while amphetamine-induced locomotion peaks during the late dark period. Our findings on the timing of amphetamine reward are consistent with prior work in rodents indicating that cocaine-induced place preference is increased during the midday as compared to dark onset or to the mid-dark period (Abarca, Albrecht, & Spanagel, 2002; Kurtuncu, et al., 2004; Sleipness, Sorg, & Jansen, 2007a) and overall argue for separate mechanisms or neural substrates underlying circadian regulation of performance vs. reward.

In conclusion, the current results demonstrate that the reward induced by both natural stimuli and drugs of abuse varies in a diurnal fashion, further reinforcing the view that time of day is a critical variable to consider when assessing appetitive or addictive behaviour in the laboratory or clinical settings. As sex and amphetamine reward rhythms differ in the precise timing of their peaks and nadirs, further work will be necessary to

elucidate whether rhythms associated with other types of reward (e.g., palatable food) correspond to either of these patterns. The current study, in conjunction with previous work, suggests that daily variation in reward may be mediated by diurnal variations in mesolimbic dopaminergic activity that are driven, in part, by local clock gene oscillations. In particular, we suggest that the peak in sex reward occurs when the drive for NAc DA release is at its highest while the peak in amphetamine-related reward coincides with a lower likelihood of evoked NAc DA release. Further work will be needed to test these hypotheses, however, and, given the anatomical and neurochemical complexity of the mesolimbic reward system, it is likely that a diurnal and/or circadian regulation occurs at multiple sites of action.

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CHAPTER 3:

Prefrontal Cortex Modulates Diurnal Rhythms in Neural Activation
in the Mesolimbic Dopamine System

3.1 INTRODUCTION

Rewarding behaviours are largely mediated by the mesolimbic system (Frohman, Pitchers, Balfour, & Coolen, 2010; 2004), comprised of the ventral tegmental area (VTA), nucleus accumbens (NAc), medial prefrontal cortex (mPFC), amygdala, and other regions (Ikemoto, 2007; Spanagel & Weiss, 1999). Mesolimbic brain areas are activated during natural reward behaviours (Martel & Fantino, 1996; Noel & Wise, 1995), including sexual behaviour (Balfour, Yu, & Coolen, 2004; Pfaus et al., 1990; Pitchers et al., 2010), aggression (Wolf, Sun, Mangiavacchi, & Chao, 2004), and maternal behaviour (Champagne et al., 2004; Numan et al., 2010); and in response to conditioned cues predicting natural rewards (Balfour, et al., 2004), and drugs of abuse (Di Chiara & Imperato, 1988; Thomas, Kalivas, & Shaham, 2008). A growing body of work has revealed that many of the behavioural responses to rewarding stimuli vary substantially over the course of a day. Conditioned place preference (CPP), locomotor activation and sensitization in response to psychostimulants (Abarca, Albrecht, & Spanagel, 2002; Kurtuncu, Arslan, Akhisaroglu, Manev, & Uz, 2004; Webb et al., 2009) and natural rewards (Webb, Baltazar, Lehman, & Coolen, 2009; Webb, Baltazar, Wang, et al., 2009) depend significantly on time-of-day. The mechanisms responsible for these diurnal variations in motivation and reward remain to be fully elucidated, but there is evidence for involvement of the mesolimbic system, and particularly of the neurotransmitter dopamine. Markers of dopaminergic activity vary by time-of-day in the VTA and NAc (Webb, Baltazar, Lehman, et al., 2009), including the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis (Sleipness, Sorg, & Jansen, 2007; Webb, Baltazar, Wang, et al., 2009), of dopamine transporters

(Sleipness, et al., 2007), and of monoamine oxidase (Hampff et al., 2008), which breaks down dopamine.

The first objective of this study was to characterize diurnal fluctuations in neural activation throughout the mesolimbic system, using cFos protein expression as the marker for neural activation. The use of cFos as a marker to elucidate diurnal rhythms in neural activation is well documented for brain areas involved in sleep and wakefulness (Basheer et al., 1997; Cirelli, Pompeiano, & Tononi, 1993; Grassi-Zucconi et al., 1993), including activation of the orexin/hypocretin system (Estabrooke et al., 2001; Martinez, Smale, & Nunez, 2002). Moreover, investigation of cFos protein expression during sleep and wake states provided support for diurnal rhythms in neural activation in the mesolimbic system (Pompeiano, Cirelli, & Tononi, 1994), specifically the mPFC and NAc. However, a comprehensive characterization of neural activation in the mesolimbic system across the day has yet to be conducted.

The second objective of this study was to test the hypothesis that the mPFC plays a critical role in mediating the daily rhythms in neural activation in the mesolimbic system. The mPFC provides a major source of glutamatergic inputs to the VTA and NAc (Carr & Sesack, 1999, 2000; Omelchenko & Sesack, 2007), and glutamate receptor activation has been implicated in induction of cFos expression (Rossetti, Marcangione, & Wise, 1998; Vanhoutte et al., 1999; Xia, Dudek, Miranti, & Greenberg, 1996). Therefore, the mPFC is a potential candidate for regulating diurnal rhythms in neural

activity in its target areas. Together, these studies test the hypothesis that there are diurnal variations in neural activity within the mesolimbic system.

3.2 METHODS

3.2.1 Animals

Adult male Sprague-Dawley rats (300-350g, Charles River Laboratories, QC, CAN) were pair-housed in standard rat housing cages with *ad libitum* food and water. The animals were placed under a 12:12 light/dark cycle (~350lux L/0 lux D) and were allowed to acclimatize for 2 weeks prior to tissue collection. Rats for lesioned experiments were allowed to acclimatize for 1 week prior to surgery and 2 weeks for recovery and light cycle entrainment. All experiments were approved by the University of Western Ontario Animal Care Committee and the University Committee on Use and Care of Animals at the University of Michigan and are in compliance with the guidelines of the Canadian Council on Animal Care and the United States National Institute of Health.

3.2.2 cFos immunoreactivity analysis

Tissue Collection

At 6 different zeitgeber times (ZT 02, 06, 10, 14, 18, 22, n = 6-8 per time point, where ZT12 corresponds to lights off by convention), rats were deeply anaesthetized with sodium pentobarbital (270 mg/kg, i.p.) and perfused transcardially with 50 ml saline followed by 500 ml 4% paraformaldehyde in 0.1M phosphate buffer (PB). Brains were removed, post-fixed for one hour, and stored in 20% sucrose in 0.1M PB with 0.01% sodium azide at 4°C until sectioning. For time points coinciding with the dark phase,

tissue collection was performed under dim red light (~1 lux). Brains were sectioned with a freezing microtome into four parallel series of 35µm coronal sections and stored in cryopreservative (Watson, Wiegand, Clough, & Hoffman, 1986) at -20°C until immunohistochemical processing.

Immunohistochemistry

All incubations and rinses were performed at room temperature on free floating tissue via gentle agitation. Following each incubation, sections were rinsed thoroughly in saline buffered sodium phosphate buffer (PBS, 0.1M, pH 7.4). Sections were incubated for ten minutes in 1% H₂O₂ in 0.1M PBS and for one hour in incubation solution (0.1% bovine serum albumin and 0.4% Triton X-100 (Fisher Scientific, Ottawa, Canada) in PBS). One series of sections for each animal was incubated with a rabbit polyclonal antibody specifically recognizing cFos (1:7,500 in incubation solution; 17 hours; SC-52; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by incubation with a biotinylated donkey anti-rabbit IgG (1:500 in incubation solution; 1 hour; Jackson Immunoresearch Laboratories, West Grove, PA, USA) and avidin-horseradish peroxidase complex (1:1,000 in PBS; 1 hour; ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). Reaction product was visualized using diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO, USA) solution (0.01% DAB, 0.012% H₂O₂ in 0.1M PB) containing 0.08% nickel sulphate for 10 minutes, resulting in a black reaction product. Sections were then washed with H₂O₂ (1% in PBS; 10 minutes) and incubated with a mouse monoclonal antibody to tyrosine hydroxylase (TH; 1:400,000 in incubation solution; 17 hours; Chemicon International, Temecula, CA), a biotinylated donkey anti-mouse IgG (1:500 in

PBS; 1 hour; Jackson Immunoresearch Laboratories, West Grove, PA, USA) and ABC (1:1,000 in PBS; 1 hour). TH-immunoreactivity was visualized with DAB (0.01% DAB, 0.012% H_2O_2 in sodium phosphate buffer [PB; 0.1 M]; 10 minutes), resulting in brown reaction product. Finally, sections were thoroughly rinsed in PB, mounted on plus-charged slides (Fisher Scientific, Ottawa, Canada) dehydrated in alcohol, cleared with Citrisolv (Fisher Scientific, Ottawa, Canada), and coverslipped with Di-N-Butyl Phthalate in xylene (Electron Microscopy Sciences, Fort Washington, PA).

Cell Counting and Analysis

cFos immunoreactive (IR) neurons were counted in reward-related brain areas using a Leica DMR microscope (Leica Microsystems, Wetzlar, Germany) with a camera lucida drawing tube by an observer blinded to the experimental groups, in standard areas of analysis (Fig. 3.1): nucleus accumbens (NAc) core (400x600 μm) and shell (400x600 μm); the anterior cingulate area (ACA; 400x600 μm), the infralimbic (IL; 400x600 μm) and the paralimbic (PL; 400x600 μm) regions of the medial prefrontal cortex (mPFC); central (CeA; 400x400 μm) and basolateral (BLA; 600x400 μm) amygdala; and dentate gyrus (DG; 1800x1200 μm). In the VTA (900x600 μm), cFos-ir, TH-ir, and dual labeled neurons were counted at a rostral-middle level (Balfour, et al., 2004). More caudal levels of VTA were not included as no cFos expression was noted at any time points. Counts of cFos-IR cells in the suprachiasmatic nucleus of the hypothalamus (SCN, 400x400 μm) were performed as a positive control of circadian activation rhythms (Schwartz et al., 2000). Areas of analysis were determined in accordance with previously defined landmarks (Swanson, 1998, Fig. 3.1). For each

animal, an average of two counts (each in one hemisphere) was calculated. One way ANOVA and Kruskal-Wallis analyses were performed, to determine the effect of time-of-day on cFos in each brain area. In the VTA, Kruskal-Wallis analyses were performed to identify effects of time on c-Fos, TH, and dual cFos/TH. Dunn's post-hoc analyses were used where needed to determine the timepoints with highest (peaks) and lowest (nadirs) immunoreactivity. In the SCN, unpaired t-test was used to determine significant difference in cFos-ir between ZT6 and 18.

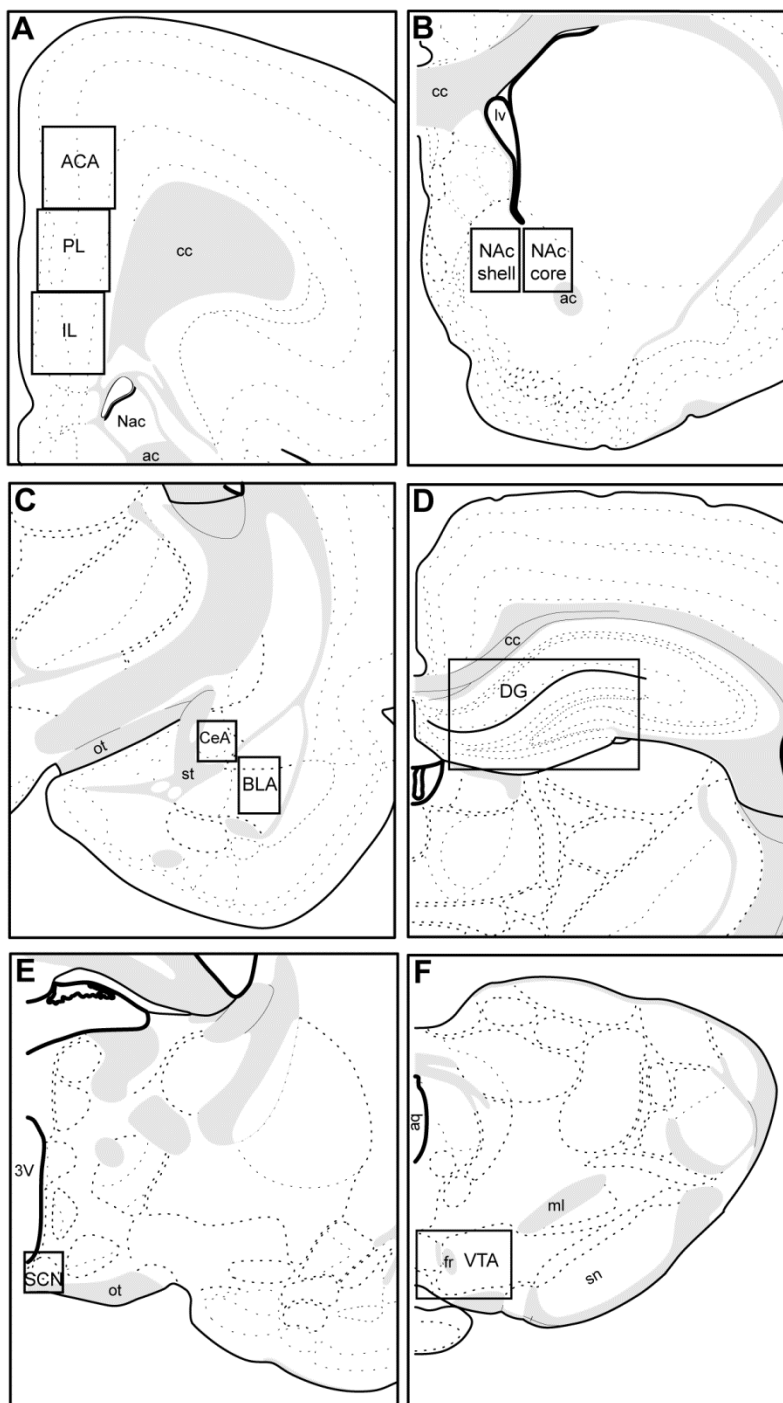


Figure 3.1: Standard areas of analyses for cell counts. Schematic representations of areas of analyses in A) anterior cingulate area (ACA), infralimbic (IL), and prelimbic (PL) regions of medial prefrontal cortex; B) nucleus accumbens (NAc) core and shell; C) central (CeA) and basolateral (BLA) amygdala; D) dentate gyrus (DG); E) suprachiasmatic nucleus (SCN); F) ventral tegmental area (VTA) (F). Abbreviations: ac, anterior commissure; aq, cerebral aqueduct; cc, corpus callosum; cp, caudate putamen; fr, fasciculus retroflexus; fx, fornix; ml, medial lemniscus; ot, optic tract; 3V, third ventricle; st, stria terminalis; lv, lateral ventricle. Drawings modified from Swanson (1998).

3.2.3 mPFC lesions

Lesion Surgery

Lesion surgeries were performed using parameters we previously reported to result in lesions restricted to IL and PL (Davis et al., 2010). Rats were anaesthetized with ketamine (87 mg/ml/kg i.p.) and xylazine (13 mg/ml/kg i.p.) and placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, California). The skull was exposed and a small burr hole was drilled and a 5 μ L Hamilton Syringe (Hamilton, Reno, Nevada) was lowered at the following coordinates relative to bregma: anterior-posterior = 2.9, medial-lateral = 0.6 mm. Ibotenic acid (2% in 0.1 M phosphate buffered saline; Sigma) or vehicle was slowly infused bilaterally at two dorsal-ventral coordinates corresponding to the infralimbic and prelimbic regions of the mPFC (from the top of skull = 5.0 and 2.5 mm; 0.25 μ L/1-minute per injection). The needle was left in place for 3 minutes following each injection to allow for optimal diffusion. Animals were allowed two weeks to recover from surgery prior to tissue collection.

cFos Immunocytochemistry

Sham- and mPFC-lesioned rats were perfused at two different time points, ZT10 and ZT18, corresponding respectively to nadirs and peaks in cFos-IR (n=6-8 per time-point). Brain sections containing the NAc core and shell, and rostral and middle VTA were processed and analyzed for cFos immunocytochemistry, as described above. Two-way ANOVA was used to analyze effects of lesion (lesion versus sham) and time-of-day (ZT10-ZT18). Fisher LSD tests were used for post-hoc analysis of time-of-day differences and lesion effects.

Lesion Verification

The locations of ibotenic lesions in the mPFC were assessed in cFos immunostained tissue. Rats with >80% tissue loss of PL and IL were considered lesioned (Davis, et al., 2010), and had minimal or no damage to the anterior cingulate area or nucleus accumbens (ZT10; n=6; ZT18; n=8). Sham animals did not have any damage (ZT10; N=6; ZT18; n=4).

3.3 RESULTS

3.3.1 Rhythmic baseline neural activation in the reward system

A significant diurnal variation in the number of cFos-IR cells was observed (Fig. 3.2) in the NAc core (K=22.99, p=0.0003, Fig. 3.3a) and shell (K=27.57, p<0.00001, Fig. 3.3b), in all three subregions of the mPFC (ACA [K=20.73, p=0.0009, Fig. 3.4a], PL [K=18.78, p =0.0021, Fig. 3.4B], and IL [K=13.70, p=0.0177], Fig. 3.4C), and in the VTA (K=23.99, p=0.0002, Fig. 3.5A).

In general, rhythms in NAc, mPFC, and VTA were similar in phase. In the NAc core and shell, cFos expression had a nadir at ZT10 and a peak from ZT18 to ZT22 (Fig. 3.2-3.3). Within the subregions of the mPFC, cFos was at a nadir at ZT10 and at a peak at ZT18 (IL, PL) or from ZT14-ZT18 (ACA; Fig. 3.2, 3.4).

In the VTA, cFos nadir was at ZT10 and peak from ZT18 to ZT22 (Fig. 3.2, 3.5). The number of TH cells co-expressing cFos immunoreactivity also varied diurnally (F=34.27, p<0.0001, Fig. 3.5C) with a nadir at ZT10 and peak at ZT18. Moreover, at peak

times, 24% of total VTA TH-IR cells also expressed cFos, and 20% of cFos-ir cells expressed TH (Fig. 3.5D). Finally, a rhythm in the number of TH expressing cells was observed with a nadir at ZT10 and peaks at ZT06, 14, and 22 (Fig. 3.5B).

In contrast, no diurnal variations in numbers of cFos-IR cells were observed in the dentate gyrus or the central and basolateral amygdala (Fig. 3.6A,B,C). In the SCN, the number of cFos-IR cells was significantly greater at ZT06 than ZT18 ($p=0.0022$, fig. 3.6D) consistent with previous reports (Schwartz, et al., 2000).

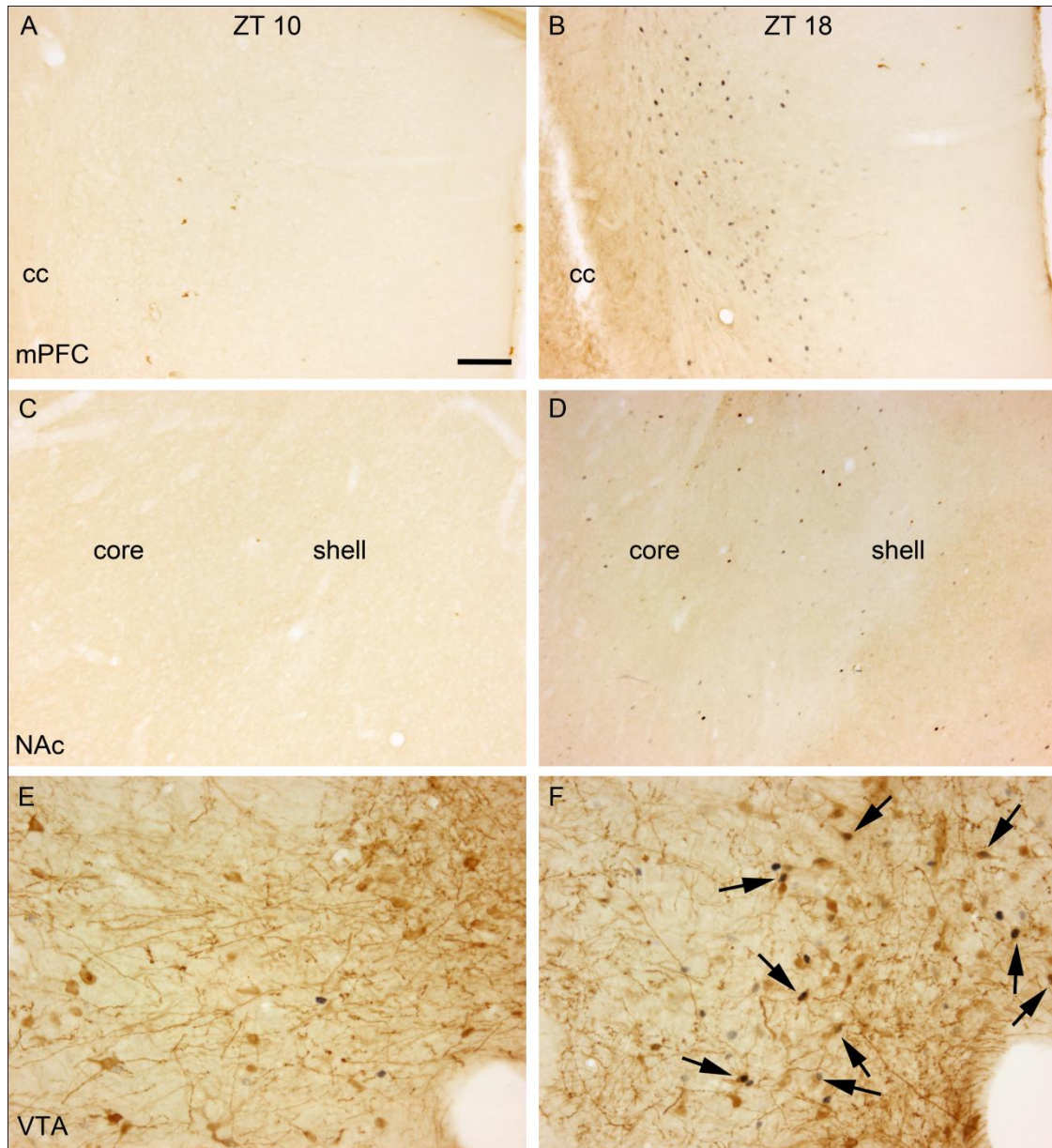


Figure 3.2: cFOS and TH immunoreactivity. Images showing Fos-ir (black) and TH-ir (brown) in mPFC (prelimbic area), NAc (core and shell), and VTA in representative animals at ZT10 (A, C, E) and ZT18 (B, D, F). Arrows in F indicate cells co-expressing Fos and TH-ir. Scale bar depicts 100 (A-D) or 50 (E,F) μm . cc= corpus callosum

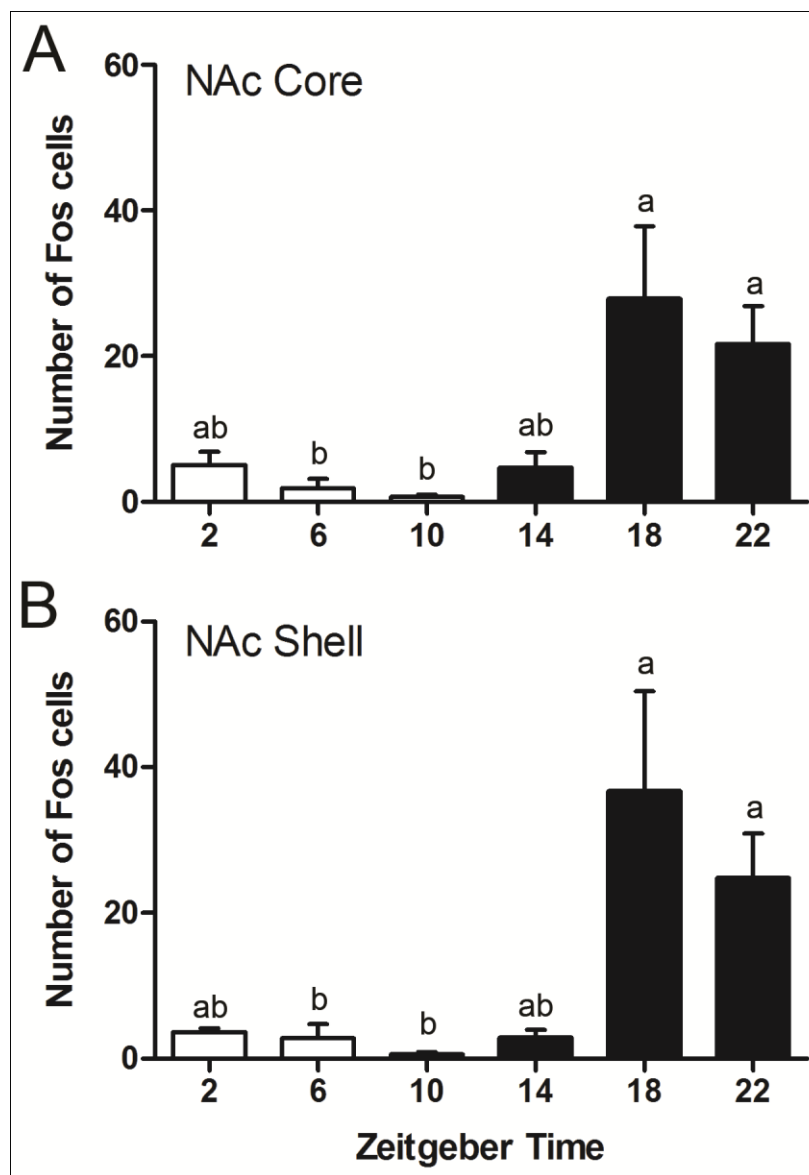


Figure 3.3: Diurnal variation in neural activation in the nucleus accumbens (NAc).

Number of cFos-IR cells in the NAc shell (A) and core (B). Data are expressed as means \pm SEM. Different letters denote significant differences between time points.

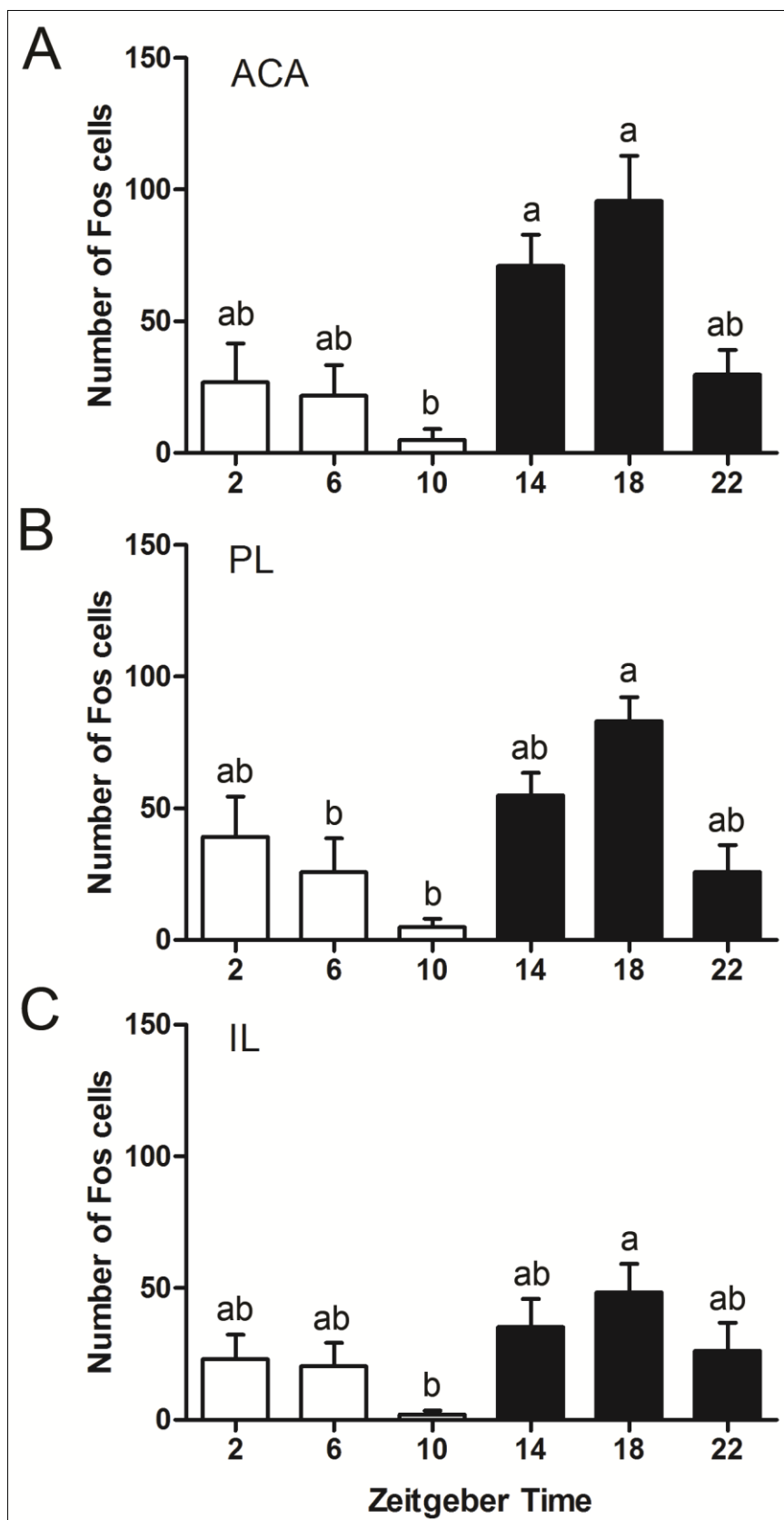


Figure 3.4: Diurnal variation in neural activation in the prefrontal cortex. Number of cFos-IR anterior cingulate area (ACA; A), infralimbic region (IL; B), and prelimbic region (PL; C) of the medial prefrontal cortex. Data are expressed as means \pm SEM. Different letters denote significant differences between time points.

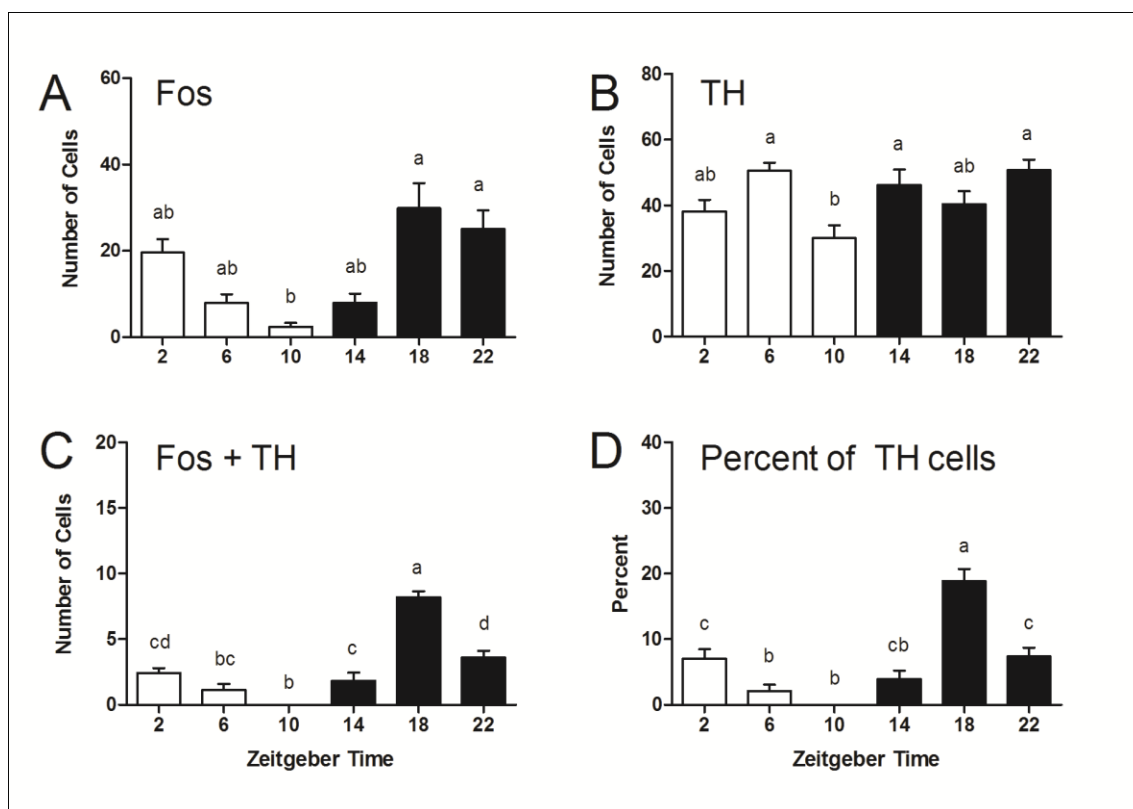


Figure 3.5: Diurnal variation in activation of dopaminergic and non-dopaminergic neurons in the ventral tegmental area. Diurnal variation in number of cFos, TH, dual cFos/TH immunoreactive cells, and percentage of TH cells co-expressing cFos in the ventral tegmental area. Data are expressed as mean number \pm SEM. Different letters denote significant differences between time points.

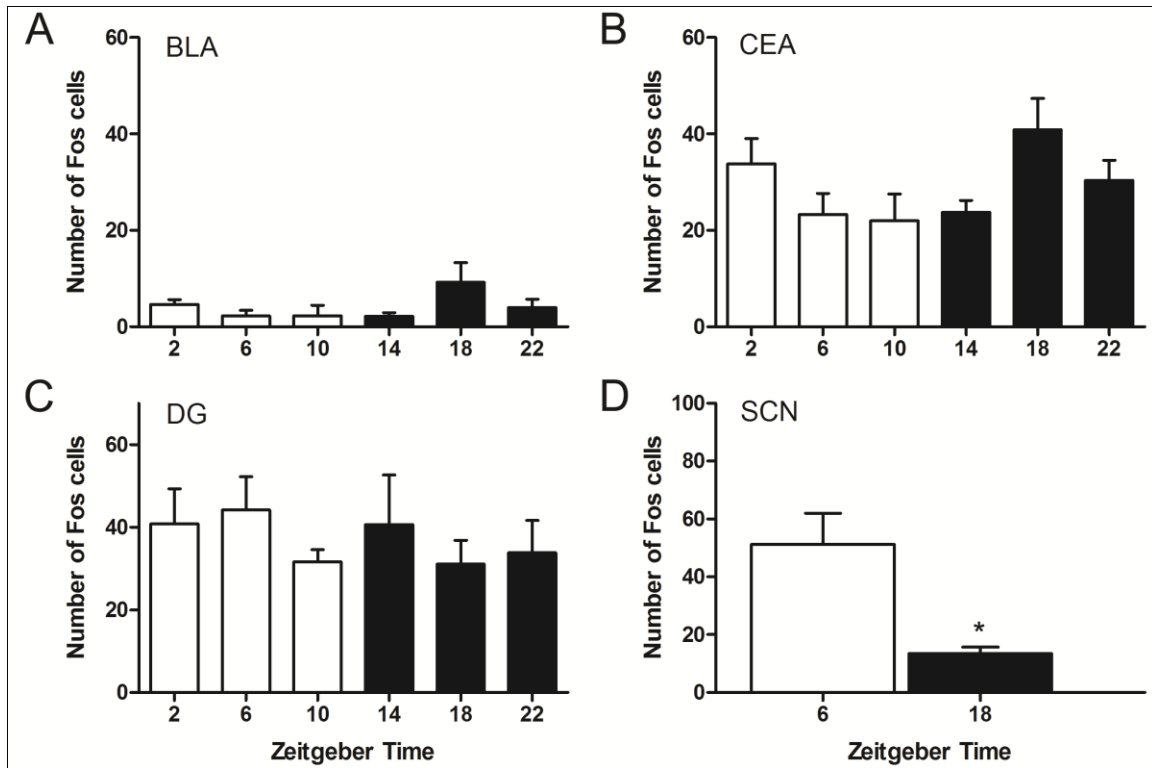


Figure 3.6: Diurnal variation in neural activation in the amygdala, hippocampus

and suprachiasmatic nucleus. Diurnal variation in number of cFos immunoreactive

cells in the basolateral amygdala (BLA; A), the central amygdala (CeA; B), dentate

gyrus (C), and suprachiasmatic nucleus (SCN; D). Data are expressed as mean \pm SEM. *

denotes significant differences between time points.

3.3.2 mPFC lesions attenuate cFOS-IR in NAc

mPFC lesions had a significant effect on cFos rhythms in NAc shell (Fig. 3.7A) and core, Fig. 3.7B), but not in the VTA (Fig. 3.7C). In the NAc, mPFC lesions had a significant effect on numbers of cFos-IR cells in both the core ($F=22.5$, $p=0.0002$) and shell ($F=22.57$, $p=0.0001$). Moreover, significant interactions were found between time-of-day and lesion status for the NAc shell ($F=50.0$, $p<0.0001$) and core ($F=32.94$, $p<0.0001$). Post-hoc analysis revealed that mPFC lesions reduced the number cFos-IR cells at ZT18 (core: $p=0.0003$; shell: $p<0.0001$), compared to sham group, and increased cFos-ir at ZT10, in the shell ($p=0.019$), but not the core. Changes in numbers of cFos-IR cells with mPFC lesions resulted in a loss of time-of-day differences in the NAc shell, and an attenuation of the difference in the NAc core (ZT10 versus 18: $p=0.0258$). In contrast to the NAc, mPFC lesions did not affect the number of cFos-IR cells in the VTA and a significant time-of-day difference was observed in lesioned animals (ZT10 versus 18: $p<0.0001$). Finally, sham treatment did not disrupt diurnal rhythms in cFos-ir and significant time-of-day differences were detected in NAc core ($p<0.0001$), NAc shell ($p<0.0001$), and VTA ($p=0.0161$), consistent with the findings described above. These results suggest that mPFC inputs may mediate neural activity rhythms in NAc, but not in VTA.

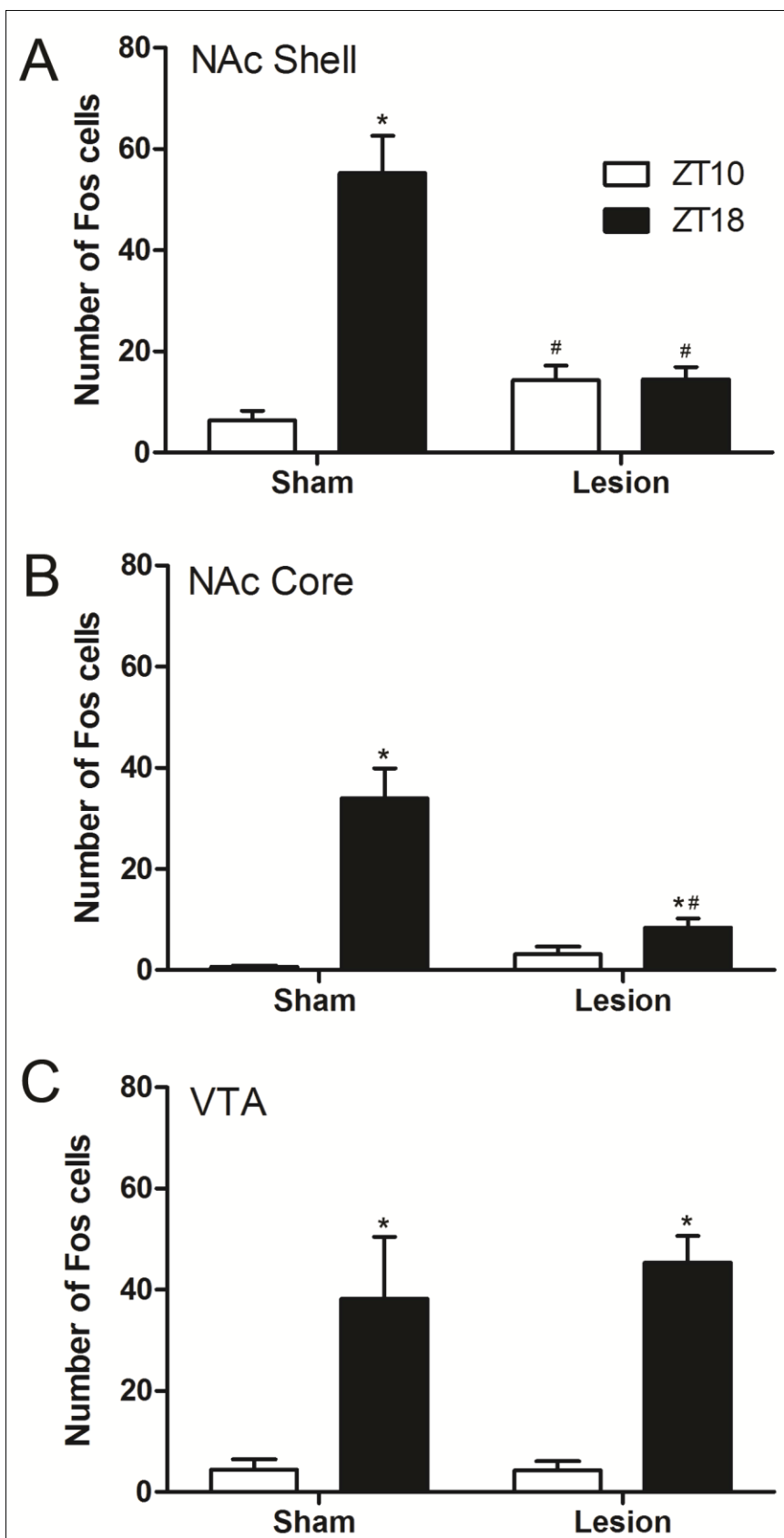


Figure 3.7: The prefrontal cortex modulates neural activity in the mesolimbic reward system. Effects of sham surgery and mPFC lesions on cFos immunoreactivity in the nucleus accumbens (NAc) core (A) and shell (B), and ventral tegmental area (VTA; C). Data are expressed as mean \pm SEM. * denotes significant effect of time-of-day within treatment group, and # denotes significant lesion effect within time of day.

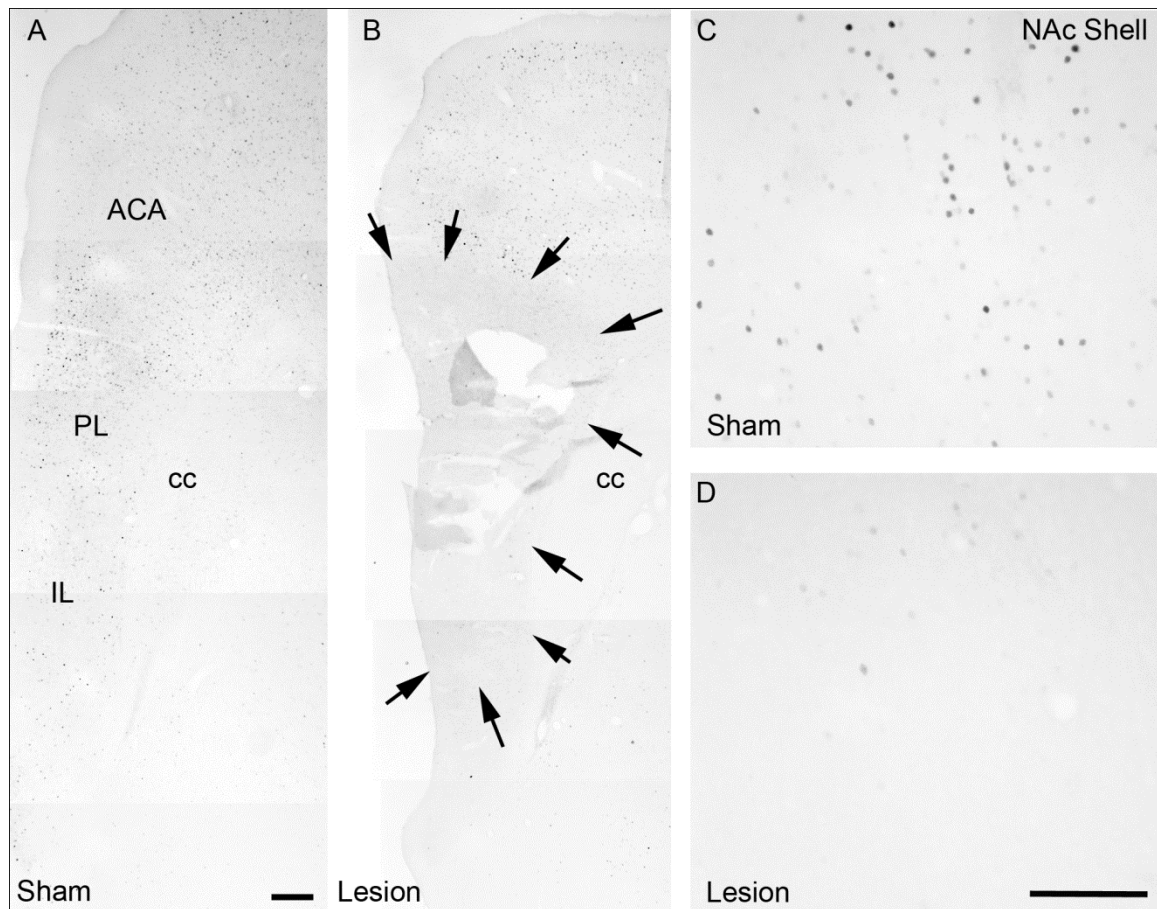


Figure 3.8: cFos immunoreactivity in prefrontal-cortex-lesioned brains. Images showing mPFC in representative Sham (A) and Lesion (B) animals, in brain sections immunoprocessed for cFos immunoreactivity. Arrows in B indicate the extent of the lesion site encompassing prelimbic (PL) and infralimbic (IL) areas, with sparing of the anterior cingulate area (ACA). Images in C and D illustrate cFos-IR in NAc shell at ZT18 in sham (C) and mPFC-lesioned (D) males. Scale bars indicate 250 (A, B) or 100 (C, D) μm . Abbreviations: cc, corpus callosum.

3.4 DISCUSSION

The current study demonstrated temporal patterns of neural activation in the VTA, NAc, and mPFC, which were generally elevated during the dark phase (ZT18-ZT22), with nadirs during the end of the light phase (ZT10). In the VTA, this rhythm in neural activation was observed in dopaminergic as well as non-dopaminergic cells. One major question deriving from these observations was: what is driving these rhythms in neural activation? Since both NAc and VTA receive glutamatergic inputs from mPFC (Carr & Sesack, 2000; Gabbott, Warner, Jays, Salway, & Busby, 2005; Sesack & Grace, 2010), we tested the hypothesis that mPFC glutamatergic inputs contribute to the neural activity rhythms. Indeed, ablation of the mPFC eliminated diurnal variation in NAc cFos-IR by eliminating the peak expression at ZT18, but did not affect rhythms in VTA activity.

This study conducted a comprehensive characterization of diurnal rhythms in cFos-IR in the mesocorticolimbic reward system. Diurnal rhythms with peaks during the middle-end of the dark phase and nadirs during the end of the light phase were demonstrated in the NAc, VTA, and mPFC, but not in the CeA, BLA, or DG. There have been previous papers using cFos as a marker for daily changes in neural activity, however, these included only a few time points or were limited to a particular portion of the day. Overall, the phases of these rhythms were consistent with our own observations. Specifically, a qualitative investigation of cFos protein expression during sleep and wake states reported peak cFos-IR at ZT18 (dark phase) compared to ZT6-9 (light phase) in the mPFC and NAc (Pompeiano et al., 1994). Additionally, data from studies of food anticipatory behaviour also reported variations in cFos-IR in the NAc, mPFC and CeA,

over a period of several hours (Angeles-Castellanos, Mendoza, & Escobar, 2007; Verwey, Khoja, Stewart, & Amir, 2007). The absence of rhythms in neural activation in the hippocampus and amygdala under basal conditions suggests that these regions may not be involved in mediating rhythms in reward. However, there is evidence to support their role as accessory regions, sensitive to the timing of rewards in the environment. Salient stimuli such as palatable food and restricted feeding schedules alter patterns of neural activation and clock gene expression throughout the reward system (Mendoza, Angeles-Castellanos, & Escobar, 2005) and can generate rhythmicity in the DG and amygdala (Verwey, et al., 2007). As these regions project to the mPFC, NAc and VTA (Kelley, 2004), under these conditions, they may convey timing information throughout the reward system and to other brain areas.

The mPFC, NAc and VTA are tightly interconnected, and form a major component of the neural circuits involved with motivation and reward (Hyman, Malenka, & Nestler, 2006; Kelley & Berridge, 2002)). The finding that rhythms in neural activity are similar within this network forms the basis for several hypotheses regarding the generation of these activity rhythms. First, it is possible that glutamatergic and dopaminergic inter-connections originating from the mPFC and VTA (Carr & Sesack, 2000; Sesack, Carr, Omelchenko, & Pinto, 2003) modulate the activity within this network. Indeed, one main finding of the current study is that mPFC inputs are critical for the diurnal rhythms in activity in the NAc, suggesting that these rhythms are influenced by glutamatergic mPFC outputs. These findings are consistent with previous reports of circadian rhythms in glutamate in NAc dialysate (Castaneda, de Prado, Prieto,

& Mora, 2004; Marquez de Prado et al., 2000), with peaks around ZT18 and remains low throughout the middle light phase.

Conversely, VTA rhythms were not dependent on mPFC inputs. Therefore, a second hypothesis is that rhythms in activation of VTA, mPFC, and potentially NAc neurons, instead derive via inputs from other brain areas, including via the master circadian clock, the SCN, in the hypothalamus. The VTA is positioned to receive timing information from the SCN via two indirect circuits; one through the medial preoptic nucleus (Luo & Aston-Jones, 2009), the other by way of orexinergic neurons in the hypothalamus, (Deurveilher & Semba, 2005; Korotkova, Sergeeva, Eriksson, Haas, & Brown, 2003; Yoshida, McCormack, Espana, Crocker, & Scammell, 2006). There is currently little evidence to support a functional role of the former pathway, while the latter, orexinergic pathways appears ideally positioned to convey circadian information to the reward system. A large number of orexinergic efferents contact the VTA (Fadel & Deutch, 2002), and in addition to its familiar role in arousal and wakefulness (Sakurai, 2007), the orexin system has been implicated in the regulation of reward and addiction (Aston-Jones, Smith, Moorman, & Richardson, 2009; Harris, Wimmer, & Aston-Jones, 2005) via its role in mediating reward induced activation of VTA dopamine neurons (Borgland, Taha, Sarti, Fields, & Bonci, 2006; Zheng, Patterson, & Berthoud, 2007). The influence of orexinergic inputs to VTA dopamine activity fluctuates over the course of the day, with greater activation during the dark phase (Moorman & Aston-Jones, 2010). Moreover, orexin neurons receive direct inputs from the SCN (Cutler et al., 1999; McGranaghan & Piggins, 2001). Perturbations in SCN activity with phase shifting or SCN ablation alters (Akiyama et al., 2004; Marston et al., 2008; Zhang et al., 2004) the

normal diurnal rhythms of orexin activation and signalling (Estabrooke, et al., 2001; Lee, Hassani, & Jones, 2005). Interestingly, clock gene expression in forebrain regions is attenuated in mice lacking orexin neurons (Akiyama, et al., 2004). Together these findings suggest an integrated functional relationship between the circadian, orexigenic, and reward systems.

Moorman and Aston-Jones (2010) recently showed that the activity of VTA DA neurons during the dark phase is modulated by the mPFC and orexinergic afferents, with a gating role of orexin on enhancing mPFC excitatory control over DA neurons. Glutamatergic projections from the mPFC reach both dopaminergic and non-dopaminergic neurons of the VTA (Omelchenko & Sesack, 2007; Rossetti, et al., 1998). Modulation of corticolimbic glutamatergic inputs by orexin has been suggested to occur via increased NMDA receptor trafficking (Borgland, et al., 2006) and via orexin receptor mediated changes in phosphoinositol signalling (Moorman & Aston-Jones, 2010). In the current study, we did not observe an effect of mPFC lesions on peak expression of VTA neural activity, suggesting that mPFC inputs did not have a major contribution to baseline excitation. However, it is possible that orexin inputs in mPFC lesioned animals are sufficient to maintain the VTA activity rhythms.

We hypothesized that the observed rhythms in neural activation in the NAc and mPFC were in part mediated by dopamine originating from the VTA. Indeed, a diurnal pattern of activated dopaminergic cells was evident in the VTA, consistent with the neural activity rhythms observed in the NAc and mPFC. Dopaminergic afferents to the

mPFC primarily originate from the VTA (Kelley & Berridge, 2002; Sesack & Grace, 2010), and may be responsible for conveying timing information to the mPFC, and thus indirectly to the NAc. A significant time-of-day effect has been previously reported in mPFC dopamine clearance in mPFC, a measure of synaptic dopamine (Sleipness, Jansen, Schenk, & Sorg, 2008). The rhythm in PFC dopamine clearance is consistent with the pattern in VTA dopaminergic activity (Webb, Baltazar, Wang, et al., 2009), with peak at ZT04, and nadir at ZT0. However, the dopamine clearance rhythm does not match the rhythm in cFos activity; hence may not be causing the rhythms in neural activity. In addition, a subportion of the rhythmic cFos-IR in the VTA neurons may be glutamatergic. Luo et al. (2008) reported that VTA neurons that fire selectively during the active night-phase express mRNA for the glutamate cell marker, vGLUT2, and account for 14% of all active cells during the dark phase. Moreover, a subset of dopaminergic neurons also synthesize and release glutamate into the NAc (Tecuapetla et al., 2010). These findings suggest that VTA neural activation may contribute to rhythms in NAc and mPFC, via release of glutamate and/or dopamine.

A final hypothesis for generating neural activity rhythms in the mPFC, VTA, and NAc, is that these originate from oscillation in core clock genes. Core clock genes have been localized to many reward-related brain areas (Angeles-Castellanos, et al., 2007; McClung et al., 2005; Ramanathan, Stowie, Smale, & Nunez, 2010; Verwey, et al., 2007; Webb, Baltazar, Wang, et al., 2009), and oscillate in the NAc and PFC (Angeles-Castellanos, et al., 2007) but do not appear to be rhythmically expressed in the VTA (Webb, Baltazar, Wang, et al., 2009). It is clear that core clock genes play a role in

regulating reward as clock gene disruptions alter typical behavioural responses to drugs of abuse. For instance, *per1* and *per2* knockout mice display increased and decreased cocaine conditioned place preference, respectively (Abarca, et al., 2002); intracerebroventricular injections of a DNzyme that interferes with *per1* impairs morphine CPP (Liu et al., 2005); arrhythmic *per2* mutant mice exhibit increased alcohol consumption; and mice lacking a functional Clock gene are hypersensitive to cocaine (McClung, et al., 2005). Clock genes disruptions also affect the normal functioning of the reward system. For instance, Clock gene knockout mice have increased excitability of dopamine neuron and increased TH expression in the VTA (McClung, et al., 2005); *per2* knockout mice exhibit altered dopamine activity, with decreased transcription of the monoamine oxidase gene throughout the mesolimbic dopamine system, and increased extracellular levels of dopamine and altered neural activity in the striatum (Hampp, et al., 2008); VTA specific knockdown of clock genes in mice results in increased dopamine cell firing and altered expression of genes associated with reward, such as increased TH, dopamine receptor, and Homer2, and decreased ionotropic glutamate receptor subunit 2, and dopamine beta-hydroxylase (Mukherjee et al., 2010).

In summary, the current study demonstrates diurnal rhythms in neural activity in areas within the mesolimbic pathway with a causal role for mPFC outputs to generate such rhythms in the NAc. These neural activity rhythms in turn may regulate diurnal differences in reward behaviours. The nadir cFos-IR during the late light phase coincides with a nadir in expression of psychostimulant reward (Baird & Gauvin, 2000; Roberts & Andrews, 1997; Roberts, Brebner, Vincler, & Lynch, 2002; Webb, Baltazar, Wang, et al.,

2009). In contrast, the peak in cFos expression correlates with high expression of reward for the natural reward behaviour mating and for amphetamine (Webb, Baltazar, Wang, et al., 2009). Future studies will be needed to test if these activity rhythms and rhythms in dopamine and glutamate signaling are indeed critical for rhythms in reward behaviour.

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CHAPTER 4:**Medial Prefrontal Cortex Modulates Diurnal Rhythms
in Amphetamine Reward**

4.1 INTRODUCTION

Behavioural responses to drugs of abuse vary by time-of-day. This includes diurnal variations in self-administration, behavioural sensitization, and conditioned place preference (CPP) for psychomotor stimulants (Abarca, Albrecht, & Spanagel, 2002; Baird & Gauvin, 2000; Webb et al., 2009). The timing of peak reward for these substances is typically in the early light phase of the light/dark cycle, coinciding with the normal inactive period for rodents. The rewarding effects of drugs of abuse are mediated by the mesolimbic reward system, of which dopamine is one of the key neurotransmitters (Ikemoto, 2007; Spanagel & Weiss, 1999). Dopamine cell bodies are located in the ventral tegmental area (VTA) and project to other structures involved in the processing of rewards, including the medial prefrontal cortex (mPFC), the nucleus accumbens (NAc), the amygdala, and the hippocampus (Kelley & Berridge, 2002). Daily changes in reward likely reflect oscillations in mesolimbic activity. Indeed, time-of-day differences have been reported for several markers of dopamine synthesis, release, and metabolism, throughout the mesolimbic system (Castaneda, de Prado, Prieto, & Mora, 2004; Sleipness, Jansen, Schenk, & Sorg, 2008; Sleipness, Sorg, & Jansen, 2007; Webb, et al., 2009). There are also reports of diurnal rhythms in the extracellular concentration of other reward-related neurotransmitters, including glutamate and gamma-aminobutyric acid (GABA) (Castaneda, et al., 2004), as well as in neural activity (Luo, Georges, & Aston-Jones, 2008) and neural activation in this system (see chapter 3). The nature of the timing signal that drives the observed rhythms in reward and mesolimbic activity has yet to be determined.

Clock genes drive rhythms in circadian oscillators, including the master circadian oscillator in the suprachiasmatic nucleus (SCN) (Albrecht, 2012; Dibner, Schibler, & Albrecht, 2010), and have been reported to be rhythmically expressed in reward-related brain areas (Angeles-Castellanos, Mendoza, & Escobar, 2007; Li, Liu, Jiang, & Lu, 2009). However, in the VTA, clock genes appear to be insufficient to drive rhythms in dopamine activity (Webb, et al., 2009), neural activation (see chapter 3) and neural activity (Luo, et al., 2008), which occur despite a lack of intrinsic clock gene oscillations (Abe et al., 2002; Webb, et al., 2009). Thus, rhythms in reward may also be driven by a timing signal originating elsewhere. The SCN master clock is responsible for entraining rhythms in extra-SCN oscillators (Yamazaki et al., 2000), and two putative signal pathways between the SCN and the VTA have been identified (Deurveilher & Semba, 2005; Korotkova, Sergeeva, Eriksson, Haas, & Brown, 2003; Luo & Aston-Jones, 2009). However, rhythms in reward have been shown to persist in the absence of an SCN signal (Iijima, Nikaido, Akiyama, Moriya, & Shibata, 2002; Sleipness, et al., 2007), suggesting that a timing signal originates from within the mesolimbic system.

The purpose of this study is to demonstrate the role of the mPFC in mediating the diurnal differences in behavioural measures of amphetamine reward. The mPFC is important for the execution of reward behaviour, with a role in reward-based learning, motivation, decision-making, and response inhibition (Hyman, Malenka, & Nestler, 2006; Miller & Cohen, 2001; Ridderinkhof, van den Wildenberg, Segalowitz, & Carter, 2004; Rolls, 2004). Moreover, the mPFC exhibits diurnal rhythms in dopamine, glutamate, GABA activity (Castaneda, et al., 2004), neural activation (see chapter 3), and clock gene

expression (Angeles-Castellanos, et al., 2007; Li, et al., 2009), which in turn may drive rhythms in other mesolimbic areas. Glutamatergic efferents of the mPFC innervate many of the reward related brain areas that exhibit diurnal oscillations in protein and gene expression, including the NAc and VTA (Gabbott, Warner, Jays, Salway, & Busby, 2005). Neurotoxic ablation of the mPFC dampens neural activation in the NAc, eliminating time-of-day differences (see Chapter 3). These observations led us to hypothesize that the mPFC is orchestrating the timing of reward rhythms. To test this hypothesis, we lesioned the mPFC and determined the resulting changes to the daily rhythm in amphetamine reward, using a CPP paradigm. Similarly, we pharmacologically inactivated the mPFC with GABA agonists during CPP conditioning or post-test to determine if mPFC modulation of reward rhythms was occurring during the acquisition or expression of CPP.

4.2 METHODS

4.2.1 Animals

Adult male Sprague-Dawley rats (300-350g, Charles River Laboratories, QC, CAN) were single housed in standard rat housing cages under a 12:12 light/dark cycle (~350lux L/0 lux D) with *ad libitum* food and water. Following arrival, animals were allowed to acclimatize for 1 week prior to surgery and 2 weeks for recovery and light cycle entrainment. All experiments were approved by the University of Western Ontario Animal Care Committee and the University Committee on Use and Care of Animals at

the University of Michigan and are in compliance with the guidelines of the Canadian Council on Animal Care and the U.S. National Institute of Health.

4.2.2 Prefrontal cortex lesion surgery

Rats were anaesthetized with ketamine (87 mg/ml/kg i.p.) and xylazine (13 mg/ml/kg i.p.) and placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, California) with the skull leveled horizontally relative to bregma and lamda. Skull was exposed and a small burrhole was drilled above the injection site at the following coordinates relative to bregma: anterior-posterior = 2.9, medial-lateral = 0.6 mm. Ibotenic acid (2% in phosphate buffered saline) or vehicle was slowly infused bilaterally at two dorsal-ventral coordinates corresponding to the infralimbic and prelimbic regions of the mPFC (from the top of skull = 5.0 and 2.5 mm; 0.25 μ L/1-minute per injection) using a 5 μ L Hamilton Syringe (Hamilton Reno Nevada). Rats were given two weeks for post-operative recovery before any behavioral testing.

4.2.3 Prefrontal cortex cannulae placement surgery:

Rats were anaesthetized and placed into stereotaxic apparatus as above. Two small burrholes were drilled at anterior-posterior = 3.0 mm, and medial-lateral = \pm 1.7 mm . Stainless steel cannulae (5mm, 22 gauge, Plastics One) were implanted bilaterally using a cannula insertion tool mounted to the stereotaxic frame and lowered at a 15 degree angle to a depth of -3.7 mm from the skull to target the IL/PL junction. The cannulae were fixed in place using layered dental acrylic and small screws affixed to the skull at three locations. Dummy cannulae (5mm, Plastics one) were screwed into the

guide cannulae to maintain tract cleanliness and keep out foreign objects. Rats were given two weeks for post-operative recovery before any behavioral testing.

4.2.4 Amphetamine conditioned place preference

Sham-, mPFC-lesioned, and cannulated rats received a week of habituating subcutaneous injections of saline immediately prior to behavioural testing for amphetamine-induced conditioned place preference (CPP) as described in Webb et al., 2009. Cannulated rats received additional handling for 1 week prior to CPP to habituate them to conditions of the drug microinfusion and to check guide cannula blockages. Each animal was conditioned and tested at either ZT11 or ZT23, consistent with the previously observed nadirs and peaks in amphetamine CPP, respectively (Webb, et al., 2009). During a pretest, animals freely roamed the CPP apparatus (Med Associates, St. Albans, Vermont) for 15 minutes to determine the time each animal spent more in chamber than the other. As a group, animals do not show a preference for either chamber, and rats with an initial protracted preference for a given chamber (>120 seconds), were excluded from the study (<15% of subjects). Rats underwent conditioning on days 2 and 3; the initially un-preferred chamber was paired for 30 minutes with amphetamine (2.5 mg/kg, subcutaneous at 1ml/kg, D-amphetamine sulfate in sterile 0.9% saline, calculated based on the free base), while the preferred chamber was paired for 30 minutes with vehicle (0.9% saline, subcutaneous at 1 ml/kg) on alternate days in a counterbalanced manner. Next, a post-conditioning preference test (post test), identical to pretest was conducted to determine reward-induced changes in chamber preference. CPP scores, the change in time spent in the amphetamine-paired-chamber before and after conditioning, were calculated and analyzed for lesion- and time-of-day effects via two-way ANOVA

with bonferonni post-hoc analysis. Difference scores (the difference in time spent in the paired versus unpaired chamber) were also calculated for all groups and analyzed for pre- and post-conditioning effects by paired T-Test.

4.2.5 Intracranial drug infusions

A drug cocktail was prepared consisting of 1ug/ul of GABA_A agonist muscimol (Sigma) and 0.2 ug/ul of GABA_B agonist baclofen (Sigma) dissolved in sterile saline. Prior to microinfusion, dummy caps were removed and a 6mm 28 gauge injector cannula was lowered into each implanted guide cannula and screwed in place. 0.5 µl of drug or saline was infused into each hemisphere, simultaneously, at 0.25µl/min. Microinfusions were made by means of a microinjector pump (Harvard Apparatus) and 10 µl glass syringes (Hamilton) connected to the injector cannulae via plastic tubing (Plastic One). Following drug infusion, injector cannulae were left in place for an additional minute to maximize diffusion of drug. Rats were returned to home cage for 10 minutes prior to CPP. Rats were either infused with inhibitor cocktail (drug) or vehicle on both conditioning days to test effects during the acquisition phase of CPP, or during the post-test day only to test effects on the expression of CPP. Moreover, infusions and CPP testing occurred either at ZT11 or at ZT23.

4.2.6 Verification of lesion and cannula placement

Following post-test, cannulated, sham- and mPFC-lesioned rats, were deeply anaesthetized with sodium pentobarbital (270 mg/kg, i.p.), decapitated, brains were

rapidly removed, frozen on dry ice, and stored at -80°C , sectioned coronally ($35\ \mu\text{m}$) using a cryostat (Leica Microsystems, Wetzlar), mounted directly onto plus-charged slides (Fisher Scientific, Ottawa, Canada) in series and post fixed in paraformaldehyde (4% in 0.1M PB) for one hour. Mounted sections containing the mPFC were stained for nissl (.625% Cresyl Violet Acetate (Sigma-Aldrich, St. Louis, MO, USA) in .0625% sodium acetate trihydrate (Fisher Scientific, Ottawa, Canada) in ddH₂O, pH 3.14), dehydrated in alcohol, cleared with Citrisolv (Fisher Scientific, Ottawa, Canada), and coverslipped with Di-N-Butyl Phthalate in xylene (Electron Microscopy Sciences, Fort Washington, PA).

The extent and locations of the lesions were assessed as previously described (Davis et al., 2010), Animals with $>80\%$ loss of PL and IL were considered lesioned animals; 18 sham-operated, 19 lesioned. Lesioned animals had minimal or no damage to the anterior cingulate area or nucleus accumbens. Moreover, sham animals did not have any cell loss in mPFC or neighbouring brain regions. Successful cannula placements were those where tracts terminated at the IL/PL interface, in accordance with known landmarks (Swanson, 1998); for acquisition phase injections, $n=13$ vehicle, 24 drug; for expression phase, 17 vehicle, 20 drug.

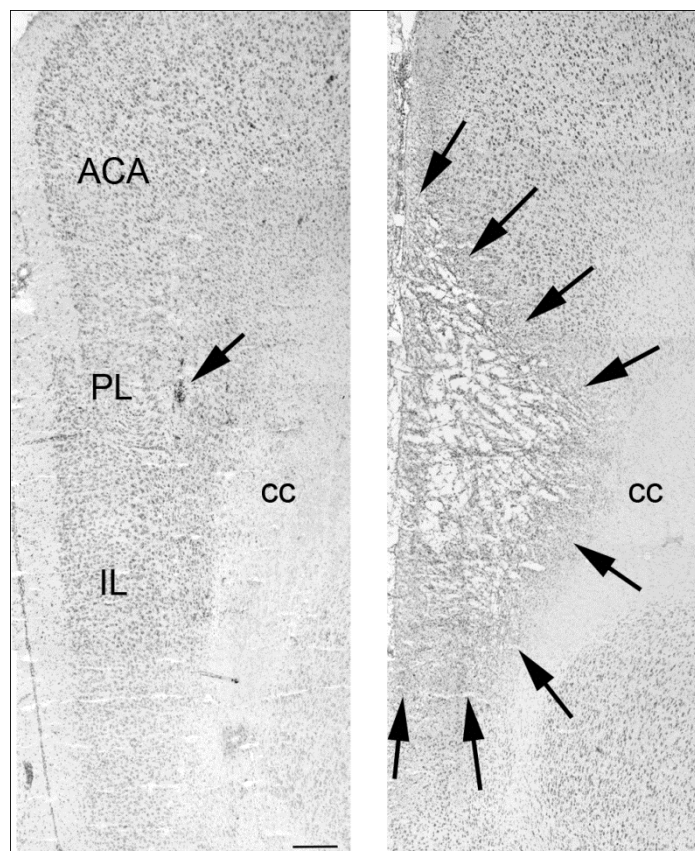


Figure 4.1: cresyl violet stain in lesioned and sham-operated prefrontal cortex.

Images show mPFC in representative sham-operated (left panel) and lesion (right panel), in brain sections stained with Cresyl Violet. Arrows on indicate extent of the lesion site; on the right, encompassing the infralimbic (IL) and prelimbic (PL) areas, with sparing of the anterior cingulate cortex area (ACA); on the left, sparing the IL, PL, and ACA. Scale bars indicate 250 μ m. Abbreviations: cc, corpus callosum.

4.3 RESULTS

4.3.1 mPFC lesion CPP

mPFC lesions had a significant effect on diurnal rhythms in amphetamine-induced CPP. Sham animals had a significantly higher CPP score at ZT 23 compared to ZT11 ($t=2.365$, $p<0.05$), consistent with our previous findings (Webb et al, 2009). There was a significant effect of lesion on CPP score ($F(1,33) = 7.038$, $p<0.05$, Figure 4.1), such that lesioned males had a significant increase in CPP score at ZT11 ($t=2.8$, $p<0.05$) compared to sham animals. There were no differences between sham and lesioned males at ZT23. Due to the increase in CPP score at ZT11 in lesioned rats, there was no significant difference in CPP score between ZT11 and ZT23. Similar effects were observed for difference scores. At ZT11, lesioned, but not sham males, had a significant increase in time spent in the amph-paired chamber ($t=3.525$, $p<0.05$). At ZT23, both mPFC lesion ($t=4.658$, $p<0.05$) and sham ($t=2.372$, $p<0.05$) males formed a significant CPP. Thus, amphetamine-induced CPP was formed at ZT11 in the lesion group but not the sham-operated group, and in both groups at ZT23.

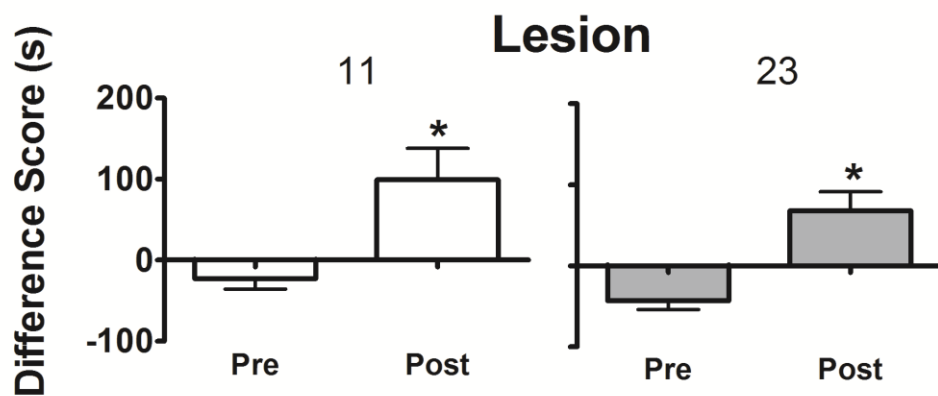
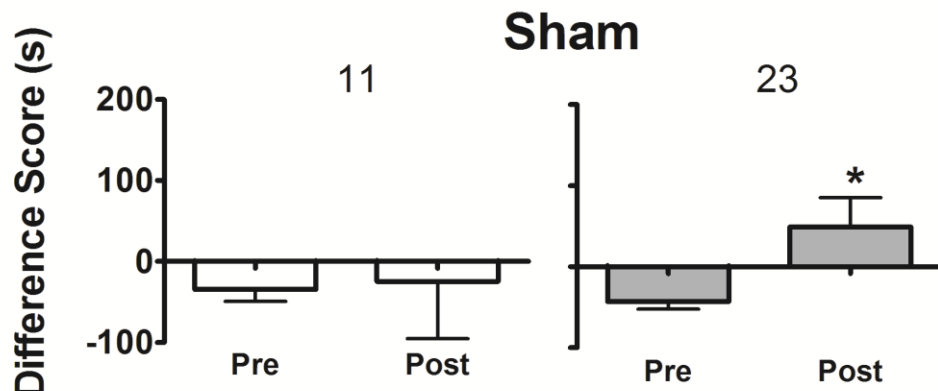
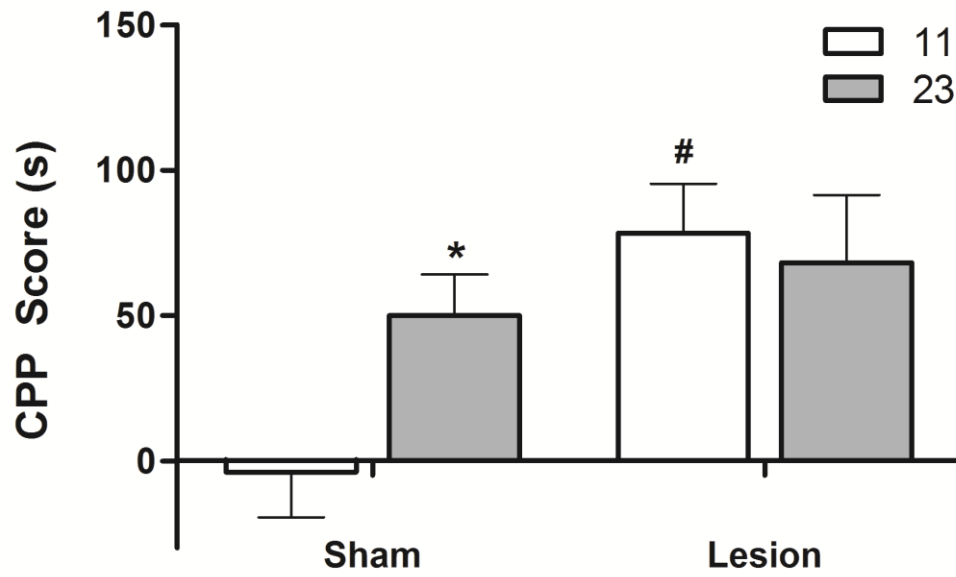


Figure 4.2 The mPFC modulates diurnal rhythms in amphetamine-induced conditioned place preference. Time-of-day differences in amphetamine-induced conditioned place preference in mPFC lesioned (lesion) and sham-operated (sham) rats. Data expressed as mean \pm SEM in (A) CPP score: the change in time spent in amphetamine paired-chamber after before and after conditioning; and (B) difference score: the difference in time between paired and un-paired chamber. * denotes significant differences in CPP score between ZT11(11) and ZT23(23), or in difference score between post (post) and pre (pre) test days of the CPP procedure; # denotes significant differences in CPP score between sham and lesion at ZT11 .

4.3.2 mPFC GABA agonist infusions

Overall, infusion of GABA agonists into the mPFC prior to acquisition or expression of amphetamine CPP mimicked the effects caused by lesions and resulted in an increased CPP at ZT11 and a subsequent loss of time-of-day differences. For infusions during the acquisition phase, there was a significant effect of the drug on CPP score ($F(1,33)= 4.393$, $p<0.05$) that was dependent on the time-of-day ($F(1,33)=7.191$, $p<0.05$, interaction effect), while no overall time-of-day effect was observed. In vehicle infused rats, there was a significant difference in CPP score between ZT11 and ZT23 ($t=2.688$, $p<0.05$) that was not observed with drug infusions. The loss of the time-of-day difference in the drug infused group was mediated by an increase in CPP score at ZT11 in the drug infused groups ($t=3.344$, $p<0.05$) compared to the vehicle group. CPP score at ZT23 was not affected by drug infusions and there were no differences between vehicle and drug groups. Similar effects were observed with difference scores. Significant pre- and post-test differences were observed at ZT11 ($t=2.956$, $p<0.05$) following drug infusion but not with saline infusions. At ZT23, significant pre- and post-test differences were observed irrespective of drug ($t=1.195$, $p<0.05$) or saline ($t=2.067$) infusion. Thus, amphetamine-induced CPP was formed at ZT11 in the drug infused group but not the vehicle group, while CPP at ZT23 was not affected and formed in both groups.

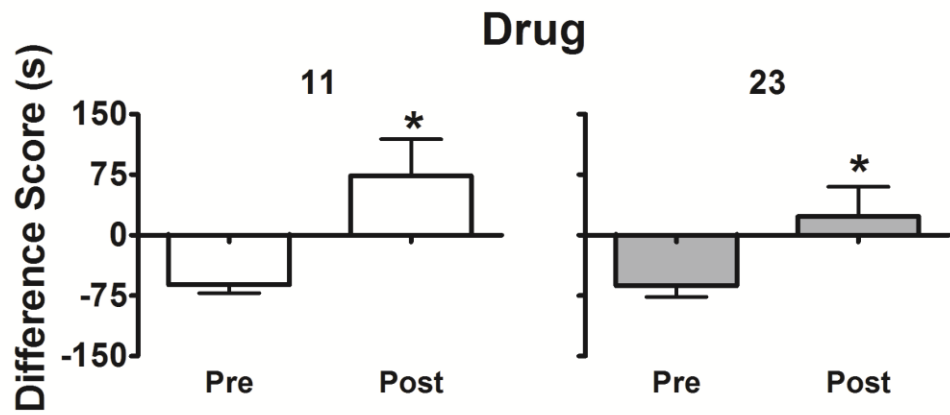
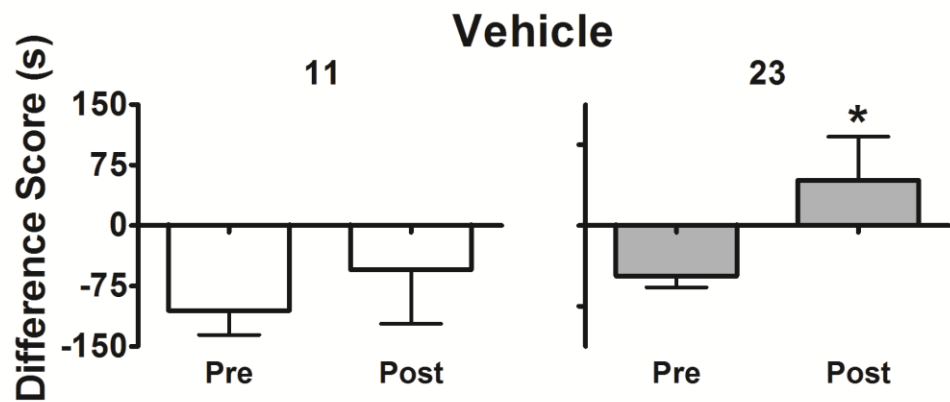
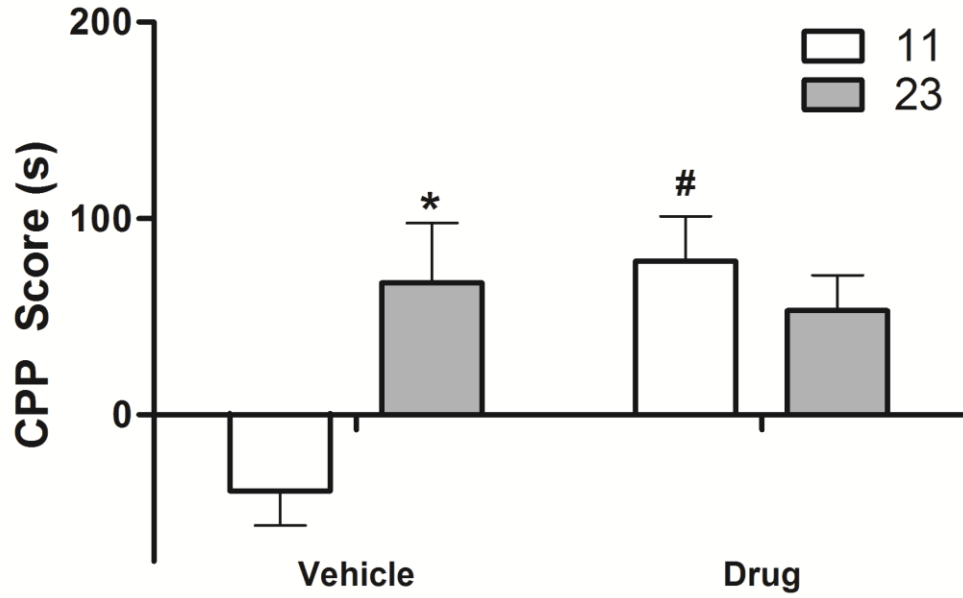


Figure 4.3: mPFC inactivation during acquisition phase prevents time-of-day differences in amphetamine-induced conditioned place preferences. Time-of-day differences in amphetamine-induced conditioned place preference in rats receiving mPFC infusions of GABA agonist cocktail (DRUG) or vehicle during conditioning. Data expressed as mean \pm SEM in (A) CPP score: the change in time spent in amphetamine paired-chamber after before and after conditioning; and (B) difference score: the difference in time between paired and un-paired chamber. * denotes significant differences in CPP score between ZT11(11) and ZT23(23), or in difference score between post-test (post) and pre-test (pre); # denotes significant differences in CPP score between sham and lesion at ZT11.

For infusions during the expression phase, there were unexpected effects of infusions of either vehicle or drug. The time that animals spent in the middle chamber across all groups was increased irrespective of drug treatment or time-of-day. As a result, the CPP score was not calculated as it is affected by middle chamber time. However, effects of drug infusions on the difference scores were consistent with those observed for acquisition phase infusions. Significant increases in time spent in the amphetamine-paired chamber were observed in drug, but not vehicle-infused males at ZT11 ($t=3.227$, $p<0.05$). At ZT23, there were no effects of drug, and both drug ($t=3.093$, $p<0.05$) or vehicle ($t=5.988$, $p<0.05$)-treated males formed a significant CPP. Again, amphetamine-induced CPP was formed at ZT11 in the drug infused group but not the vehicle group, and mPFC inhibition did not affect CPP at ZT23.

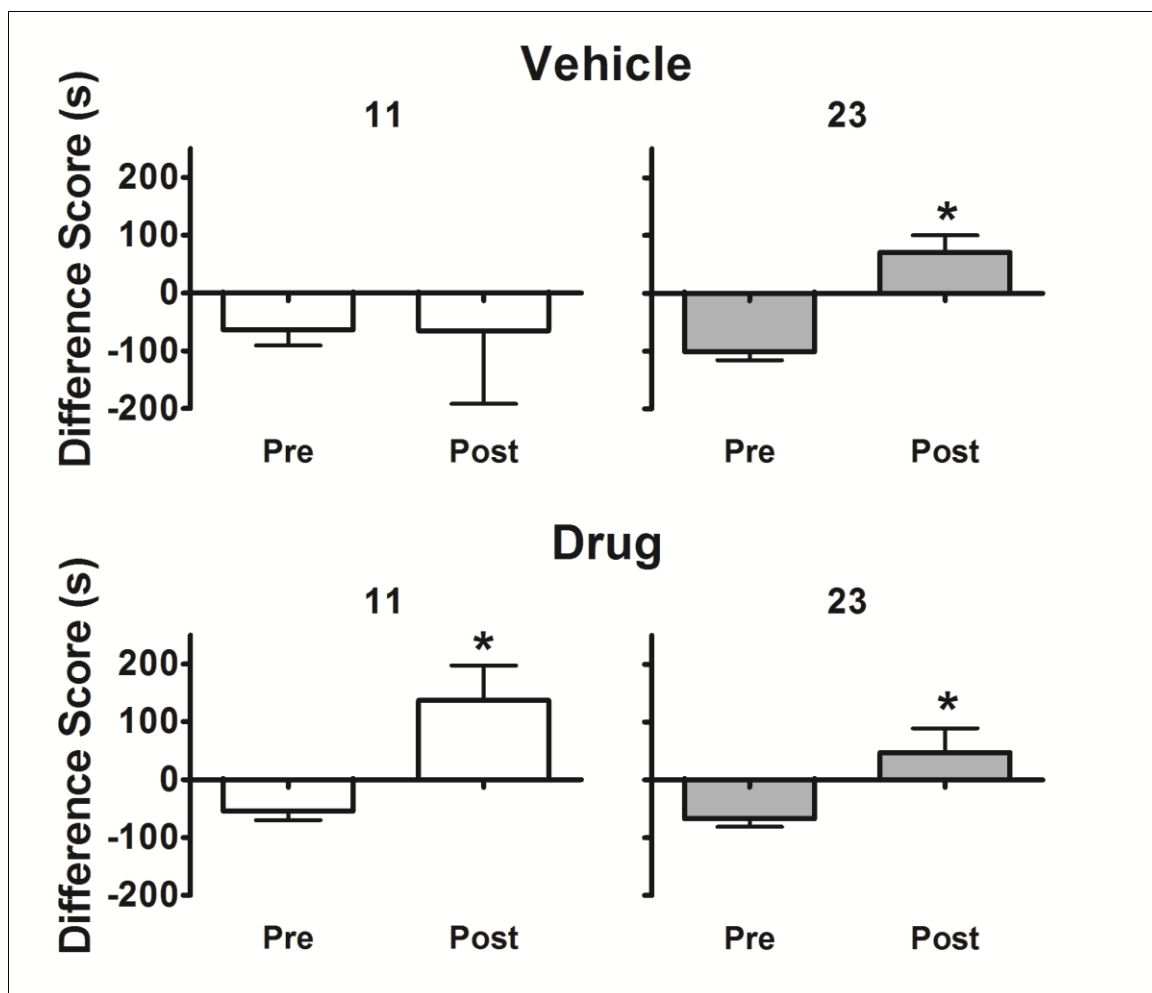


Figure 4.4: mPFC inactivation during expression phase prevents time-of-day differences in amphetamine-induced conditioned place preferences. Time-of-day differences in amphetamine-induced conditioned place preference in rats receiving mPFC infusions of GABA agonist cocktail (Drug) or vehicle on post-test day. Data expressed as mean \pm SEM in difference score: the difference in time between post (post) and pre (pre) test days of the CPP procedure; * denotes significant change in difference score between post- and pre-test .

4.4 DISCUSSION

The aim of this study was to determine the role of the mPFC in regulating diurnal rhythms in reward. Our findings indicate that the mPFC is an important modulator of rhythmic behavioural output from the reward system, as the diurnal rhythm in amphetamine reward (Webb, et al., 2009) is lost with lesioning or pharmacological inactivation of the mPFC. With either mPFC manipulation, the loss of rhythmicity occurred via an increase in amphetamine reward at the nadir point, suggesting that amphetamine becomes equally rewarding at all times of the day. Furthermore, the mPFC appears to be exerting its rhythmic control over reward during both the acquisition and expression of learned reward-place associations. mPFC infusion of GABA agonists during either phase had an equal effect on the amphetamine CPP rhythm, a loss of time-of-day differences.

Several studies have investigated the role of the mPFC in CPP for drugs of abuse (Tzschentke, 2007). Neurotoxic quinolinic acid lesions of the mPFC have little effect on amphetamine CPP (Tzschentke, 1998b; Tzschentke & Smith, 1998), while prelimbic (PL) but not infralimbic (IL) lesions block cocaine-induced CPP during the light phase, (Tzschentke & Smith, 1998b) but not during the dark phase (Zavala, Weber, Rice, Alleweireldt, & Neisewander, 2003). Conversely, IL but not PL lesions block opiate-induced CPP during the light phase (Tzschentke & Smith, 1998). mPFC lesions have similar effects on other drug-related behaviours. Neurotoxic lesions of the PL impair cocaine-induced sensitization, lesions of the IL impair morphine sensitization, while whole mPFC lesions did not affect amphetamine sensitization (Li & Wolf, 1997; Li,

Wolf, & White, 1999; Tzschentke & Schmidt, 2008). Tzschentke & Schmidt (2008) conclude that the mPFC does not mediate the rewarding effects of amphetamine, and processing of morphine and cocaine reward is region specific. Here we report a significant increase in amphetamine CPP with IL/PL lesions during the late dark phase (ZT23), but not during the late-light phase (ZT11). The discrepancy between our findings and previous reports with respect to the effects of mPFC lesions on drug CPP are not immediately obvious but may reflect important procedural differences.

Firstly, previous CPP paradigms used at least three reward-paired trials during conditioning. Under these conditions, significant psychostimulant-induced CPP was observed in sham-operated animals irrespective of time-of-day (Tzschentke & Smith, 1998b; Zavala, et al., 2003). A single pairing with amphetamine was chosen to prevent inducing circadian anticipatory behaviour and to minimize drug-induced changes to basal mesolimbic activity. Roberts et al. (2002) previously reported that a daily rhythm in cocaine self-administration disappears at high doses, suggesting a breakdown in circadian regulation of the reward system and the development of an addictive phenotype. Multiple pairings with the drug may have had the same effect as an increased dose, disrupting baseline rhythms in reward and mPFC activity.

A second challenge in interpreting other studies investigating effects of mPFC lesion or inactivation on reward (Franklin & Druhan, 2000; Ishikawa, Ambroggi, Nicola, & Fields, 2008; Peters, LaLumiere, & Kalivas, 2008; Tzschentke & Smith, 1998a;

Tzschentke & Smith, 1998b; Tzschentke, 1999), is that behavioural testing was conducted over long periods of time. Rats were typically placed on a 12hr light/dark cycle with lights on at 07:00 (ZT0), and experiments carried out during the light phase. Presumably, behavioural testing coincides with the normal work period, or roughly ZT2 to ZT10. Significant time-of-day differences in amphetamine reward during this interval with a peak (ZT5) and nadir (ZT11) have been reported (Webb, et al., 2009). mPFC lesions caused a significant increase in amphetamine reward at nadir times, but not at peak times. Thus, the lack of mPFC lesion effects on amphetamine CPP (T. M. Tzschentke & W. J. Schmidt, 1998b) in other studies may reflect a testing interval that included peaks and nadirs, causing time-dependent effects to be masked. Despite the possible masking effects caused by long testing intervals, mPFC lesions still impair CPP induced by another psychostimulant, cocaine (Tzschentke & Smith, 1998b; Tzschentke & Schmidt, 1999). Tzschentke & Schmidt (1999) attributes the lack of mPFC lesion effects on amphetamine CPP to evidence that amphetamine reward is predominantly mediated by mesolimbic dopamine. Here we observe that the mPFC plays an important role in regulating amphetamine CPP, while others have reported that mPFC inactivation by GABA agonists increases methamphetamine self-administration and impairs methamphetamine-seeking behaviour (Rocha & Kalivas, 2010). Thus, effects of mPFC lesions on amphetamine CPP may be influenced by time-of-day and other procedural parameters.

To our knowledge, this is the first study to have investigated the effects of acute mPFC GABA agonist infusions on reward CPP. mPFC inactivation has been reported to

impair several other reward-related behaviours. For instance, inactivation of the IL/PL by GABA agonists induces cocaine-seeking behaviour in extinguished rats (Peters, LaLumiere, et al., 2008). Alternatively, IL/PL inactivation does not impair the suppression of cocaine-seeking behaviour during extinction training, nor reinstatement of cocaine-seeking behaviour following abstinence (Peters, Vallone, Laurendi, & Kalivas, 2008). IL/PL inactivation also impairs learned goal-directed behaviours with decreased cue-evoked operant bar-pressing for sucrose acquisition, and increased bar-pressing to non-rewarded cues (Ishikawa, et al., 2008). It was shown that dorsal mPFC (ACA and dorsal PL) inactivation is responsible for the former, while inactivation of ventral mPFC (IL and ventral PL) the latter. Several researchers have suggested specialized roles of the ventral and dorsal mPFC in goal-directed behaviours (Ghazizadeh, Ambroggi, Odean, & Fields, 2012; Ishikawa, et al., 2008; LaLumiere, Smith, & Kalivas, 2012; Peters, LaLumiere, et al., 2008; Peters, Vallone, et al., 2008). The ventral mPFC, specifically the IL, is important for inhibiting inappropriate or non-rewarding responses, and similarly, extinction of goal-directed behaviour. The dorsal mPFC, including the PL, is important for the initiation of conditioned drug responses. It is possible that diurnal rhythms in reward are controlled in a similar way, by daily oscillations in the underlying neural mechanisms that mediate initiation and inhibition of reward behaviour. mPFC lesions or inactivation disrupt these rhythms leading to constitutively elevated amphetamine reward. However, the specific role of each subregion in mediating diurnal rhythms in reward has yet to be determined. The influence of the IL and PL on goal-directed behaviour is thought to be mediated by projections to the NAc shell and NAc core, respectively (Berendse, Graaf, & Groenewegen, 2004; Sesack, Deutch, Roth, & Bunney, 1989). We

previously observed a loss of the rhythm in neural activation in the NAc core and NAc shell (chapter 3) with IL/PL lesions. However, the loss of rhythmicity in accumbal activation occurred via a decrease in cFos-IR during the dark phase (ZT18), and thus appears to be unrelated to the effects of mPFC lesions on amphetamine reward, which are mediated by increased amphetamine CPP during the light phase (ZT11).

The loss of time-of-day differences in amphetamine CPP with mPFC lesions and inactivation suggests that a rhythmic signal is emanating from the mPFC, which may be driven by intrinsic clock gene oscillations (Angeles-Castellanos, et al., 2007; Li, et al., 2009). Another possibility is that the mPFC is not intrinsically rhythmic but mediates a rhythmic signal that originates elsewhere. This hypothesis is in line with the well-established role of the mPFC in integrating and modulating inputs from various reward areas including the amygdala, hippocampus and VTA (Bechara, 2005), where a temporal signal could be another kind of input. The VTA is a likely candidate for conveying a timing signal to the mPFC as it is implicated in the regulation of reward (Kelley & Berridge, 2002), and expresses diurnal rhythms in neural activation (see chapter 3). Dopaminergic projections originating in the VTA innervate the mPFC (Thierry, Blanc, Sobel, Stinus, & Golwinski, 1973), where diurnal differences in markers of dopamine release have been reported (Sleipness, et al., 2008). Mesocortical dopamine is critical for reward related learning (Wise, 2004), and exerts precise control over mPFC excitability by regulating dopamine D1 and D2 receptor mediated feed forward inhibition (Tierney, Thierry, Glowinski, Deniau, & Gioanni, 2008; Xu & Yao, 2010). Thus, an exogenous

timing signal may be conveyed through diurnal variations in dopamine signalling and D1/D2 dependent excitability.

The specific changes to reward behaviour following mPFC manipulation have led many to suggest that the mPFC exerts top-down control over reward behaviour, activating or inhibiting learned responses and shifting strategies in the face of changing information (Bechara, 2005; Kroener & Lavin, 2010; Miller & Cohen, 2001; Peters, LaLumiere, et al., 2008). This conception of the mPFC is consistent with the current findings. We observed that amphetamine reward becomes persistently elevated throughout the day with mPFC impairment, causing a loss of the daily reward rhythm. These findings are consistent with the hypothesis that rhythms in reward are mediated by a mechanism of negative regulatory control by the mPFC.

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CHAPTER 5:

Summary and Future Directions

5.1 SUMMARY AND FUTURE DIRECTIONS

5.1.1 Diurnal rhythms in amphetamine and sex reward

A diurnal rhythm in reward, measured by conditioned place preference (CPP), for sex (Fig. 2.1) and amphetamine (Fig. 2.2) was observed (Webb, Baltazar, Lehman, & Coolen, 2009). The daily peak in amphetamine reward occurred during the early day (ZT5), and was coincident with previously reported peaks for cocaine reward. The time-of-day difference between the peak and nadir in amphetamine reward persists in constant darkness, suggesting that diurnal rhythms in reward are circadian in nature. In comparison to amphetamine, the peak in sex reward was observed during the early night (ZT17). The reasons for the phase disparity between drug and sex rhythms are not yet known, but suggest multiple levels of regulation in the underlying reward circuits.

5.1.2 Diurnal rhythms in mesolimbic activity

Rhythms in reward were likely mediated by oscillations in the underlying neural circuits. Thus, we characterized daily changes in mesolimbic dopamine activity, and neural activation. Diurnal rhythms for the marker of dopamine synthesis, tyrosine hydroxylase (TH), were observed in the VTA and the NAc (Fig. 2.4)(Webb et al., 2009), but with different patterns of expression. The peak and nadir in TH protein in the NAc is observed during the early night (ZT14) and during the mid day (ZT6), and coincides with those of sex reward (Fig. 2.6). The rhythm in VTA TH expression was opposite that of the NAc, with a peak in the mid day (ZT6) and a nadir just before the transition to dark (ZT10), and overlaps with the daily rhythm in amphetamine reward (Fig. 2.6).

Neural activation in the NAc (Fig.3.3), PFC (Fig. 3.4), and VTA (Fig. 3.5), was also observed to vary with a diurnal rhythm, the phases of which were coincident. The peaks in cFos immunoreactivity occurred during the mid night (ZT18), and the nadirs near the light to dark transition (ZT10). Moreover, the number of activated dopamine cells also varied by time-of-day in the VTA with a similar phase, and accounted for approximately 20% of total activation. Overall, these findings demonstrate diurnal regulation of the mesolimbic system, which is likely linked to the observed rhythms in reward behaviour.

It is hypothesized that the peak in sex reward occurs when neural activation and the potential for dopamine release in the NAc is highest (Fig. 2.6). For rats, this coincides with the normal active period at night, when encounters with biologically relevant stimuli are most likely. While correlations in TH and neural activation potentially explain circadian regulation of natural rewards, the peak in amphetamine reward coincides with a decreased likelihood for evoked NAc dopamine release (Fig. 2.6). Amphetamine artificially increases extracellular dopamine in the NAc by blocking the dopamine transporter (Seiden, Sabol, & Ricaurte, 1993; Wise & Bozarth, 1987). Thus, the peak in amphetamine reward may be linked to increased dopamine receptor sensitization at a time when extracellular dopamine is normally low. However, these hypotheses derive from the activity of mesolimbic dopamine projections alone. Given the anatomical and neurochemical complexity of the reward system (Kelley & Berridge, 2002; Koob, 2006; Sesack, Carr, Omelchenko, & Pinto, 2003), circadian regulation of reward likely occurs at multiple sites of action. Research on the circadian regulation of

the mesolimbic system has mainly focused on markers of dopamine signalling (McClung et al., 2005; Sleipness, Jansen, Schenk, & Sorg, 2008; Sleipness, Sorg, & Jansen, 2007; Webb, Baltazar, Wang, et al., 2009), while the role of the non-dopaminergic elements has largely been unexplored. Future experiments will focus on characterizing diurnal rhythms in activity of other major neurotransmitters in the mesolimbic system, with a particular focus on glutamate signalling.

5.1.3 The medial prefrontal cortex modulates rhythms in reward and mesolimbic activity

Diurnal rhythms in reward led us to investigate the nature of the timing mechanism that drives them. Clock genes drive molecular oscillations in the master circadian clock, the SCN (Ko & Takahashi, 2006), and have been localized to some reward-related brain regions (Angeles-Castellanos, Mendoza, & Escobar, 2007; Iijima, Nikaido, Akiyama, Moriya, & Shibata, 2002; Li, Liu, Jiang, & Lu, 2009; Uz, Akhisaroglu, Ahmed, & Manev, 2003). Here, we report diurnal fluctuations in the concentration of clock proteins (Per1 and BMAL1) in the NAc with differential temporal expression (Fig.2.5), but not in the VTA, suggesting that observed rhythms mesolimbic and neural activity in this region is mediated by a timing signal originating elsewhere.

The role of the mPFC in regulating observed rhythms in amphetamine-induced CPP, and in neural activation in the NAc and VTA was investigated. The mPFC is a good candidate for conferring rhythmicity upon the reward system as it is important for the regulation of reward (Hyman, Malenka, & Nestler, 2006; Miller & Cohen, 2001; Ridderinkhof, van den Wildenberg, Segalowitz, & Carter, 2004) and expresses rhythms

in neurotransmitter activity (Castaneda, de Prado, Prieto, & Mora, 2004) and clock gene expression (Angeles-Castellanos, et al., 2007; Li, et al., 2009). Lesions of the mPFC caused an attenuation of neural activation at peak time (ZT18) in the NAc core and shell (Fig.3.7), such that the diurnal rhythm was altogether lost, with no effect on the rhythms in the VTA. These observations suggest that the NAc may be an important convergence point for timing signals conveyed by corticoaccumbal glutamatergic and mesolimbic dopaminergic projections.

As rhythms in VTA activity do not appear to be driven by local clock gene activity or by the PFC, the timing signal responsible for VTA rhythmicity must originate elsewhere. As previously mentioned, a potential signalling pathway between the SCN and the VTA has been identified via hypothalamic orexin neurons (Deurveilher & Semba, 2005; Korotkova, Sergeeva, Eriksson, Haas, & Brown, 2003), which are ideally positioned to transmit an entrainment signal to the mesolimbic system. Again, the orexin system has been implicated in reward (Aston-Jones, Smith, Moorman, & Richardson, 2009; Harris, Wimmer, & Aston-Jones, 2005) via its regulation of VTA dopamine neurons, which been shown to vary by time-of-day (Moorman & Aston-Jones, 2010). Moreover, phase shifting or eliminating SCN output alters diurnal rhythms of orexin signalling (Marston et al., 2008; Zhang et al., 2004). This initial evidence suggests that hypothalamic orexin neurons are important for modulating rhythms in mesolimbic activity, however additional experiments will be necessary to elucidate the underlying mechanism and behavioural consequences.

As the PFC was important for modulating rhythms in mesolimbic activity, we investigated its capacity for effecting rhythms in reward behaviour. Similarly, PFC lesions affected a significant increase in amphetamine reward at the nadir point (ZT11), resulting in a loss of time-of-day differences (Fig. 4.2). Next, we determined if the role of the PFC in modulating reward rhythmicity was differentially exerted during the acquisition of conditioned reward-place association or upon its recall (Fig. 4.3-4). Pharmacological inactivation of the mPFC has the same effect as lesions irrespective of the phase of conditioning. The mPFC is important for the precise top-down control of goal-directed behaviours (Miller & Cohen, 2001) and thus, may relegate reward to times when encountering a rewarding stimulus is increased. Based on these findings, it is hypothesized that mPFC is exerting its influence over the reward system via a mechanism of negative regulatory control, which is consistent with the overall role of the mPFC of inhibitory control (Peters, Kalivas, & Quirk, 2009; Peters, LaLumiere, & Kalivas, 2008; Peters, Vallone, Laurendi, & Kalivas, 2008; Van den Oever, Spijker, Smit, & De Vries, 2010). Consistent with this hypothesis, the in peak amphetamine reward may coincide with the nadir in mPFC inhibition. Thus, lesioning or inactivating the mPFC results in amphetamine being rewarding at all times of day.

This body of work has provided an extensive characterization of diurnal rhythms in reward and mesolimbic activity. It provides the first example of such rhythms for natural reinforcers, and extends those of psychostimulants to include multiple time-points across the day. Furthermore, it has provided some potentially meaningful correlations between reward behaviours and rhythms in mesolimbic activity, and established the PFC

as an important locus for regulating the timing of reward. This work has investigated the relationship between rhythms in mesolimbic activity and reward under baseline conditions. Additional experiments will be necessary to determine if rewarding stimuli have a differential effect on inducing neuroplastic changes to the structure and function of mesolimbic circuits across the day. Furthermore, there are other questions that need to be answered. For instance, what is the role of the SCN in regulating or entraining rhythms in reward?; how do rhythmic glutamate and dopamine signals converging on the nucleus accumbens interact to generate rhythms in reward?; what is the nature of the timing mechanism driving mPFC rhythms?

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APPENDIX A



Jan. 1, 2009

***This is the 1st Renewal of this protocol**

***A Full Protocol submission will be required in 2011**

Dear Dr. **Lehman**:

Your Animal Use Protocol form entitled:

The Effects of the Pacemaker in the Eye on Circadian Rhythms

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **Jan. 1, 2009 to Dec. 31, 2009**

The protocol number for this project remains as **2007-115**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. Approved Protocol - M. Lehman, L. Coolen, W. Lagerwerf
Approval Letter - M. Lehman, L. Coolen, W. Lagerwerf



January 1, 2010

***This is the 2nd Renewal of this protocol**

***A Full Protocol submission will be required in 2011**

Dear Dr. **Lehman**

Your Animal Use Protocol form entitled:

The Effects of the Pacemaker in the Eye on Circadian Rhythms

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **January 1, 2010 to December 31, 2010**

The protocol number for this project remains as **#2007-115**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. Approved Protocol - M. Lehman, L. Coolen, W. Lagerwerf
Approval Letter - M. Lehman, L. Coolen, W. Lagerwerf

APPENDIX B



The University of Michigan
University Committee on Use and Care of Animals (UCUCA)

PI: Lehman, Michael
Protocol Number: #10507
Approval Period: 02/14/2011 - 02/14/2014
Funding Agency: Canadian Institutes of Health Research
Title: Interactions Between the Eye and Central Circadian Clock
funded by Natural Sciences and Engineering Research Council of Canada (NSERC)
DRDA Number:

Date: 02/14/2011

Dear Principal Investigator,

The University of Michigan Committee on Use and Care of Animals (UCUCA) has reviewed your application to use vertebrate animals (Application #10507). This project has been approved. The proposed animal use procedures are in compliance with University guidelines, State and Federal regulations, and the standards of the "Guide for the Care and Use of Laboratory Animals."

When communicating with the UCUCA Office please refer to the Approval Number #10507. The approval number must accompany all requisitions for animals and pharmaceuticals.

The approval date is 02/14/2011. The approval period is for three years from this date. However, the United States Department of Agriculture (USDA) requires an annual review of applications to use animals. Therefore, each year of this application prior to the anniversary of its approval date, you will be notified via email to submit a short annual review. Your continued animal use approval is contingent upon the completion and return of this annual review. You will also be notified 120 days prior to the expiration of the approval period so that your renewal application can be prepared, submitted and reviewed in a timely manner in the eSirius program and an interruption in the approval status of this project avoided.

UCUCA approval must be obtained prior to changes from what is originally stated in the protocol. An amendment must be submitted to the UCUCA for review and approved prior to the implementation of the proposed change.

The University's Animal Welfare Assurance Number on file with the NIH Office of Laboratory Animal Welfare (OLAW) is A3114-01, and most recent date of accreditation by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC, Intl.) is November 06, 2009.

If you receive news media inquiries concerning any aspect of animal use or care in this project, please contact James Erickson, News and Information Services, 647-1842. If you have security concerns regarding the animals or animal facilities, contact Bill Bess, Director of Public Safety, 763-3434.

Sincerely,

Richard Keep, Ph.D.
Professor, Neurosurgery
Chairperson, University Committee on Use and Care of Animals

CURRICULUM VITAE

Ricardo M Baltazar

Education:

PhD in Neuroscience: Western University
Department of Anatomy and Cell Biology
Sept. 2007 - present

Bachelor of Science University of Toronto
Faculty of Arts and Science: Human Behavioral Biology
Honors project in circadian rhythms
Sept. 2002- May 2007

Teaching Experience:

Department of Anatomy and Cell Biology, TA for 'Neuroscience for Rehabilitation Sciences', Fall 2007 & 2008.

Scholarships:

National Science and Engineering Council, Post-graduate Scholarship, 2010-2013.

Western Graduate Research Scholarship 2007-2012

Ontario Graduate Scholarship in Science and Technology, 2009-2010

Federation of Portuguese Canadian Businesses, graduate scholarship 2007, 2009

University of Toronto Excellence Award in Natural Sciences and Engineering, 2007

Awards:

Margaret Moffat research day poster award, 2008

Altman Award, 2008

Publications:

Ricardo M Baltazar, Lique M Coolen, Ian C Webb (2013). Diurnal rhythms in neural activation in the mesolimbic system: critical role of the medial prefrontal cortex. European Journal of Neuroscience, In Press.

Meghan E Reale, Ian C Webb, Xu Wang, Ricardo M Baltazar, Lique M Coolen, Michael N Lehman (2013). The transcription factor Runx2 is under circadian control in the suprachiasmatic nucleus and functions in the control of rhythmic behavior. PLoS One, In Press

Gina Glickman, Ian C Webb, Jeffrey A Elliott, Ricardo M Baltazar, Meghan E Reale, Michael N Lehman, Michael R Gorman (2012). Photic sensitivity for circadian response to light varies with photoperiod. Journal of Biological Rhythms, 27(4):308-18

Ricardo M Baltazar, Ian C Webb, Xu Wang, Lique M Coolen, Michael N Lehman (2009). Diurnal Variations in Natural and Drug Reward, Mesolimbic Tyrosine Hydroxylase and Clock Gene Expression in the Male Rat. Journal of Biological Rhythms, 24 (6), 465-76.

Ian C Webb, Ricardo M Baltazar, Michael N Lehman, Lique M Coolen (2009). Bidirectional interactions between the circadian and reward system: is restricted food access a unique zeitgeber? European Journal of Neuroscience, 30(9),1739-48.

Conference Abstracts:

Ricardo M. Baltazar, Ian C. Webb, Lique M. Coolen & Michael N. Lehman. (2011) The prefrontal cortex modulates diurnal rhythms in cFos immunoreactivity in the mesolimbic reward system. Society for Neuroscience, Washington DC, November 14th 2011.

Ricardo M. Baltazar, Ian C. Webb, Lique M. Coolen & Michael N. Lehman. (2011) Diurnal rhythms in cFos immunoreactivity in the mesolimbic reward system. Canadian Conference for Study of Biological Rhythms, Montreal, June 2011

Ricardo M. Baltazar, Ian C. Webb, Lique M. Coolen & Michael N. Lehman. (2010) Diurnal rhythms in cFos immunoreactivity in the mesolimbic reward system. Society for Biological Rhythms, Florida, May 2010.

Ian C. Webb*, Ricardo M. Baltazar, Lique M. Coolen & Michael N. Lehman. (2008) Diurnal Variation in Natural and Drug-Induced Reward in the Male Rat. Annual Society for Neuroscience Meeting, Washington D.C. November 15th 2008.

Ian C. Webb, Ricardo M. Baltazar, Lique M. Coolen & Michael N. Lehman. (2008) Diurnal Rhythms in the Response to Natural Rewards in the Male Rat. Conference for Study of Biological Rhythms, Florida, May 18th 2008.

Ricardo M. Baltazar, Ian C. Webb, Lique M. Coolen & Michael N. Lehman. (2008)
Diurnal Rhythms in the Response to Natural Rewards in the Male Rat. Southern Ontario
Neuroscience Association, University of Western Ontario, May 2008.

Ricardo M. Baltazar, Ian C. Webb, Lique M. Coolen & Michael N. Lehman. (2008)
Diurnal Rhythms in the Response to Natural Rewards in the Male Rat. Margaret Moffat
Research day, University of Western Ontario, March 20th 2008.