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Diurnal rhythms in neural activation in the mesolimbic reward system: critical role of the medial prefrontal cortex

Ricardo M. Baltazar,[1] Lique M. Coolen[1,2,3] and Ian C. Webb[4]
[1]Department of Anatomy and Cell Biology, University of Western Ontario, London, ON, Canada
[2]Department of Molecular & Integrative Physiology, University of Michigan, Ann Arbor, MI, USA
[3]Department of Physiology & Biophysics, University of Mississippi Medical Center, Jackson, MS, USA
[4]Department of Neurobiology and Anatomical Sciences, University of Mississippi Medical Center, 2500 North State St, Jackson, MS, 39216-4505, USA

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Abstract

Previous evidence suggests a circadian modulation of drug-seeking behavior and responsiveness to drugs of abuse. To identify potential mechanisms for rhythmicity in reward, a marker of neural activation (cFos) was examined across the day in the mesolimbic reward system. Rats were perfused at six times during the day [zeitgeber times (ZTs): 2, 6, 10, 14, 18, and 22], and brains were analysed for cFos and tyrosine hydroxylase (TH)-immunoreactive (IR) cells. Rhythmic expression of cFos was observed in the nucleus accumbens (NAc) core and shell, in the medial prefrontal cortex (mPFC), and in TH-IR and non-TH-IR cells in the ventral tegmental area (VTA), with peak expression during the late night and nadirs during the late day. No significant rhythmicity was observed in the basolateral amgydala or the dentate gyrus. As the mPFC provides excitatory input to both the NAc and VTA, this region was hypothesised to be a key mediator of rhythmic neural activation in the mesolimbic system. Hence, the effects of excitotoxic mPFC lesions on diurnal rhythms in cFos immunoreactivity at previously observed peak (ZT18) and nadir (ZT10) times were examined in the NAc and VTA. mPFC lesions encompassing the prelimbic and infralimbic subregions attenuated peak cFos immunoreactivity in the NAc, eliminating the diurnal rhythm, but had no effect on VTA rhythms. These results suggest that rhythmic neural activation in the mesolimbic system may contribute to diurnal rhythms in reward-related behaviors, and indicate that the mPFC plays a critical role in mediating rhythmic neural activation in the NAc.

Introduction

Rewarding behaviors are largely mediated by the mesolimbic system (Wise, 2004; Frohmader *et al.*, 2010a), composed of the ventral tegmental area (VTA), nucleus accumbens (NAc), medial prefrontal cortex (mPFC), amygdala, and other regions (Spanagel & Weiss, 1999; Ikemoto, 2007). Mesolimbic brain areas are activated during natural reward behaviors (Noel & Wise, 1995; Martel & Fantino, 1996), including sexual behavior (Pfaus *et al.*, 1990; Balfour *et al.*, 2004; Frohmader *et al.*, 2010b; Pitchers *et al.*, 2010), aggression (Wolf *et al.*, 2004), and maternal behavior (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 *et al.*, 2008). A growing body of work has revealed that many of the behavioral responses to rewarding stimuli vary substantially over the course of a day. Conditioned place preference, locomotor activation and sensitisation in response to psychostimulants (Abarca *et al.*, 2002; Kurtuncu *et al.*, 2004; Webb *et al.*, 2009) and natural rewards (Webb *et al.*, 2009) depend significantly on the time of day. The mechanisms responsible for these diurnal rhythms in motivation and reward remain to be fully elucidated, but there is evidence for involvement of the mesolimbic system, and particularly for the neurotransmitter dopamine. Markers of dopaminergic activity vary by time of day in the VTA and NAc, including the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis (Sleipness *et al.*, 2007; Webb *et al.*, 2009), dopamine transporters (Sleipness *et al.*, 2007), and monoamine oxidase (Hampp *et al.*, 2008).

Here, we characterised diurnal fluctuations in neural activation throughout the mesocorticolimbic system, using cFos protein expression as the marker. The use of cFos to elucidate diurnal rhythms in neural activation is well documented for brain areas involved in sleep and wakefulness (Pompeiano *et al.*, 1995; Bentivogolio, 1999), including activation of the orexin–hypocretin system (Estabrooke *et al.*, 2001; Martinez *et al.*, 2002). Moreover, investigation of cFos protein expression during sleep and awake states provided support for diurnal rhythms in neural activation in the mesolimbic system (Pompeiano *et al.*, 1994). However, a comprehensive characterisation of neural activation in the mesolimbic system across the day has yet to be conducted.

We also tested the hypothesis that the mPFC plays a critical role in mediating the daily rhythms in neural activation in the mesolim-

Correspondence: Ian C. Webb, as above.
E-mail: iwebb@umc.edu

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bic system. The mPFC provides a major source of glutamatergic inputs to the VTA and NAc (Carr & Sesack, 1999, 2000; Balfour *et al.*, 2006; Omelchenko & Sesack, 2007), and glutamate receptor activation has been implicated in the induction of cFos expression (Xia *et al.*, 1996; Rossetti *et al.*, 1998; Vanhoutte *et al.*, 1999; Dominguez *et al.*, 2007). Therefore, the mPFC is a potential candidate for regulating diurnal rhythms in neural activation in its target areas.

Materials and methods

Animals

Young adult male Sprague-Dawley rats (300–350 g; Charles River Laboratories, Quebec, Canada) were pair-housed in standard rat housing cages with *ad libitum* food and water. The rats were placed under a 12 : 12-h light/dark cycle (~350 lux light/0 lux dark), and were allowed to acclimatise and entrain to the light/dark cycle for 2 weeks prior to tissue collection or surgery. 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 were performed in compliance with the guidelines of the Canadian Council on Animal Care and the United States National Institutes of Health.

Tissue collection

At six different zeitgeber times (ZTs) (ZT2, ZT6, ZT10, ZT14, ZT18, and ZT22, where ZT12 corresponds to lights off, by convention), rats (n = 6–8 per time point) were deeply anesthetised with intraperitoneal sodium pentobarbital (270 mg/kg) and perfused transcardially with 50 mL of saline followed by 500 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were removed, post-fixed for 1 h, and stored in 20% sucrose in 0.1 M 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 35-μm coronal sections, and stored in cryopreservative (Watson *et al.*, 1986) at −20 °C until immunohistochemical processing.

Immunohistochemistry

All incubations and rinses were performed at room temperature on free-floating tissue with gentle agitation. Following each incubation, sections were rinsed thoroughly in phosphate-buffered saline (PBS) (0.1 M, pH 7.4). One series of brain sections from all experimental animals were stained simultaneously. The tissue was first incubated for 10 min in 1% H_2O_2 in 0.1 M PBS and for 1 h in incubation solution [0.1% bovine serum albumin and 0.4% Triton X-100 (Fisher Scientific, Ottawa, Ontario, Canada) in PBS]. Next, the tissue was incubated with a rabbit polyclonal antibody specifically recognising cFos (1 : 7500 in incubation solution; 17 h; SC-52; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by biotinylated donkey anti-rabbit IgG (1 : 500 in incubation solution; 1 h; Jackson Immunoresearch Laboratories, West Grove, PA, USA) and avidin–biotin–horseradish peroxidase complex (ABC) (1 : 1000 in PBS; 1 h; ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA). Antibody binding was visualised with diaminobenzidene (DAB) (Sigma-Aldrich, St Louis, MO, USA) solution (0.01% DAB, 0.012% H_2O_2 in 0.1 M PB) containing 0.08% nickel sulfate for 10 min, resulting in a black reaction product. Sections were then washed with H_2O_2 (1% in PBS; 10 min), and incubated with a mouse monoclonal antibody against TH (1 : 400 000 in incubation solution; 17 h; Chemicon International, Temecula, CA, USA), a biotinylated donkey anti-mouse IgG (1 : 500 in PBS; 1 h; Jackson Immunoresearch), and ABC (1 : 1000 in PBS; 1 h). TH immunoreactivity was visualised with DAB (0.01% DAB and 0.012% H_2O_2 in PB (0.1 M; 10 min), resulting in a brown reaction product (Fig. 2). Finally, sections were thoroughly rinsed in PB, mounted on plus-charged slides (Fisher Scientific), dehydrated in alcohol, cleared with Citrisolv (Fisher Scientific), and coverslipped with di-*N*-butyl phthalate in xylene (Electron Microscopy Sciences, Fort Washington, PA, USA). The cFos antibody has been extensively validated, and results in a single band at the appropriate mass on western blot.

Cell counting and analysis

cFos-immunoreactive (IR) neurons in reward-related brain areas were counted in one or two sections per rat, with standard areas of analysis determined in accordance with previously defined landmarks (Swanson, 1998) (Fig. 1). The areas were as follows: the NAc core (400 × 600 μm) and shell (400 × 600 μm); the anterior cingulate area (ACA) (400 × 600 μm), the infralimbic (IL) (400 × 600 μm) and the prelimbic (PL) (400 × 600 μm) regions of the mPFC; the central amygdala (CeA) 400 × 400 μm) and basolateral amygdala (BLA) (600 × 400 μm); and the dentate gyrus (DG) (1800 × 1200 μm). For each rat, counts were averaged to derive the mean number of cFos cells per unilateral structure. In the VTA (900 × 600 μm), cFos-IR, TH-IR and dual-labeled neurons were counted in two sections at the rostral–middle level (Balfour *et al.*, 2004). More caudal levels of the VTA were not included, as no cFos expression was noted at any time points. Counts of cFos-IR cells in the suprachiasmatic nucleus (SCN) (400 × 400 μm) in two sections were performed as a positive control for circadian activation rhythms (Schwartz *et al.*, 2000). Cell counts were carried out by two observers blinded to the experimental groups, using a Leica DMR microscope (Leica Microsystems,Wetzlar, Germany) with a camera lucida drawing tube.

Statistical analysis

One-way ANOVA followed by Newman–Keuls comparisons, Kruskal–Wallis ANOVA followed by Dunn's comparisons or independent t-tests were utilised, where appropriate, to examine time-of-day effects. To confirm diurnal rhythmicity, data were also analysed with CIRCWAVE software (v. 1.4, courtesy of R. Hut; http://www.euclock.org). CIRCWAVE uses harmonic regression to fit a sinusoidal curve to the data and to test its significance against a fitted horizontal line.

mPFC lesion surgery and verification

Rats were anesthetised with intraperitoneal ketamine (87 mg/mL/kg) and intraperitoneal xylazine (13 mg/mL/kg), and placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA). The skull was exposed, and two small burr holes were drilled at the following coordinates relative to bregma: anterior–posterior, +2.9 mm; medial–lateral, ± 0.6 mm. A 5-μL Hamilton Syringe (Hamilton, Reno, NV, USA) was used to bilaterally infuse ibotenic acid (2% in 0.1 M PBS; Sigma-Aldrich) or vehicle at two

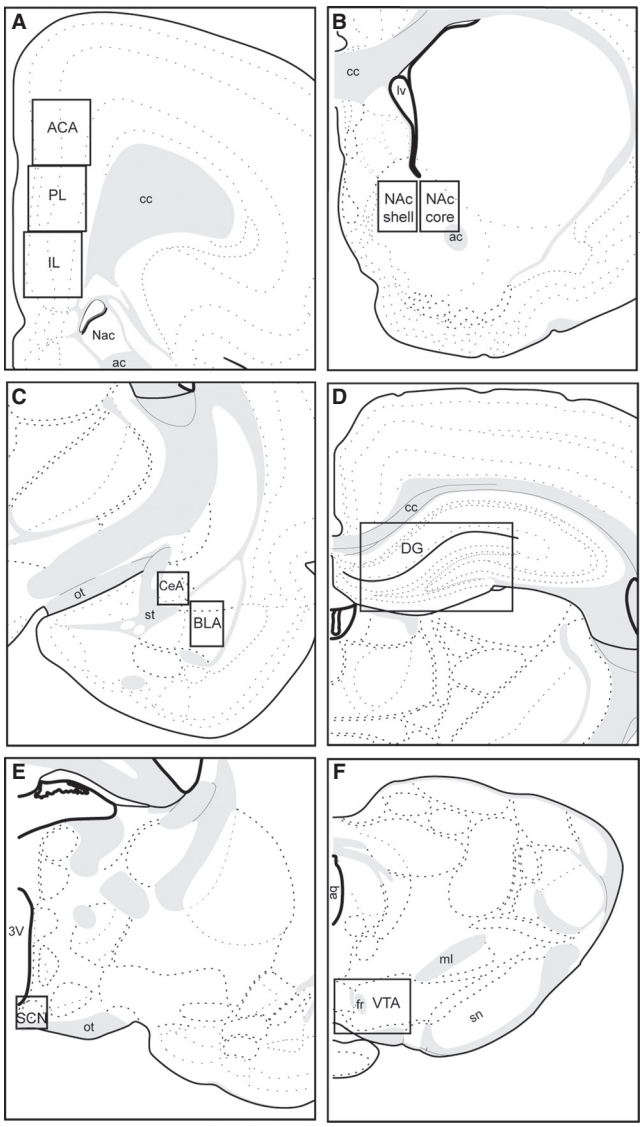

FIG. 1. Areas of analyses for cell counts. Schematic representations of areas of analyses in: (A) the ACA, IL and PL regions of the mPFC; (B) the NAc core and shell; (C) the CeA and BLA; (D) the DG; (E) the SCN; and (F) the VTA. ac, anterior commissure; aq, cerebral aqueduct; cc, corpus callosum; fr, fasciculus retroflexus; lv, lateral ventricle; ml, medial lemniscus; ot, optic tract; sn, substantia nigra; st, stria terminalis; 3V, third ventricle. Drawings modified from Swanson (1998).

dorsal–ventral coordinates corresponding to the IL and PL regions of the mPFC (from the top of skull, −5.0 and −2.5 mm; 0.25 μL per 1 min per injection). The needle was left in place for 3 min following each injection, to allow for adequate diffusion. Rats were allowed 2 weeks to recover from surgery prior to tissue collection.

Sham-lesioned and mPFC-lesioned rats were perfused at two different time points, ZT10 and ZT18, corresponding, respectively, to nadirs and peaks in cFos immunoreactivity. One series of brain sections containing the NAc core and shell and the rostral and middle VTA from all sham-operated and lesioned rats were processed simultaneously for cFos immunoreactivity, and analysed as described above. The location and size of the lesions were determined by examining the area in cFos-immunostained mPFC sections showing tissue damage and lack of staining. Lesions of the mPFC typically spanned a distance from anterior to posterior of +4.85 mm to +1.70 mm relative to bregma, and encompassed the entire IL region and the majority of the PL region (ZT10, $n = 6$; ZT18, $n = 8$). Sham-operated rats did not have any damage (ZT10, $n = 6$; ZT18, $n = 4$).

Statistical analysis

Two-way ANOVAs followed by Bonferroni comparisons or independent *t*-tests were used to assess lesion and time-of-day effects.

Results

Rhythmic neural activation in the mesolimbic reward system

A significant diurnal rhythm in the number of cFos-IR cells was observed in the NAc core (ANOVA, $K_5 = 22.99$, $P = 0.0003$; CIRCWAVE, $F_{4,35} = 10.11$, $P < 0.0001$) and shell (ANOVA, $K_5 = 27.57$, $P < 0.0001$; CIRCWAVE, $F_{4,35} = 10.54$, $P < 0.0001$; Figs 2C and D and 3), in all three subregions of the mPFC [ACA (ANOVA, $K_5 = 20.73$, $P = 0.0009$; CIRCWAVE, $F_{4,38} = 8.87$, $P < 0.0001$), PL (ANOVA, $K_5 = 18.78$, $P = 0.0021$; CIRCWAVE, $F_{4,38} = 7.19$, $P = 0.0002$), and IL (ANOVA, $K_5 = 13.70$, $P = 0.0177$; CIRCWAVE, $F_{2,40} = 3.29$, $P = 0.048$); Figs 2A and B and 4], and in the VTA (ANOVA, $K_5 = 23.99$, $P = 0.0002$; CIRCWAVE, $F_{2,33} = 22.56$, $P < 0.0001$; Figs 2E and F and 5A). In general, rhythms in the NAc, mPFC and VTA were similar in phase, with peaks during the night and nadirs during the late day. In the NAc core and shell, cFos expression had a nadir at ZT6–10 and peaks at ZT18–22 (Fig. 3). Within the subregions of the mPFC, cFos expression had a nadir at ZT10 (ACA and IL region) or ZT6-10 (PL region) and a peak at ZT18 (IL and PL regions) or ZT14–18 (ACA; Fig. 4). In the VTA, cFos expression had a nadir at ZT10 and peaks at ZT18–22 (Fig. 5A).

The number of VTA TH-IR cells co-expressing cFos also showed a diurnal rhythm (ANOVA, $F_{5,30} = 34.27$, $P < 0.001$; CIRCWAVE, $F_{4,31} = 28.82$, $P < 0.0001$; Figs 2E and F and 5C), with a nadir at ZT10 and peaks at ZT18–22. Moreover, at the peak time (ZT18), 18.8% ± 1.8% of total VTA TH-IR cells also expressed cFos, and 23.8% ± 3.9% of cFos-IR cells expressed TH (Fig. 5D). The number of TH-IR cells also varied over the day ($F_{5,35} = 4.68$, $P = 0.003$), but in an ultradian fashion. Multiple peaks were observed at ZT6, ZT14, and ZT22, and a nadir at ZT10 (Fig. 5B).

In contrast, no diurnal rhythms in the numbers of cFos-IR cells were observed in the BLA or the DG (Fig. 6A and C). A marginal diurnal rhythmicity was observed in the CeA (CIRCWAVE, $F_{2,39} = 3.25$, $P = 0.049$; Fig. 6B), and ANOVA failed to detect significant differences between different time points (ANOVA, $F_{5,36} = 2.19$, $P = 0.076$). Finally, consistent with previous reports (Schwartz *et al.*, 2000), the number of SCN cFos-IR cells was significantly greater at ZT6 than at ZT18 ($P = 0.0022$; Fig. 6D).

mPFC lesions attenuate rhythmic neural activation in the NAc without influencing the VTA

mPFC lesions altered cFos rhythms in the NAc shell and core (Figs 7A and B and 8), but not in the VTA (Fig. 7C). In the NAc, mPFC lesions had a significant effect on the numbers of cFos-IR cells in both the core ($F_{1,19} = 22.25$, $P = 0.0002$) and the shell ($F_{1,19} = 22.57$, $P = 0.0001$). Moreover, significant interactions were found between time of day and lesion status for the NAc

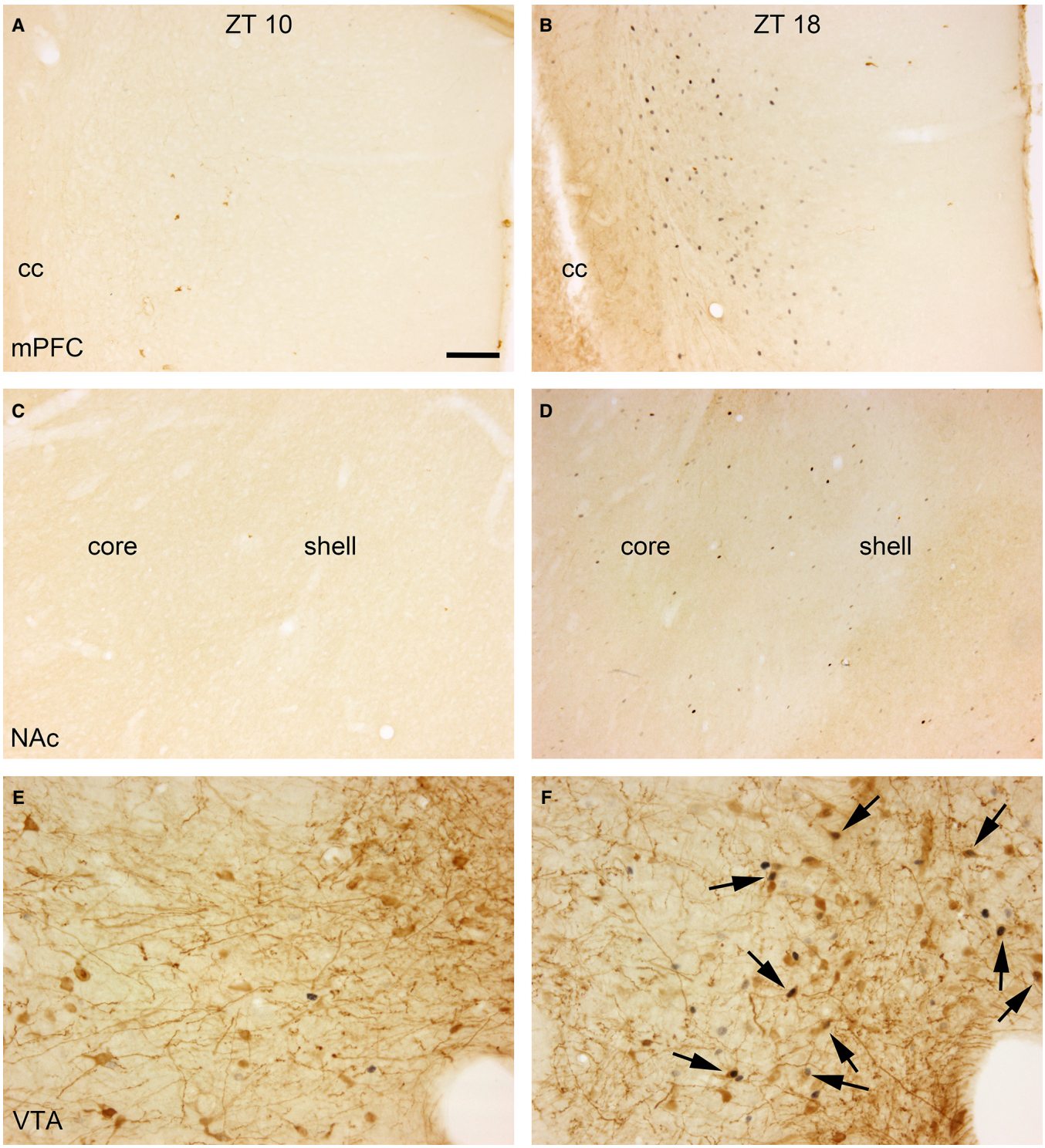

FIG. 2. cFos and TH immunoreactivity. Images showing cFos immunoreactivity (black) and TH immunoreactivity (brown) in the mPFC (prelimbic area), NAc (core and shell) and VTA in representative animals at ZT10 (A, C, and E) and ZT18 (B, D, and F). Arrows in F indicate cells co-expressing cFos and TH. Scale bars: 100 μm (A–D) or 50 μm (E and F). cc, corpus callosum. [Color version of figure available online].

shell ($F_{1,19} = 50.01$, $P < 0.0001$) and core ($F_{1,19} = 32.94$, $P < 0.0001$). *Post hoc* analysis revealed that mPFC lesions reduced the number of cFos-IR cells at ZT18 (core, $P = 0.0003$; shell, $P < 0.0001$), as compared with the sham group, and increased the number of cFos-IR cells at ZT10 in the shell ($P = 0.019$), but not in the core. Changes in the 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 vs. ZT18: $P = 0.0258$). 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 rats (ZT10 vs. ZT18: $P < 0.0001$). Finally, sham treatment did not disrupt

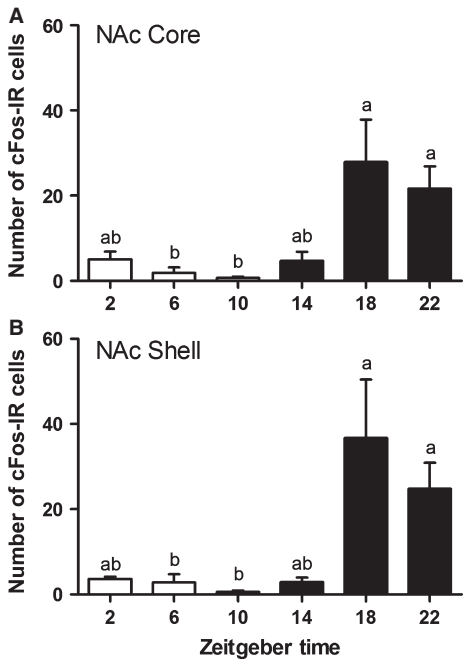

FIG. 3. Diurnal rhythms in cFos expression in the NAc: numbers of cFos-IR cells in the NAc core (A) and shell (B). Data are expressed as means ± SE of the mean. The statistical relationship between the groups is indicated by lower-case letters; groups that share a common letter do not differ significantly.

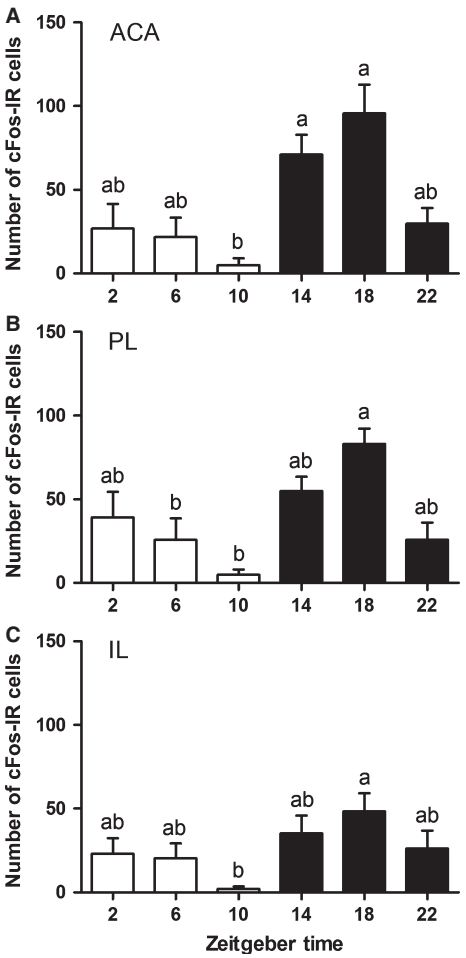

FIG. 4. Diurnal rhythms in cFos expression in the mPFC: numbers of cFos-IR cells in the ACA (A), IL region (B), and PL region (C) of the mPFC. Data are expressed as means ± SE of the mean. The statistical relationship between the groups is indicated by lower-case letters; groups that share a common letter do not differ significantly.

diurnal rhythms in cFos immunoreactivity, and significant time-of-day differences were detected in the 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 mediate neural activation rhythms in the NAc, but not in the VTA.

Discussion

The current study demonstrated diurnal rhythms of neural activation in the VTA, NAc, and mPFC, which were generally elevated during the dark phase (ZT18–ZT22), and had nadirs during the end of the light phase (ZT10). In the VTA, this rhythm in neural activation was observed in both dopaminergic and non-dopaminergic cells. One major question arising from these observations concerns the origin of the signal driving these rhythms in neural activation. As both the NAc and the VTA receive glutamatergic inputs from the mPFC (Carr & Sesack, 2000; Gabbott *et al.*, 2005; Sesack & Grace, 2010), we tested the hypothesis that mPFC inputs contribute to the observed neural activation rhythms. Indeed, ablation of the mPFC eliminated the diurnal rhythm in NAc cFos immunoreactivity by attenuating the peak expression at ZT18, but did not affect rhythms in VTA activity.

A comprehensive characterisation of the daily expression of cFos in the mesocorticolimbic reward system revealed diurnal rhythms, with peaks during the middle–end of the dark phase and nadirs during the end of the light phase, in the NAc, VTA, and mPFC, but not in the BLA or DG. Marginal rhythmicity was detected in the CeA. Although the expression of cFos is a useful indicator of neuronal activation, it is not always synonymous with membrane depolarisation, as dissociations have been observed between *in vivo* single-unit recordings and expression of cFos (Hoffman & Lyo, 2002). Nonetheless, previous studies have also utilised cFos as a

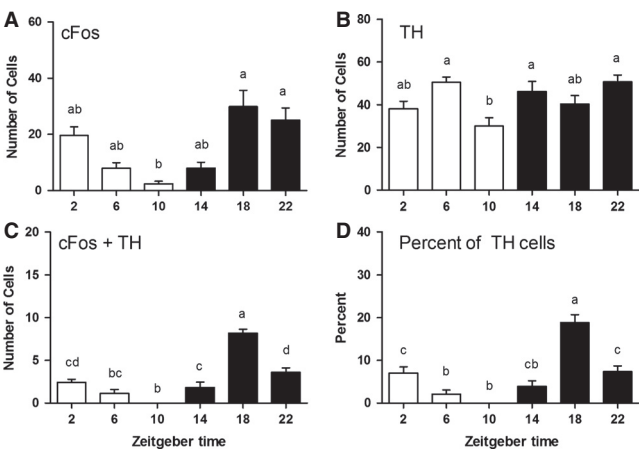

FIG. 5. Diurnal rhythms in cFos expression by dopaminergic and non-dopaminergic neurons in the VTA: diurnal variation in the numbers of cFos-IR cells (A), TH-IR cells (B), and dual cFos/TH-IR cells (C), and the percentage of TH cells co-expressing cFos (D), in the VTA. Data are expressed as mean number ± SE of the mean. The statistical relationship between the groups is indicated by lower-case letters; groups that share a common letter do not differ significantly.

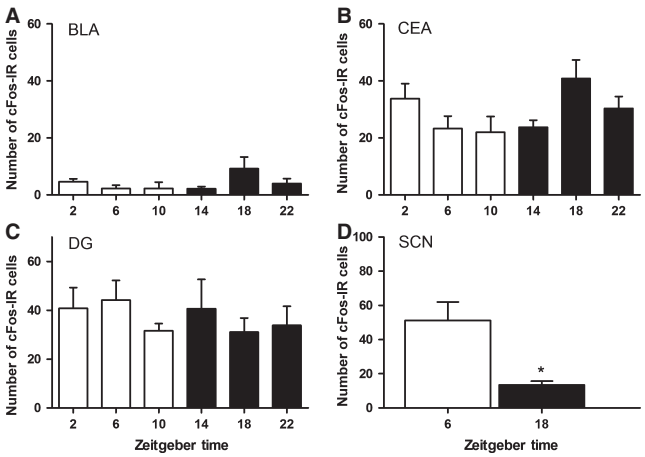

FIG. 6. Diurnal variation in neural activation in the amygdala, hippocampus and SCN: numbers of cFos-IR in the BLA (A), the CeA (B), the DG (C), and the SCN (D). Data are expressed as mean ± SE of the mean. *Significant differences between time points.

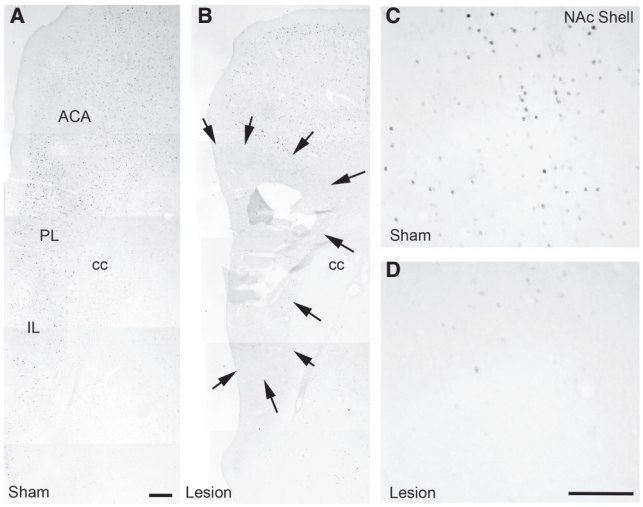

FIG. 8. cFos immunoreactivity in mPFC-lesioned brains: images showing the mPFC in representative sham (A) and lesioned (B) rats, in brain sections immunoprocessed for cFos immunoreactivity. Arrows in B indicate the extent of the lesion site encompassing the PL and IL regions, with sparing of the ACA. The images in C and D illustrate cFos immunoreactivity in the NAc shell at ZT18 in sham (C) and lesioned (D) rats. Scale bars: 250 μm (A and B) or 100 μm (C and D). cc, corpus callosum.

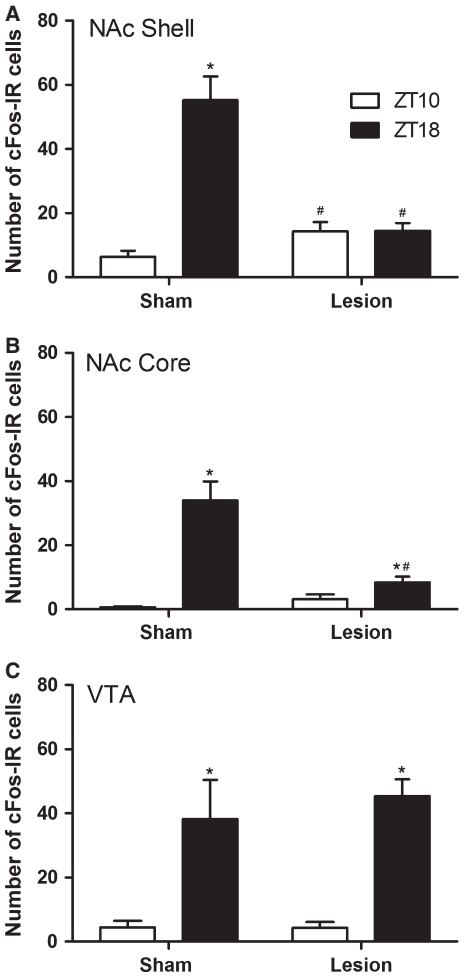

FIG. 7. The mPFC modulates neural activation in the mesolimbic reward system: effects of sham surgery and mPFC lesions on cFos immunoreactivity in the NAc core (A), NAc shell (B), and VTA (C). Data are expressed as mean ± SE of the mean. *Significant effect of time of day within the treatment group. #Significant lesion effect within the time of day.

marker for daily changes in neural activation; 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 awake states reported peak cFos immunoreactivity at ZT18 (dark phase) as compared with ZT6–9 (light phase) in the frontal cortex and NAc (Pompeiano *et al.*, 1994). Additionally, data from studies of food anticipatory behavior also showed rhythms in cFos immunoreactivity in the NAc and PFC, over a period of several hours (Angeles-Castellanos *et al.*, 2007; Verwey *et al.*, 2007). The absence of rhythms in neural activation in the DG and BLA 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 that are 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 *et al.*, 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 in motivation and reward (Kelley & Berridge, 2002; Hyman *et al.*, 2006). The finding that rhythms in neural activation 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 interconnections originating from the mPFC and VTA (Stuber *et al.*, 2012) modulate the activity within this network. Indeed, a main finding of the current study is that mPFC inputs contribute to the diurnal rhythms in neural activation 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 the NAc or striatal dialysate (Marquez de Prado *et al.*, 2000; Castaneda *et al.*, 2004), with peaks around ZT18 and nadirs throughout the mid-light phase.

Conversely, VTA rhythms were not dependent on mPFC inputs. Therefore, an alternative hypothesis is that rhythms in activation of VTA, mPFC and, potentially, NAc neurons instead derive from inputs from other brain areas, including 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), and the other by way of orexinergic neurons in the hypothalamus (Korotkova *et al.*, 2003; Deurveilher & Semba, 2005; Yoshida *et al.*, 2006). There is currently little evidence to support a functional role of the former pathway, whereas the latter pathway appears to be 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 (Harris *et al.*, 2005; Aston-Jones *et al.*, 2009) via its role in mediating reward-induced activation of VTA dopaminergic neurons (Borgland *et al.*, 2006; Zheng *et al.*, 2007). The influence of orexinergic inputs on 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 alter (Akiyama *et al.*, 2004; Zhang *et al.*, 2004; Marston *et al.*, 2008) the normal diurnal rhythms of orexin activation and signaling (Estabrooke *et al.*, 2001; Lee *et al.*, 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 & Aston-Jones (2010) recently showed that the activity of VTA dopaminergic neurons during the dark phase is regulated by the mPFC and orexinergic afferents, with a gating role of orexin in enhancing mPFC excitatory control over dopaminergic neurons. Glutamatergic projections from the mPFC reach both dopaminergic and non-dopaminergic neurons of the VTA (Rossetti *et al.*, 1998; Omelchenko & Sesack, 2007). Modulation of corticolimbic glutamatergic inputs by orexin has been suggested to occur via increased *N*-methyl-D-aspartate receptor trafficking (Borgland *et al.*, 2006) and via orexin receptor-mediated changes in phosphoinositol signaling (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 make 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 hypothesised 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 activation 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, a measure of synaptic dopamine (Sleipness *et al.*, 2008). The rhythm in PFC dopamine clearance is consistent with the pattern in VTA dopaminergic activity (Webb *et al.*, 2009), with a peak at ZT4 and a nadir at ZT12. However, the dopamine clearance rhythm does not match the rhythm in cFos activity, and thus may not be causing the rhythms in neural activation. In addition, a subportion of the rhythmic cFos immunoreactivity in the VTA neurons may be glutamatergic. Luo *et al.* (2008) have reported that VTA neurons that fire selectively during the active night phase express mRNA for the glutamate cell marker vesicular glutamate transporter 2, and account for 14% of all active cells during the dark phase. Moreover, a subset of dopaminergic neurons also synthesise glutamate and release it into the NAc (Tecuapetla *et al.*, 2010). These findings suggest that VTA neural activation may contribute to rhythms in the NAc and mPFC, via release of glutamate and/or dopamine.

A final hypothesis for the generation of neural activation rhythms in the mPFC, VTA and NAc is that these originate from oscillations in core circadian clock genes. Clock genes have been localised to many reward-related brain areas (McClung *et al.*, 2005; Angeles-Castellanos *et al.*, 2007; Verwey *et al.*, 2007; Webb *et al.*, 2009; Ramanathan *et al.*, 2010), and oscillate in the NAc and mPFC (Angeles-Castellanos *et al.*, 2007), but do not appear to be rhythmically expressed in the VTA (Webb *et al.*, 2009). It is clear that clock genes play a role in regulating reward, as clock gene disruptions alter typical behavioral responses to drugs of abuse. For instance, *period1* knockout mice show increased cocaine conditioned place preference (Abarca *et al.*, 2002); intracerebroventricular injections of a DNAzyme that interferes with Period1 impairs morphine conditioned place preference (Liu *et al.*, 2005); *period2* mutant mice show increased alcohol consumption (Brager *et al.*, 2011); and mice lacking a functional *clock* gene are hypersensitive to cocaine (McClung *et al.*, 2005). Clock gene disruptions also affect the normal functioning of the reward system. For instance, clock gene knockout mice have increased excitability of dopamine neurons and increased TH expression in the VTA (McClung *et al.*, 2005); *Period2* knockout mice show altered dopamine activity, with decreased transcription of the monoamine oxidase gene throughout the mesolimbic dopamine system, 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 expression of the genes encoding TH, dopamine receptor, and Homer2, and decreased expression of the genes encoding ionotropic glutamate receptor subunit 2 and dopamine β-hydroxylase (Mukherjee *et al.*, 2010). The necessity of mesolimbic clock gene oscillations for rhythms in reward-related behaviors remains to be determined.

In summary, the current study demonstrates diurnal rhythms in neural activation in areas within the mesolimbic pathway, with a causal role for mPFC outputs in the generation of such rhythms in the NAc. These neural activation rhythms, in turn, may regulate diurnal differences in reward behaviors. The nadir in cFos immunoreactivity during the late light phase coincides with a nadir in the expression of psychostimulant reward (Roberts & Andrews, 1997; Baird & Gauvin, 2000; Roberts *et al.*, 2002; Webb *et al.*, 2009), whereas the peak in cFos expression correlates with high expression of reward for sexual behaviors (Webb *et al.*, 2009). Future studies will be needed to determine whether these neural activation rhythms are, indeed, critical for rhythmic reward behavior.

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Abbreviations

ABC, avidin–biotin–horseradish peroxidase complex; ACA, anterior cingulate area; BLA, basolateral amgydala; CeA, central amgydala; DAB, diaminobenzidene; DG, dentate gyrus; IL, infralimbic; IR, immunoreactive; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; PB, phosphate buffer; PBS, phosphate-buffered saline; PL, prelimbic; SCN, suprachiasmatic nucleus; TH, tyrosine hydroxylase; VTA, ventral tegmental area; ZT, zeitgeber time.

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