Regulation by endogenous dopamine of the expression of the clock protein, PERIOD2, in the forebrain of the male Wistar rat.

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ABSTRACT

Regulation by endogenous dopamine of the expression of the clock protein, PERIOD2, in the forebrain of the male Wistar rat.

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Daily exposure to light is the most powerful means of entraining circadian rhythms of clock gene expression in the suprachiasmatic nucleus (SCN), the body's master timekeeper, and in other brain regions and peripheral tissues; however, a variety of non-photic stimuli have also been found to entrain rhythmic clock gene expression in the brain, including motivationally significant events such as stressors and rewards. The present experiments investigated the role of the neurotransmitter, dopamine (DA), in entraining the daily rhythm of the clock protein, PERIOD2 (PER2), in the SCN of the male Wistar rat, as well as in several regions of the limbic forebrain that regulate motivational states: namely, the dorsal striatum, the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), the central nucleus of the amygdala (CEA), the basolateral amygdala (BLA), and the dentate gyrus (DG). In the dorsal striatum, the normal daily peak of PER2 expression was blunted following selective destruction of midbrain DA neurons with 6-hydroxydopamine (6-OHDA) and after blockade of D2 DA receptors with the antagonist, raclopride, whereas daily injections of a D2 agonist but not a D1 agonist restored and entrained the PER2 rhythm in the 6-OHDA-lesioned striatum. Disruption of catecholamine signaling in general using systemic injections of the tyrosine hydroxylase inhibitor, alpha-methyl-para-

tyrosine (AMPT), or daily morphine injections and withdrawal of morphine also blunted the normal PER2 peak in the dorsal striatum. Together, these results suggest that daily stimulation of D2 receptors is necessary for the striatal PER2 rhythm. In the BNSTov and CEA, daily injections of a D2 agonist or morphine increased PER2 expression near the time of injection, but other manipulations of DA signaling or catecholamines in general had no effect on PER2. In the BLA and DG, disruption of catecholamine signaling using AMPT or morphine injections blunted the normal PER2 peak in these regions but DA-selective manipulations had no effect on PER2. None of these manipulations affected the normal PER2 rhythm in the SCN. Taken together, these findings indicate that daily stimulation of D2 receptors regulates the PER2 rhythm in the dorsal striatum, and that DA does not directly contribute to the normal PER2 rhythm in the SCN or in any other forebrain region examined.

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INTRODUCTION

Circadian rhythms are 24-h fluctuations in cellular, physiological, and behavioral processes that are expressed by essentially all life forms on Earth. Familiar examples of these rhythms include the sleep-wake cycle, feeding, drinking, and locomotor activity. Circadian rhythms provide a mechanism for organisms to anticipate daily changes in the environment and to co-ordinate multiple, sometimes conflicting, physiological processes within the day. The ubiquitous expression of circadian rhythms and high degree of conservation of their genetic basis across species suggest the importance of these rhythms to the survival of organisms. The impact of circadian rhythms on normal physiological activity is also clearly demonstrated by the distressing symptoms experienced by individuals whose normal rhythms are disrupted, as in the case of jet lag or nighttime shift work.

In mammals, circadian rhythms are regulated at a systems level by a master circadian pacemaker in the brain, the suprachiasmatic nucleus (SCN), and at a cellular level by several genes, referred to as clock genes, which are cyclically expressed in a series of feedback loops. The precise timing of these feedback loops is adjusted by daily exposure to light and darkness, which is conveyed from the eyes to the SCN via the retinohypothalamic tract (RHT)(for reviews see, Reppert and Weaver, 2001; Antle and Silver, 2005). Although the SCN is considered the master timekeeper, other brain structures and peripheral

tissues also rhythmically express clock genes (Abe et al., 2002; Balsalobre, 2002; Shieh, 2003); however, signals from the SCN are thought to be required to entrain and co-ordinate the rhythms within other tissues and throughout the whole organism (Yoo et al., 2004).

In addition to light, non-photic events can also entrain daily rhythms. Notably, these signals can shift rhythmic activity in the brain and periphery without significantly affecting the SCN clock. Among the events known to be effective non-photic time givers, or zeitgebers (ZTs), emotionally arousing stimuli, such as rewards, stressors, or stimuli paired with aversive events, can significantly influence normal rhythmic activity (Mrosovsky, 1996; Amir and Stewart, 2009). Interestingly, the impact of these stimuli on rhythms of clock gene expression outside of the SCN can be seen within brain regions that regulate motivated behaviors. Although the mechanisms by which emotional states affect circadian rhythms are not well understood, there is increasing interest in the role of the neurotransmitter dopamine (DA), long associated with the expression of motivated behaviors, in the regulation of circadian rhythms. The present thesis investigates the role of DA in the entrainment of circadian rhythms in the SCN and the limbic forebrain in the rat.

The SCN is the master circadian clock in mammals

The rat SCN comprises approximately 10 000 cells in each hemisphere and is located just above the optic chiasm in the hypothalamus and immediately adjacent to the third ventricle. It is roughly divided along a dorsolateral to

ventromedial axis into core and shell regions that differ in terms of innervation by the RHT, the kinds of peptides expressed in cells, and sites to which efferents project (Van den Pol, 1980; Moga and Moore, 1997; Leak et al., 1999). The core region, located in the ventrolateral portion of the nucleus, is heavily innervated by glutamatergic fibers of the RHT and is the primary region in the SCN to be affected by an organism's exposure to light (Tanaka et al., 1997; Morin and Allen, 2006). Cells in the core contain the neurotransmitter GABA (gamma-amino-butyric acid) that can be co-localized with various peptides that play a critical role in the regulation of rhythmic activity in the SCN, such as vasoactive intestinal polypeptide (VIP), gastrin-releasing peptide (GRP), and calbindin, as well as substance P (SP) and neurotensin (Moore et al., 2002). In contrast to the core, the shell region, which is located in the dorsomedial portion of the nucleus, is only sparsely innervated by the RHT. The cells of the shell region are also GABAergic, but these co-localize the peptides arginine vasopressin (AVP), calretinin, enkephalin (ENK), and somatostatin (Moore et al., 2002). Within the SCN, VIP- and GRP-positive cells in the core region project heavily into the shell and make synaptic contacts with AVP-positive cells; however, relatively few projections from the shell innervate the core (Daikoku et al., 1992; Drouyer et al., 2010). Instead, efferents from the shell region project outside of the SCN, with the majority of fibers innervating other nuclei in the medial hypothalamus (Watts and Swanson, 1987; Leak and Moore, 2001).

The SCN displays a 24-h rhythm in several activities, including the firing rate of individual cells (Welsh et al., 1995; although see Webb et al., 2009a) the

synthesis and release of peptides expressed in the core and shell (Yamase et al., 1991; Hofman and Swaab, 1993; Shinohara et al., 1995), and expression of the immediate early gene, *c-fos* (Kornhauser et al., 1990; Colwell and Foster, 1992). Adjusting the timing of these activities in the SCN to changes in environmental light occurs by way of the RHT that projects from the retina to the core of the SCN. In the retina, light stimulates photoreceptors such as melanopsin, which is expressed in a widely distributed subset of retinal ganglion cells (Panda et al., 2002; Provencio et al., 2002), the photopigment cryptochrome (Miyamoto and Sancar, 1998), and the opsin-based rod and cone receptors (reviewed in Lowrey and Takahashi, 2000). Stimulation of these receptors by light is conveyed to the SCN via fibers in the RHT and results in the release of glutamate, pituitary adenylate cyclase activating polypeptide (PACAP), and SP within the SCN core (Chen et al., 1999; Hamada et al., 1999; Hannibal et al., 2000). These signals can then adjust the phase of the SCN clock to a slightly earlier time (a phase advance) or to a later time (a phase delay), depending on when these signals occur in relation to the existing time in the cycle of the SCN clock. These adjustments in the phase of the SCN clock are evident in the timing of the daily locomotor activity rhythm in rodents housed in conditions of constant darkness. For example, exposure to a light pulse during the early subjective nighttime results in a phase delay in the SCN clock, such that the normal daily increase in activity occurs slightly later on the following day. In contrast, a light pulse in the late subjective nighttime produces a phase advance and results in a slightly earlier onset of activity on the following day. Synchronization of cellular activity

within and across the subregions of the SCN in response to this photic information is then achieved by communication from the core to the shell via electrical signals through gap junctions and activity-dependent release of GABA, VIP, and GRP in the shell (reviewed in Antle and Silver, 2005; Aton and Herzog, 2005).

Several lines of evidence demonstrate conclusively that the SCN serves as the master circadian clock in mammals. For example, the firing of individual SCN neurons continues to follow a circadian rhythm under conditions of constant darkness and persists when cells are cultured in isolation (Welsh et al., 1995; Herzog et al., 1998). The timing of activity within the SCN remains constant across a range of temperatures, a property that is referred to as temperature compensation and allows for maintenance of clock `speed` irrespective of temperatures changes that would otherwise affect rates of cellular activity (Herzog and Huckfeldt, 2003). Furthermore, lesioning of the SCN in rodents abolishes circadian rhythms of behavior and hormone secretion, but can be restored in part by transplantation of SCN tissue from another animal (Lehman et al., 1987; Ralph et al., 1990). Remarkably, the temporal characteristics of the restored rhythm (e.g., its period) in the recipient of the transplanted tissue are not the same as the rhythm expressed before the loss of the SCN tissue, but rather match those of the donor animal. For example, following transplantation of SCN tissue from a *tau* mutant hamster, which displays a locomotor activity rhythm with a period shorter than 24 h (Ralph and Menaker, 1988), into an SCN-lesioned wild-type hamster, the wild-type hamster develops a locomotor activity rhythm

with a shortened period that matches that of the mutant donor, instead of the 24-h rhythm it expressed before the SCN lesion (Ralph et al., 1990). This result indicates that the properties of the SCN tissue itself determine the circadian rhythm of locomotor activity.

To transmit time-of-day information to other tissues in the body, the SCN uses both synaptic transmission and diffusible signals. For example, severing axonal projections from the SCN using knife cuts disrupts some rhythmic functions, but not others (Inouye and Kawamura, 1979; Honma et al., 1984). Conversely, transplantation of SCN tissue that is encapsulated in plastic, which thereby prevents the growth of new projections from the transplanted cells, restores some rhythms in SCN-lesioned rats, but not all rhythms (e.g., Lehman et al., 1987; Silver et al., 1996). GABAergic axons from the shell region of the SCN project primarily to the subparaventricular zone and nuclei within the medial hypothalamus, such as the paraventricular nucleus, dorsomedial and ventromedial nuclei of the hypothalamus, and medial preoptic area, and stimulation of these regions by the SCN regulates several rhythmic functions (Morin and Allen, 2006). For example, activity-dependent release of AVP from SCN terminals in the paraventricular nucleus regulates the rhythm of activity of the hypothalamic-pituitary-adrenal axis (Kalsbeek et al., 2010). Several humoural factors from the SCN have also been identified, such as transforming growth factor alpha (TGF-alpha), and prokineticin-2 (PK-2), and evidence suggests that secretion of these signals from the SCN regulate the locomotor activity rhythm in rodents (Kramer et al., 2001; Cheng et al., 2002).

Clock genes as the basis of circadian rhythms

The molecular mechanisms underlying the expression of circadian rhythms consist of a group of genes, referred to as clock genes, which regulate their own transcription in a series of feedback loops that takes approximately 24 h to complete. A core set of clock genes was initially characterized in the fruit fly, *drosophila melanogaster*, and homologues for most of these genes have been identified in many other species (for reviews, see Lowrey and Takahashi, 2000; Reppert and Weaver, 2001; Ko and Takahashi, 2006). Although ongoing research is likely to uncover new and critical components to this system, in mammals the core group of genes known at this time includes the following: the PER-ARNT-SIM (PAS)-domain containing basic helix-loop-helix transcription factors, *circadian locomotor cycles kaput* (*clock*) and *brain and muscle ARNT-like protein 1* (*bmal1*); the *Cryptochrome* (*Cry1* and *Cry2*) and *Period* (*Per1*, *Per2*, and *Per3*) genes; and the orphan nuclear receptors, *Rev-erb-α* and *Ror-α*. The importance of each of these genes in the maintenance of normal circadian rhythmicity is clearly illustrated by the consequences of mutating individual clock genes. For example, mice having a single mutation of *clock*, *bmal1*, *Per1*, or *Per2*, or a double mutation of *Cry1* and *Cry2*, display arrhythmic locomotor activity when housed in the absence of light cues (i.e., constant darkness) (Kume et al., 1999; van der Horst et al., 1999; Vitaterna et al., 1999; Bunger et al., 2000; Albrecht et al., 2001). In humans, a mutation of the *Per2* gene is associated with a condition known as familial advanced sleep phase syndrome, in which

individuals fall asleep and awaken much earlier than is usual in the general population (Toh et al., 2001).

The following is a simplified overview of the operation of the transcription-translation feedback loops of these genes within the mammalian SCN: the protein product of the transcription factor *clock* (CLOCK), which is constitutively expressed, and the protein product of *bmal1* (BMAL1, also known as MOP3) form heterodimers in the cytoplasm through interaction at their PAS sites (Hogenesch et al., 1998). Transcription of the *bmal1* gene is rhythmic and is regulated in part by the nuclear receptors, REV-ERB-α (which inhibits *bmal1* transcription) and ROR-α (which enhances its transcription) (Guillaumond et al., 2005), as well as by the protein products of the *Cry* (CRY) and *Per2* (PER2) genes (Shearman et al., 2000; Yu et al., 2002). The CLOCK:BMAL1 heterodimers translocate to the nucleus and initiate transcription of *Rev-erb* α, *Ror-α*, and the *Cry* and *Per* genes by binding to E-box sites on the promoter regions of these genes. In turn, the proteins CRY1, CRY2, PER1, PER2, and PER3 form multimeric complexes with each other in the cytoplasm, which then translocate into the nucleus and inhibit their own transcription by interfering with the binding of CLOCK:BMAL1 to their promoter regions (Griffin et al., 1999; Kume et al., 1999). Although previous research has indicated that this inhibitory feedback by the CRY:PER multimer is mediated primarily through an interaction of CRY1 with CLOCK, recent evidence suggests that it is PER2 that serves as this interface (Chen et al., 2009). In addition to inhibiting their own transcription, the CRY1, CRY2, and PER2 proteins initiate transcription of *bmal1*. Thus, the

molecular clockwork contains both positive (CLOCK:BMAL1-mediated transcription of *Rev-erb-α*, *Ror-α*, *Cry*, and *Per* genes; CRY- and PER2-mediated upregulation of *bmal1* expression; ROR-α upregulation of *bmal1* expression) and negative (downregulation of *Cry* and *Per* transcription by their proteins; REV-ERB-α inhibition of *bmal1* transcription) loops that maintain cyclic expression of these genes over 24 h in individual SCN neurons.

The interaction of these feedback loops produces distinct and predictable peaks and troughs in the expression of most of the clock genes and their proteins over 24 h (Reppert and Weaver, 2001). In the early morning (circadian time, or CT, 0), CLOCK: BMAL1 dimers accumulate in the nucleus of SCN neurons and initiate transcription of the *Cry* and *Per* genes, as well as the *Rev-erb-α* and *Ror-α* genes. Among these genes, the expression of *Per1* and *Rev-erb-α* mRNA peaks first, near midday (CT4-6), followed by *Per3* (CT4-8), *Ror-α* (CT6-10), *Per2* (CT8-10), and *Cry1* (CT8-10). The expression of *clock* is constitutive, as previously noted, and the expression of *Cry2* mRNA is weak and does not have a clearly defined rhythm (Lowrey and Takahashi, 2000). Although the time of peak mRNA expression of each of these genes is different, their time of peak protein expression coincides around dusk (CT12-14). The accumulation of REV-ERB-α protein during the daytime decreases BMAL1 levels through inhibition of *bmal1* transcription. As CRY and PER complexes translocate to the nucleus, they inhibit the transcriptional activity of CLOCK:BMAL1 dimers and result in decreasing levels of CRY and PER throughout the night. In addition to inhibiting their own transcription, CRY1, CRY2, and PER2 proteins provide a positive drive

on the expression of *bmal1*. Along with ROR-α, these proteins enhance the transcription of *bmal1*, such that its mRNA increases mid-nighttime (CT15-18) and its protein levels peak near the end of the night (CT22-24). The accumulation of BMAL1 protein and its subsequent dimerization with CLOCK marks the completion of the feedback loops and the beginning of another day.

In addition to these genes, enzymes that regulate post-translational modifications of clock proteins are also considered critical components of the core clock loop because they adjust the precise timing of gene expression, in part by regulating the localization and rate of degradation of clock proteins. For example, casein kinase I epsilon (CKIε) phosphorylates PER1 and PER2, which modulates the ability of these proteins to dimerize with CRY1 and CRY2, to move between cellular compartments (i.e., into the nucleus), and their rate of ubiquitylation (Lowrey et al., 2000; Vielhaber et al., 2000). One illustration of the significant role that CKIε has in the clock is found in *tau* mutant hamster, which results from a mutation of the gene encoding CKIε (*tau).* This mutation results in a reduced ability of CKIε to phosphorylate PER and results in a more rapid translocation of the CRY:PER multimers into the nucleus to repress their own transcription (Lowrey et al). As described above, the *tau* mutant hamster exhibits a shortened circadian period in which locomotor activity rhythms are less than 24 h and PER protein expression in the SCN peaks earlier than in wild-type hamsters. Hamsters heterozygous for the *tau* mutation show a shortening of the rhythm by approximately 2 h, whereas homozygous mutants have rhythms that are approximately 4 h shorter than wild-type. Another enzyme, glycogen

synthase kinase 3 beta (GSK3ß), has also been implicated in the post-translational modification of multiple clock proteins, including PER2 and BMAL1 (Martinek et al., 2001; Iitaka et al., 2005; Sahar et al., 2010). Disruption of the activity of this enzyme results in a lengthened circadian period of locomotor activity and rhythm of expression of the clock proteins PER2 and BMAL1 that is believed to be due to a disruption of the normal rate of their nuclear translocation and ubiquitylation.

In addition to their roles in maintaining the circadian clockwork, several clock genes have been found to regulate functions other than those involved in rhythmicity. In particular, the *Per* genes have been found to contribute to several different processes, such as regulation of tumour growth, metabolism of catecholamines in the brain, and extracellular glutamate uptake (for a review, see Albrecht et al., 2007). Of particular relevance to this thesis, *Per2* is reported to modulate the expression of the gene for monoamine oxidase A (*maoa*) synthesis and the expression of the excitatory amino acid transporter (*eaat*), in astrocytes, which contributes to the re-uptake of glutamate from the extracellular space (Spanagel et al., 2005; Hampp et al., 2008). Changes in these proteins following mutation of the *Per2* gene are believed to underlie the enhanced extracellular levels of DA and glutamate in the ventral striatum of *Per2* knock-out mice and the increased responsiveness of these mutants to drugs of abuse, such as cocaine and alcohol (Abarca et al., 2002; Hampp and Albrecht, 2008).

How does light entrain clock gene expression?

Photic entrainment of the SCN is manifested at the level of clock genes and involves a glutamatergic-mediated induction of *Per* expression. In the early nighttime, when exposure to light results in a phase delay of the locomotor activity rhythm, activation of NMDA receptors by glutamate release in the SCN core leads to the rapid upregulation of *Per1* mRNA levels within approximately 60 min via CREB-mediated transcription (Shigeyoshi et al., 1997; Gau et al., 2002; Travnickova-Bendova et al., 2002). *Per2* mRNA is also increased in response to a light pulse at this time of day, although its expression increases more slowly than does *Per1* (peaking after approximately 2 h) (Zylka et al., 1998; Yan and Silver, 2002). Interestingly, previous reports indicate that there are no changes in PER2 protein expression in the SCN following a light pulse (Beaule et al., 2003). In the late nighttime, when a light pulse induces a phase advance, *Per1* mRNA is also induced in response to light via a CREB-dependent mechanism, but not *Per2* (although see Zylka et al.; Yan and Silver). Given the differential pattern of *Per1* and *Per2* induction following a light pulse, it has been suggested that the increase in *Per2* mRNA during the early nighttime mediates phase delays, whereas *Per1* expression in the late nighttime mediates phase advances (Albrecht et al., 2001).

Rhythmic clock gene expression outside of the SCN

Although the SCN is considered the dominant or master pacemaker in mammals, many other brain regions and peripheral tissues exhibit rhythmic

activity and clock gene expression. One of the earliest demonstrations of rhythmic activity in mammalian tissues outside of the SCN was in the retina of the mouse, in which it was observed that melatonin synthesis followed a circadian rhythm that persisted even in conditions of constant darkness (Tosini and Menaker, 1996). More recently, several studies using luminescent reporter genes linked to either the *Per1* (*Per1*::Luc) or *Per2* (PER2::Luc) gene in mice have demonstrated that these components of the clock are rhythmically expressed in brain regions including (but not limited to) the olfactory bulbs, cortex, limbic forebrain, hypothalamic nuclei, pineal gland, pituitary, cerebellum, as well as in peripheral tissues such as bone, skeletal muscle, liver, lung, kidney, cornea, and testes (Yamazaki et al., 2000; Abe et al., 2002; Shieh, 2003; Yoo et al., 2004).

Consistent with the role of the SCN as the primary timekeeper for the body, the peak or phase of clock gene expression in extra-SCN tissues typically lags behind that of the SCN (Zylka et al., 1998; Yoo et al., 2004; Davidson et al., 2009). However, each tissue exhibits its own characteristic period of activity that is evident in PER2::Luc samples cultured in isolation (Yoo et al., 2004) and capacity to re-entrain to shifts in the LD cycle (Yamazaki et al., 2000; Davidson et al., 2009). These tissue-specific characteristics suggest that these extra-SCN regions have intrinsic oscillating properties in and of themselves and their rhythms are not driven exclusively by the SCN (Yoo et al., 2004; Guilding and Piggins, 2007).

Signals from the SCN are required, however, to synchronize clock gene expression within a given tissue, as well as to re-entrain the phase of rhythmic gene expression in these tissues after a change in the light schedule. In support of this, clock gene expression in the SCN shifts rapidly in response to an altered light schedule, whereas the adjustment of clock gene rhythms in extra-SCN tissues occurs more slowly, in some cases on the order of several days (e.g., Yamazaki et al., 2000; Davidson et al., 2009). Extra-SCN tissues explanted from *Per1*::Luc or PER2::Luc mice to *in vitro* conditions will show rhythmic expression of the fluorescent reporter for several days, but the strength of this signal gradually diminishes over cycles, whereas in vitro cultures of the SCN continue to exhibit robust rhythms in luminescence for weeks (Yamazaki et al., 2000; Abe et al., 2002; Yoo et al., 2004). Importantly, the reduction in reporter expression within extra-SCN tissues over time does not appear to be attributable to a shutting down of the molecular clockwork, but rather reflects a loss of coherence in the phase of the rhythm across individual cells in the absence of a synchronizing input. Indeed, changing of the in vitro medium, or the addition of dexamethasone or forskolin to the medium after several days in culture can reinstate a rhythm in PER2 fluorescence in extra-SCN tissues (Yamazaki et al., 2000). Furthermore, tissues harvested from SCN-lesioned animals killed several weeks after the lesion was made appear arrhythmic in the expression of the reporter (Sakamoto et al., 1998), although rhythmic activity is still evident in individual cells (Yoo et al., 2004). These results suggest that the SCN is necessary to synchronize the phase of activity within each tissue.

Among these extra-SCN regions, the retina and the olfactory bulbs are unique in that the rhythms of cellular activity and clock gene expression in these tissues are independent of entraining signals from the SCN. In fact, studies of the retina and olfactory bulbs indicate that the oscillatory mechanisms in these regions meet the defining criteria for independent or master clocks (for a review, see Guilding and Piggins, 2007): their rhythms free-run in constant conditions, they exhibit temperature compensation, they can be entrained to daily cues other than light, and they can entrain rhythms of activity and clock gene expression in other tissues (Tosini and Menaker, 1996; Granados-Fuentes et al., 2004; Tosini et al., 2008). In the retina of rodents and non-mammalian vertebrates, functions such as melatonin synthesis, as noted above, outer disc segment shedding, and synthesis and release of neurotransmitters including DA follow a circadian rhythm that persists following the removal of potential entraining signals from the SCN (for a review, see Tosini et al., 2008). In most vertebrates studied, including the rat, clock genes in the retina are rhythmically expressed within cells of the inner nuclear and inner plexiform layers (Ruan et al., 2006). Clock gene expression can be directly modulated by light itself, as well as by non-photic cues, such as injections of the D2/3 DA receptor agonist quinpirole (e.g., Steenhard and Besharse, 2000; Besharse et al., 2004; Ruan et al., 2008).

In the olfactory bulbs, clock genes are rhythmically expressed by GABAergic mitral cells and rhythmic activity in this region is thought to regulate day-night differences in olfactory sensitivity (Funk and Amir, 2000; Abe et al., 2002; Granados-Fuentes et al., 2006). Rhythmic gene expression in the

olfactory bulbs persists in rodents housed in conditions of constant light (which disrupt rhythmic activity in the SCN and other tissues), following lesions of the SCN, and when the olfactory bulbs themselves are isolated in vitro (Granados-Fuentes et al., 2004). The olfactory bulbs and SCN do not project directly to one another, but receive indirect projections via brainstem nuclei (Krout et al., 2002). Interestingly, evidence suggests that the olfactory bulbs modulate rhythmic functions controlled by the SCN, as well as rhythmic activity within the SCN itself. For example, olfactory stimulation can modulate light pulse-induced phase shifts in locomotor activity and FOS protein expression in the SCN, whereas olfactory bulbectomy speeds re-entrainment to a 6-h phase advance in the LD cycle (Amir et al., 1999; Granados-Fuentes et al., 2006).

Non-photic entrainment

Although light is the most powerful zeitgeber to entrain daily rhythms, a number of non–photic entraining stimuli have also been identified. These include restricted feeding (Stephan, 2002; Waddington Lamont et al., 2007), metabolic signals (Diaz-Munoz et al., 2000), steroid hormones (Amir and Robinson, 2006; Perrin et al., 2006; Segall et al., 2006), physical activity (Salgado-Delgado et al., 2008), and motivationally relevant cues, such as fear-associated stimuli, stressors, and rewards (Mrosovsky, 1996; Amir et al., 1999; Amir and Stewart, 1999b; Takahashi et al., 2001; Angeles-Castellanos et al., 2008). Interestingly, the effect of non-photic stimuli in entraining the circadian clock is not limited to the SCN; rather, non-photic cues can entrain rhythms in extra-SCN tissues independently of the activity of the SCN clock. Indeed, the presentation of non-

photic stimuli to animals housed in a regular LD schedule can shift rhythmic activity in the brain and periphery such that these rhythms diverge entirely from the rhythm in the SCN, which remains entrained to the LD cycle (e.g., Masubuchi et al., 2000; Waddington Lamont et al., 2007).

The most prominent example of a non-photic entraining stimulus in rodents is the restriction of food availability to a time of day when eating does not normally occur (i.e., the light phase). Under regular LD conditions, restricted feeding schedules can entrain rhythms of locomotor activity, secretion of hormones including corticosterone, insulin, leptin, and expression of clock genes in both the brain and peripheral tissues, without affecting the rhythm of the SCN (Honma et al., 1983; Diaz-Munoz et al., 2000; Wakamatsu et al., 2001; Stephan, 2002; Angeles-Castellanos et al., 2007; Waddington Lamont et al., 2007). Interestingly, clock gene expression in the SCN can be entrained by a restricted feeding schedule, but in conditions of constant light, in which the activity of the SCN is otherwise arrhythmic (Lamont et al., 2005b). One of the remarkable features of entrainment by restricted feeding schedules is the resulting synchronization of clock gene expression in several different tissues to a common phase that is timed to the presentation of food. For example, in rats given ad libitum food access, the rhythm of expression of the PER2 protein in the oval nucleus of the bed nucleus of the stria terminalis (BNSTov) and central nucleus of the amygdala (CEA) peaks near the transition from the light phase to the dark phase (Amir et al., 2004; Lamont et al., 2005a), whereas this rhythm in the basolateral amygdala (BLA) and dentate gyrus of the hippocampus (DG)

peaks at the beginning of the light phase, approximately 12 h later than the BNSTov and CEA (Lamont et al., 2005a). When food presentation is limited to 2 h in the middle of the light phase, the peak of PER2 expression in all of these regions shifts to the middle of the dark phase, occurring approximately 12 h after food is presented, whereas the rhythm in the SCN is unaffected (Waddington Lamont et al., 2007).

The specific mechanisms by which restricted feeding schedules entrain rhythms in the brain and periphery remain unknown, although evidence suggests that this effect is not solely attributable to metabolic deficits resulting from reduced food intake or to the reward value of food presentation (Waddington Lamont et al., 2007). Several studies have implicated the dorsomedial nucleus of the hypothalamus (DMH) as a critical site mediating entrainment by restricted feeding (Gooley et al., 2006; Fuller et al., 2008). Although controversial, some evidence indicates that lesions of the DMH prevent the entrainment of the locomotor activity rhythm by a restricted feeding schedule (Gooley et al., 2006). Clock genes are not rhythmically expressed in the DMH under ad libitum feeding conditions, but rhythms in PER1 expression have been found to develop in this region in rats fed on a restricted schedule (Verwey et al., 2009).

Although not as potent as restricted feeding schedules, motivationally significant or arousing stimuli have also been demonstrated to entrain circadian rhythms of behavior, physiology, and clock gene expression (for reviews, see Mrosovsky, 1996; Amir and Stewart, 2009). Evidence for an association between motivational state and circadian rhythms arises in part from the

circadian disturbances observed in a number of psychiatric conditions involving mood, such as bipolar disorder and seasonal affective disorder (for reviews, see McClung, 2007; Harvey, 2008; Falcon and McClung, 2009; Mendlewicz, 2009). Powerful demonstrations of the effect of motivational state on the circadian system have also been previously reported by Amir and Stewart, who showed in rats that presenting a light pulse in an environment previously paired with shock, or a light that has itself been paired with shock, suppresses the induction of FOS protein in the SCN and reduces the phase shift in wheel-running activity that normally results from light exposure (Amir and Stewart, 1998, 1999b). Importantly, the presentation of a shock-associated light pulse enhances FOS protein expression in primary visual cortex, indicating a differential impact of the light on the visual and circadian systems of light perception (Amir and Stewart, 1999a, 2009).

Additional studies have demonstrated an effect of arousing stimuli on the entrainment of rhythmic clock gene expression in brain regions outside of the SCN that are involved in the regulation of motivated behaviors. In rodents, exposure to stressors such as intermittent restraint or injection of lipopolysaccharide can shift the expression of *Per1* mRNA within the paraventricular nucleus of the hypothalamus (Takahashi et al., 2001) and repeated restraint stress shifts the expression of PER2 in regions of the extended amygdala, specifically in the BNSTov and CEA (Robinson et al., 2005). Importantly, research from the Amir laboratory and others has shown that corticosterone, the primary stress hormone in rodents, can modulate the

expression of clock genes in several tissues including the BNSTov and CEA without affecting the SCN (Balsalobre et al., 2000; Amir et al., 2004; Segall et al., 2006; Segall and Amir, 2010). Changes in circulating corticosterone levels could be a mechanism by which stressful events affect normal rhythmic activity and disrupt clock gene expression in tissues ordinarily entrained by SCN signals.

Related to the link between motivational states and circadian rhythms are findings demonstrating an effect of drugs of abuse on rhythms of behavior and clock gene expression. Treatment with drugs such as methamphetamine, cocaine, nicotine, and opiates, can entrain rhythms of locomotor activity and clock gene expression in several brain regions and peripheral tissues in rodents (Honma et al., 1986; Shibata et al., 1994; Kosobud et al., 1998; Masubuchi et al., 2000; Nikaido et al., 2001; Iijima et al., 2002; Uz et al., 2005; Vansteensel et al., 2005; Wang et al., 2006; Kosobud et al., 2007). Importantly, these effects on locomotor activity have not been observed when drugs without abuse potential are used, such as haloperidol (Kosobud et al., 2007; Gillman et al., 2009), suggesting that the mechanisms underlying this entrainment involve the activation of reward or positive motivational, and perhaps withdrawal, states. The profound effect of drugs of abuse, particularly chronic administration of methamphetamine, on locomotor activity rhythms has led to the suggestion that there is a specialized oscillatory mechanism in the brain that provides an interface between drugs of abuse and the circadian system (for a review, see Honma and Honma, 2009). This putative oscillator, referred to as the methamphetamine-sensitive oscillator (MASCO), was posited in view of findings

that long-term delivery of methamphetamine through the drinking water to rats and mice can entrain the locomotor activity rhythm to a period greater than 24 h, even in the presence of a functional SCN (which ordinarily regulates the locomotor rhythm) and regular light cues (a potent zeitgeber and suppressor of locomotor activity) (Honma et al., 1986; Masubuchi et al., 2000; Tataroglu et al., 2006). The location of such an oscillator and the specific signals to which it responds remain a matter of speculation. Previous evidence showing that pre-treatment with the non-selective DA receptor antagonist, haloperidol, blocks the induction of *Per1* in the dorsal striatum by an acute injection of methamphetamine (Nikaido et al., 2001) and that injections of haloperidol can shift the locomotor rhythm entrained by chronic methamphetamine (Honma and Honma, 1995) suggests that DA might be involved. However, additional studies have questioned whether the expression of MASCO involves traditional genetic clock mechanisms, inasmuch as mutations of individual clock genes do not prevent the entrainment of the locomotor activity rhythm in mice by chronic methamphetamine (Honma et al., 2008; Mohawk et al., 2009).

Taken together, there is converging evidence supporting a role for motivational or emotional states in the entrainment of circadian rhythms. Importantly, many of the brain regions affected by these stimuli are those that mediate behavioral and physiological responses to motivationally significant events, which might suggest that rhythmic clock gene expression in these regions plays an important role in the organization of these responses.

DA as a non-photic zeitgeber

The evidence reviewed so far suggests that the entrainment of circadian rhythms by motivational states involves mechanisms not only upstream of the SCN, as shown by the modulation of the SCN response to light input (Amir and Stewart, 1999), but also those signals downstream of the SCN control, such as corticosterone secretion (Balsalobre et al., 2000; Amir et al., 2004; Segall et al., 2006). Another candidate signal downstream from SCN control of increasing interest in the entrainment of circadian rhythms is the neurotransmitter DA. The activity of neurons in the ventral tegmental area (VTA), site of the mesocorticolimbic DA cells, follows a circadian rhythm that is controlled by indirect inputs from the SCN and extracellular levels of DA the dorsal striatum, a terminal region of the nigrostriatal DA system, fluctuate in a 24 h rhythm (Paulson and Robinson, 1994; Castaneda et al., 2004; Luo and Aston-Jones, 2009). As reviewed in the sections below, previous research has already established a role for DA release in the entrainment of circadian rhythms, such as the entrainment of foetal rhythms in utero, as well as in the activity of the retinal clock in adulthood. More recent findings show that stimulation of DA receptors can directly modulate clock gene expression in other brain regions, suggesting a mechanism by which alterations of mood and ingestion of abused drugs might affect circadian oscillators outside the SCN.

As mentioned, some of the first evidence demonstrating a role for DA in the entrainment of circadian rhythms arose from developmental studies of circadian rhythms in foetal rats. Rhythmic *c-fos* mRNA expression in the SCN

and locomotor activity patterns in neonates are initially entrained in utero to the circadian rhythm of the mother (Reppert and Schwartz, 1986; Weaver and Reppert, 1995). A series of studies by Reppert and colleagues demonstrated that this entrainment in early life requires stimulation of D1 DA receptors in the SCN during a restricted window of time (Weaver et al., 1992). Although D1 receptors are still expressed at low levels within the SCN of the adult rat, this role for DA in the entrainment of the SCN clock is unique to early life. Indeed, injections of the D1 receptor agonist, SKF 38393, given to SCN-lesioned dams near the end of the gestation period will entrain the subsequent activity rhythm of the neonate but have no effect on the activity of the dam (Viswanathan et al., 1994).

DA also plays a critical role in the rhythmic activity of the retina. In the majority of vertebrate species studied, DA is found in amacrine cells within the inner nuclear and inner plexiform layers, where clock genes are also rhythmically expressed (Ruan et al., 2006). One of its primary functions is in mediating light adaptation and cone-based vision, in part through stimulation of D2 receptors that are expressed by both rod and cone photoreceptors (for a review, see Witkovsky, 2004). Consistent with this role, DA synthesis and release in the retina follows a rhythm that peaks in the daytime, and these rhythms are regulated by both light and melatonin (Tosini and Menaker, 1996; Green and Besharse, 2004). DA release is also believed to mediate the effects of light on the circadian system in the retina. Studies in the African clawed frog (*xenopus laevi*) and in mice demonstrate that both light and stimulation of DA receptors

can induce *Per* expression in the retina, and stimulation of DA receptors can mimic the effect of light in suppressing nighttime melatonin release (Cahill and Besharse, 1991; Steenhard and Besharse, 2000; Doi et al., 2006). The specific mechanisms by which DA exerts this effect on rhythmic activity in the retina is not yet clear, as there is evidence for both D1 receptor- and D2 receptor-mediated effects on clock gene expression. For example, Ruan et al (2008) showed that the application of D1 agonists, but not D2 agonists, to explants of retina from PER2::Luc mice shifts the PER2 rhythm and that antagonism of D1 receptors attenuates the phase shift in PER2 induced by light pulsing the tissue. In contrast, in vitro evidence shows a direct mechanism by which stimulation of D2 receptors enhances the transcriptional activity of CLOCK:BMAL1 through a CREB-dependent mechanism (Yujnovsky et al., 2006). Furthermore, D2 receptor knock-out mice show impaired light masking of locomotor activity, a blunted rhythm of *Per1* expression in the retina, and reduced induction of *Per1* in the retina following a light pulse (Doi et al., 2006; Yujnovsky et al., 2006).

Additional evidence supporting an association between dopaminergic activity and circadian rhythms derives from the disrupted rhythms of physiological functions and clock gene expression observed in pathologies involving midbrain DA systems, such as Parkinson's disease (PD) and drug addiction. Several symptoms of PD involve disturbances in normal physiological rhythms, including the sleep-wake cycle, body temperature, heart rate, cortisol secretion, and blood pressure (Bruguerolle and Simon, 2002; Willis, 2008). Furthermore, a diurnal fluctuation in the motor symptoms of PD in humans – specifically, a worsening in

the afternoon – is frequently reported. Notably, some evidence suggests that the rhythmic expression of BMAL1 protein is blunted in red blood cells of individuals with PD, which suggests that a loss of normal dopaminergic activity affects clock gene expression in peripheral cell types (Cai et al., 2010). Another association between dopaminergic signaling and modulation of circadian rhythms has also been suggested from studies of addictive drugs (Falcon and McClung, 2009). Most abused drugs have the common property of increasing extracellular DA levels in terminal regions of the mesolimbic DA system, such as the dorsal and ventral striatum, BNSTov, and amygdala (Di Chiara and Imperato, 1988; Harmer et al., 1997; Carboni et al., 2000). Conversely, withdrawal from prolonged exposure to drugs such as morphine, ethanol, and cocaine is associated with a reduction in extracellular DA content (Acquas et al., 1991; Rossetti et al., 1992; Tran-Nguyen et al., 1998; Diana et al., 1999). Abnormal rhythms of sleep, hormone secretion, and clock gene expression are observed in animals and in humans following drug exposure (Howe et al., 1980; Yuferov et al., 2003; Uz et al., 2005; Lynch et al., 2008) and during withdrawal from long-term drug use (Stinus et al., 1998; Ammon et al., 2003; Li et al., 2009a; Li et al., 2009b).

Finally, recent evidence suggests a role for DA in modulating rhythmic clock gene expression directly within extra-SCN tissues. In vitro studies indicate that DA receptor signaling at both D1 and D2 receptors can regulate clock gene expression in the mouse striatum (Imbesi et al., 2009). In vivo studies have similarly demonstrated an effect of DA receptor stimulation on clock gene expression in the rodent striatum (Masubuchi et al., 2000; Nikaido et al., 2001;

Iijima et al., 2002; Imbesi et al., 2009; Sahar et al., 2010). For example, the rhythmic expression of *Per2*, the clock-controlled gene *dbp,* and BMAL1 protein is blunted in the striatum of D2 receptor knock-out mice (Sahar et al., 2010). These findings parallel the loss of rhythmic *Per1* expression in the retina of D2 knock-out mice (Yujnovsky et al., 2006) and suggest that DA contributes to the maintenance of rhythmic clock gene expression in multiple tissues.

It is important to note that only a limited number of studies have examined the effect of DA receptor manipulations on clock gene expression in regions outside of the SCN and dorsal striatum (Masubuchi et al., 2000; Uz et al., 2005), in spite of diverse projections of midbrain DA neurons. Furthermore, none to my knowledge has studied the effects of direct manipulations of endogenous DA in vivo. The BNSTov and CEA are heavily innervated by DA fibers from the ventral tegmental area and administration of drugs of abuse alters extracellular DA levels in these regions (Freedman and Cassell, 1994; Harmer et al., 1997; Carboni et al., 2000). As noted above, clock gene expression in these regions has been found to be highly sensitive to changes in hormones, restricted feeding schedules, as well as to stressors. The BLA and DG are also innervated by DA fibers from the midbrain and activity within these regions is implicated in responses to drugs of abuse (Fallon et al., 1978; Scatton et al., 1980; Fuchs et al., 2005; Fuchs et al., 2007). Although restricted feeding schedules shift clock gene expression in these regions, other non-photic stimuli do not affect clock gene expression in the same manner observed in the BNSTov and CEA. It would be of interest to determine whether DA modulates the expression of clock

genes in multiple regions outside of the SCN and if so, whether the mechanisms by which it acts are region-specific.

Present thesis

The experiments presented in this thesis were intended to address the role of DA in the rhythmic expression of clock genes in several regions of the rat limbic forebrain. Two experimental approaches were used to this end. In the first, endogenous DA levels in the forebrain were manipulated directly using DA-selective lesions and pharmacological disruption of DA signaling. The rhythmic expression of the clock protein, PER2, was examined in the SCN and several terminal regions of the mesolimbic DA system in which clock genes are expressed rhythmically; namely, the dorsal striatum, BNSTov, CEA, BLA, and DG. Agonists selective for D1 or D2 DA receptors were also used to address the specific mechanisms by which DA modulates PER2 expression in these regions. In the second approach, the expression of PER2 in these regions was investigated following a course of daily, timed injections of morphine. Although it is well known that morphine affects the release of a number of neurotransmitters and hormones capable of affecting clock gene expression, acute injections of morphine increase extracellular levels of DA in the striatum and BNST (Di Chiara and Imperato, 1988; Carboni et al., 2000). Furthermore, as mentioned earlier, termination of chronic morphine injections, so-called morphine withdrawal, is associated with a severe reduction in DA levels in the striatum. Thus in these experiments, the expression of PER2 was examined following the withdrawal of

daily morphine injections, a period associated with disruptions in several rhythmic functions (Howe et al., 1980; Stinus et al., 1998; Li et al., 2009b).

The activity of the circadian transcription-translation feedback loops was monitored using the PER2 protein as a marker of clock activity within cells of the SCN and in regions of the limbic forebrain. As previously described, PER2 plays a pivotal role in the circadian clock in part by regulating the transcriptional activity of CLOCK:BMAL1 and the transcription of the *bmal1* gene. The *Per2* gene has also been implicated in several functions outside of the core clock feedback loops that pertain to the activity of DA systems and responses to drugs of abuse. As such, changes in PER2 expression not only reflect the state of the molecular clockwork, but might also speak to the functional implications of altered clock gene expression in regions of the limbic forebrain.

The regions of the limbic forebrain studied here, namely the dorsal striatum, the BLA, the DG, the BNSTov, and the CEA, were selected on the basis of previous research showing a unique role of these regions in responding to selected non-photic entraining cues having motivational salience. The dorsal striatum forms a critical component of the basal ganglia and plays a central role in the regulation of movement, as well as learning, reward, and other functions. The activity of the primary outputs of the dorsal striatum, GABAergic medium spiny neurons, are regulated by glutamatergic innervation from cortex and thalamic nuclei and by dopaminergic inputs arising from the substantia nigra pars compacta (for a review, see Gerfen, 2004). As noted above, *Per2* expression in this region has been found to regulate the tone of both of these neurotransmitters

within the striatum by modulating their clearance from the extracellular space. The output projections of the dorsal striatum are segregated into two different paths that are distinguishable on the basis of the sites to which they project and the receptors and peptides they each express. In general, neurons in the striatonigral projection, or direct pathway, innervate the substantia nigra pars reticulata, and express D1 DA receptors and the peptides dynorphin and substance P, whereas neurons of the striatopallidal projection, or indirect pathway, innervate the external compartment of the globus pallidus and the subthalamic nucleus, and express D2 DA receptors and the peptide enkephalin. In view of the anatomical and functional compartmentalization of circuits within the dorsal striatum, it would be of interest to determine whether the expression of clock genes in this region is widespread or confined to a particular class of striatal cells. These findings could provide further insight into the functional role of the clock. Previous experiments have demonstrated that PER rhythms in this region, which peak in the early morning approximately 12 h after the peak in the SCN, are sensitive to particular non-photic signals, such as restricted feeding schedules (Amir, unpublished observations) and drugs of abuse (Masubuchi et al., 2000; Iijima et al., 2002; Uz et al., 2005), but not to others, such as changes in thyroid hormone levels (Amir and Robinson, 2006) or melatonin (Amir et al., 2006).

Cells within the BLA and DG also express PER2 rhythms that are in phase with the dorsal striatum (that is, peaking in the early morning) and that are sensitive to restricted feeding schedules (Wakamatsu et al., 2001; Lamont et al.,

2005a). The BLA receives some innervation by midbrain DA neurons originating in the ventral tegmental area and both D1 and D2 receptors are expressed in this region (for a review, see de la Mora et al., 2010). DA release within the BLA has been implicated in the organization of motivated behavior, including the formation of associations between conditioned stimuli and rewards (Meil and See, 1997; Weiss et al., 2000; Berglind et al., 2006). Similarly, the DG receives some dopaminergic input arising from the midbrain DA cell groups (Scatton et al., 1980) and activity in the hippocampus is associated with motivational states, including responses to environments previously paired with drugs of abuse (Fuchs et al., 2005). Interestingly, evidence suggests that clock gene expression in the hippocampus is implicated in the action of antidepressants (Uz et al., 2005). The hippocampus and portions of the amygdala are closely interconnected and send reciprocal projections to each other (Petrovich et al., 2001). Notably, rhythms of PER2 expression in the DG and BLA are similarly affected by a restricted feeding schedule (whereby the phase of PER2 in each region shifts to 12 h after the presentation of food) yet are insensitive to other non-photic events, such as stressors. This insensitivity of clock gene expression in the DG to stressors and to changes in corticosterone levels is surprising, given the pivotal role of the hippocampus in regulating activity of the hypothalamic-pituitary-adrenal (HPA) axis (for a review, see Schmidt and Duman, 2007).

The PER2 rhythms in the BNSTov and CEA are unusual in comparison to the rhythms of clock gene expression in the majority of other brain regions and peripheral tissues, in that their phase is synchronized to that in the SCN (Amir et

al., 2004; Lamont et al., 2005a). The anatomical composition and functional roles of the BNSTov and CEA are closely related and these regions are often described as components of the central extended amygdala (Alheid and Heimer, 1988; Dong et al., 2001a; Dong et al., 2001b; Alheid, 2003). Furthermore, it has been noted that the morphological and neurochemical composition of the BNSTov and the CEA resembles that of the dorsal striatum (Cassell et al., 1986; Swanson and Petrovich, 1998; Swanson, 2000). DA innervation of the BNSTov and CEA arises from the ventral tegmental area (Hasue and Shammah-Lagnado, 2002) and changes in DA release in these regions are associated with responses to a variety of motivationally relevant stimuli, including drugs of abuse (Tran-Nguyen et al., 1998; Carboni et al., 2000) and stressors (Inglis and Moghaddam, 1999; Kozicz, 2002). It is notable that in spite of the associations of these regions with the striatum, their PER2 rhythms are 180 degrees out of phase. In addition to restricted feeding schedules, PER2 expression in the BNSTov and CEA is modulated by other non-photic cues including stressors (Robinson et al., 2005), corticosterone (Segall and Amir, 2010), and estrogen levels (Perrin et al., 2006).

Experiments

The first study was designed to examine the relation between diurnal changes in DA levels in the dorsal striatum and the rhythmic expression of PER2 in this region. Using immunohistochemistry and in vivo microdialysis, the pattern of PER2 expression and extracellular DA levels in the dorsal striatum over 24 h was characterized. DA levels within the dorsal striatum were then directly

modulated to examine the impact of changes in DA content on the daily fluctuation of PER2 expression in this region and in the SCN in rats housed under 12:12 LD conditions. Using DA-selective lesions by injecting 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle or systemic injections of the tyrosine hydroxylase inhibitor, alpha methyl para tyrosine (AMPT), to inhibit catecholamine synthesis, it is shown that the amplitude of rhythmic PER2 expression within the dorsal striatum is regulated by daily fluctuation in DA levels, whereas PER2 expression in the SCN does not respond to changes in DA activity. Pharmacological experiments using D1 and D2 receptor selective agonists were subsequently conducted to determine the specific mechanisms by which DA affects the pattern of PER2 expression in the dorsal striatum. Additional immunohistochemical staining studies were also undertaken to investigate the particular cell types in the dorsal striatum within which PER2 is expressed. Together, the results of the first chapter indicate that daily fluctuation in DA activity at D2 receptors contributes to the normal pattern of PER2 expression in the dorsal striatum, but does not affect PER2 expression in the SCN.

In the second chapter, the relation of DA activity to PER2 expression in the BNSTov, the CEA, the BLA, and the DG was examined using additional brain sections from the same rats used in the experiments of study 1. It is demonstrated that, compared to the dorsal striatum, depletion of DA using 6-OHDA lesions or AMPT injections produce different effects on the daily fluctuation in PER2 expression in these regions, suggesting that DA might only

indirectly regulate PER2 expression within these regions and that the role of DA in modulating PER2 expression in the dorsal striatum is perhaps specific to that tissue.

In the third chapter, changes in PER2 expression in each of the aforementioned regions was examined in response to daily injections of morphine and following withdrawal of morphine injections. The use of daily morphine injections in this experiment provided an additional means of manipulating extracellular DA levels and disrupting the normal daily rhythm of DA release in terminal regions of the mesolimbic DA pathway, such as the dorsal striatum. Although the effects of morphine are not limited to actions on DA systems, the results of this experiment corroborate the results of the preceding studies by demonstrating that daily morphine injections, limited to the early light phase in rats housed under 12:12 LD conditions, disrupt rhythms of PER2 expression in the dorsal striatum, BNSTov, CEA, BLA, and DG, without affecting the SCN. In addition, daily morphine injections disturbed rhythms of wheel-running activity, such that running during the nighttime (the normal active period of rats) was dramatically suppressed. PER2 rhythms were also disturbed during the initial period of withdrawal from daily morphine injection in each region examined, except for the SCN; however, these rhythms reverted to their normal pattern following 7 days of withdrawal from morphine.

SECTION 1: Endogenous dopamine signaling is necessary for the rhythmic expression of the clock protein, PERIOD2, in the dorsal striatum, but not in other regions of the limbic forebrain or in the suprachiasmatic nucleus.

The experiments in Chapter 1 were carried out to investigate the role of dopamine (DA) in regulating the daily pattern of expression of the clock protein, PERIOD2 (PER2), in the dorsal striatum and suprachiasmatic nucleus (SCN) in the male Wistar rat. First, endogenous DA levels in the forebrain were reduced by making unilateral lesions of midbrain DA projections with the toxin 6-hydroxydopamine; by inhibiting synthesis of DA and other catecholamines with systemic injections of the tyrosine hydroxylase inhibitor, alpha methyl para tyrosine; and by chronically infusing DA receptor antagonists via osmotic minipumps. Second, D1 or D2 DA receptors were stimulated with either timed, daily injections or continuous infusion of D1 or D2 receptor agonists in rats with unilateral 6-OHDA lesions to determine the consequences of this stimulation on the PER2 rhythms in the dorsal striatum and SCN.

Chapter 2 examines the effects of the manipulations of endogenous DA activity described in Chapter 1 on the daily pattern of PER2 expression in four other areas of the limbic forebrain; the bed nucleus of the stria terminalis (BNSTov), the central nucleus of the amygdala (CEA), the basolateral amygdala (BLA), and the dentate gyrus of the hippocampus (DG). The results described in this chapter were obtained using additional brain sections from the rats used in the experiments presented in Chapter 1.

CONTRIBUTION OF AUTHORS – CHAPTERS 1 & 2

Dr. Shimon Amir, a supervisor and a principle investigator, contributed to the design of the experiments described in chapters 1 and 2. He performed some of the analyses of wheel-running activity and carried out some of the imaging and analysis of the immunohistochemical staining for PER2 expression in the dorsal striatum described in chapter 1. He also contributed to the writing and editing of each manuscript.

Dr. Jane Stewart, a supervisor and a principle investigator, contributed to the design of the experiments described in chapters 1 and 2. She also contributed to the writing and editing of each manuscript.

Pamela Cassidy is an undergraduate student who contributed to the execution of the experiments described in chapters 1 and 2 and to the design of the alpha methyl para tyrosine experiment.

Marie-Pierre Cossette is a graduate student who contributed to the design and execution of the *in vivo* microdialysis experiment described in chapter 1.

Barry Robinson is the laboratory manager for Dr. Amir and developed the immunohistochemical protocols used in chapters 1 and 2. He also assisted with some of the 6-hydroxydopamine surgeries and carried out the double immunohistochemical staining work described in chapter 1.

Michael Verwey is a graduate student who carried out some of the 6-hydroxydopamine surgeries and analysis of PER2 immunohistochemistry described in chapter 1.

CHAPTER 1

Endogenous dopamine signaling regulates the rhythm of expression of the clock protein PER2 in the rat dorsal striatum via daily activation of D2 dopamine receptors.

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ABSTRACT

A role for dopamine (DA) in the regulation of clock genes in the mammalian brain is suggested by evidence that manipulations of DA receptors can alter the expression of some clock genes outside the suprachiasmatic nucleus (SCN), the master circadian clock. The role of endogenous DA in the regulation of clock gene expression is unknown. Here we demonstrate a direct relation between extracellular DA levels and the rhythm of expression of the clock protein PERIOD2 (PER2) in the dorsal striatum of the male Wistar rat. Specifically, we show that the peak of the daily rhythm of extracellular DA in the dorsal striatum precedes the peak of PER2 by about 6 h and that depletion of striatal DA by 6-hydroxydopamine or alpha-methyl-para-tyrosine or blockade of D2 DA receptors by raclopride blunts the rhythm of striatal PER2. Furthermore, timed daily activation of D2 DA receptors, but not D1 DA receptors, restores and entrains the PER2 rhythm in the DA-depleted striatum. None of these manipulations had any effect on PER2 rhythms in the SCN. Finally, we present evidence that PER2 is co-expressed with D2 DA receptors in the striatum. Our findings are consistent with the idea that PER2 is expressed in striatopallidal projection neurons and that the rhythm of expression of PER2 in these neurons depends on daily dopaminergic activation of D2 DA receptors. These observations may have implications for circadian abnormalities seen in Parkinson's disease.

INTRODUCTION

Circadian rhythms of physiology and behavior are generated by a core set of clock genes, which regulate their own expression in a 24-h transcriptional-translational feedback cycle (for reviews, see Reppert and Weaver, 2001; Ko and Takahashi, 2006). Among these genes, *Period2* (*Per2)* plays a critical role in the regulation of this 24-h rhythm by serving as the interface between the negative and positive limbs of the cycle (Chen et al., 2009).

In mammals, the master circadian clock resides in the suprachiasmatic nucleus (SCN), and the phase of rhythmic clock gene expression in this region is entrained primarily by environmental light (Lowrey and Takahashi, 2000). Rhythmic clock gene expression is not limited to the SCN, however, and has been characterized in several other regions of the brain and in peripheral tissues (Abe et al., 2002; Shieh, 2003; Amir et al., 2004; Lamont et al., 2005a; Guilding and Piggins, 2007). It is believed that these rhythms in clock gene expression serve to control, in a tissue specific manner, daily variations in cellular and metabolic activity and functional output. Although signals from the SCN are required to maintain and coordinate rhythmic gene expression in the brain and periphery, these extra-SCN rhythms can also be influenced independently by a variety of other signals, including steroid hormones, stressors, and restricted feeding schedules (Takahashi et al., 2001; Wakamatsu et al., 2001; Perrin et al., 2006; Segall et al., 2006; Angeles-Castellanos et al., 2007; Amir and Stewart, 2009; Verwey and Amir, 2009).

Increasing evidence suggests that the neurotransmitter dopamine (DA) may be involved in the regulation of extra-SCN clock gene expression. In particular, activation of D2 receptors has been demonstrated to modulate the circadian effects of light on locomotor activity in mice (Doi et al., 2006) and to regulate expression of clock genes in the retina (Besharse et al., 2004; Yujnovsky et al., 2006) and striatum (Imbesi et al., 2009; Sahar et al., 2010), a region important for motor control, learning, cognition and reward. There is no direct evidence, however, that endogenous DA regulates rhythmic expression of clock genes. To study this, we characterized the rhythm and the cellular expression of the clock protein PERIOD2 (PER2) in the dorsal striatum, a region richly innervated by dopaminergic projections from the substantia nigra. Furthermore, we examined the relationship between the circadian rhythms of locomotor activity, extracellular DA level, and PER2 expression in this region. To determine the role of endogenous DA in the regulation of PER2 expression in the dorsal striatum, we depleted DA or blocked activity at DA receptors by making unilateral central injections of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle (MFB), systemic injections of the tyrosine hydroxylase inhibitor alpha-methyl-para-tyrosine (AMPT), or continuous infusions of selective D1 or D2 DA receptors antagonists. Finally, we studied the effect of D1 and D2/3 DA receptor agonists, delivered either by timed daily injection or continuously, on the rhythmic expression of PER2 in the dorsal striatum of 6-OHDA-lesioned or intact rats. Our results establish endogenous DA, acting via D2 DA receptors, as a regulator of PER2 rhythms in the dorsal striatum.

MATERIALS AND METHODS

Subjects. A total of 168 male Wistar rats (Charles River, St. Constant, QC), weighing approximately 300 g at the start of each experiment, were used. Rats were housed singly in shoebox cages equipped with running wheels and kept in individual lightproof and sound-attenuated cabinets. Within each cabinet, an overhead fluorescent tube provided light on a 12:12 light-dark (LD) cycle (light intensity within the cage was approximately 300 lux) and wheel-running activity was monitored continuously by computer using VitalView software (Minimitter, Sunview, OR, USA). Rats used in the AMPT experiment were housed singly in plastic shoebox cages, but without running wheels, and were kept in a private room with a 12:12 LD cycle (lights on at 08:00 h). Throughout all experiments, rats had ad libitum access to laboratory chow (Purina Foods) and tap water. Rats were left in their home cages for at least 10 days to entrain to the LD cycle before the start of each experiment, and were randomly assigned to an experimental condition. All experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of Concordia University.

Drugs. 6-OHDA (Sigma, Oakville, Ont.) was prepared in a concentration of 2 µg/µl in physiological saline containing 0.5% ascorbic acid. Desipramine hydrochloride, quinpirole hydrochloride, SKF 81297, AMPT, SCH 23390, and raclopride tartrate were purchased from Sigma and were dissolved in distilled water.

Intra-cranial cannulation for in vivo microdialysis. Rats were anaesthetized using a mix of ketamine (Ketaset, 90 mg/kg, intraperitoneal injection (i.p.); Ayerst, Guelph, ON) and xylazine (Rompun, 10 mg/kg, i.p.; Bayer, USA). Bilateral cannulae (21 gauge, 8.1 mm below pedestal; Plastics One, Roanoke, VA, USA) were implanted in the dorsal striatum using the following stereotaxic coordinates: posterior (from bregma) – 0.1 mm, medial/lateral 3.4 mm, ventral (from skull surface) – 3.0 mm. Cannulae were secured to the skull surface using dental acrylic. As a post-operative treatment, rats were given 2 ml of saline with 2 mg/kg ketoprofen (Anafen, Merial, CAN) subcutaneously (s.c.) and 0.15 ml Procillin (Penicillin G 150 000 I.U.; Bayer) intramuscularly.

Unilateral 6-OHDA lesion. One hour before surgery, rats were injected i.p. with desipramine (2.5 mg/kg) to protect noradrenergic fibers from the 6-OHDA toxin. Rats were then anaesthetized using a mix of ketamine and xylazine, and a unilateral injection of 8 µg 6-OHDA was made over 8 min into either the right or left MFB (counterbalanced across rats) using the following stereotaxic coordinates: posterior (from bregma) – 3.0 mm, medial/lateral +/- 1.4 mm, ventral (from skull surface) – 8.2 mm. Following the infusion of the toxin, the injector was left in place for 1 min and then was slowly raised over 3 min. Ketoprofen (2mg/kg) and 0.15 ml Procillin were given post-operatively. The extent of the lesion was confirmed post-mortem with immunohistochemical staining for expression of the dopamine transporter (DAT).

Osmotic minipump surgery. Osmotic minipumps (Alzet model 2ML2, Durect Corp., Cupertino, CA) were implanted to deliver a constant 24-h infusion

of SCH 23390, raclopride, quinpirole, or SKF 81297. The pumps were filled according to the manufacturer's instructions and were left in 0.09% saline for 24 h before implantation. Rats were anaesthetized using isoflurane (Vetoquinol NA Inc., Lavaltrie, PQ) and a small incision was made between the scapulae. Using a hemostat, a small pocket was created under the skin into which a pump was inserted with the flow moderator pointing away from the incision site. The incision was then closed with wound clips. For rats in the sham surgery group, the same surgical procedure was followed but no pump was inserted.

Immunohistochemistry. At the end of each experiment, rats were given an overdose of sodium pentobarbital (Somnotol, 100 mg/kg) at their assigned zeitgeber time (ZT, where ZT1 indicates 1 h after lights on and ZT13 1 h after lights off) and perfused intracardially with 300 ml of cold 0.09% saline, followed by 300 ml of cold 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were extracted and kept in 4% paraformaldehyde solution overnight at 4° C, then sliced with a vibratome into 50 µm coronal sections. Sections were stored in Watson's cryoprotectant (Watson et al., 1986) at –20° C until immunohistochemistry was performed.

Immunohistochemical staining for PER2 and PERIOD1 (PER1) immunoreactive (IR) nuclei was carried out according to protocols described previously (Amir et al., 2004; Verwey et al., 2009). Briefly, PER2 staining was carried out using a polyclonal rabbit primary antibody (concentration 1:1000, ADI, San Antonio, TX, USA) in a Triton Trizma-buffered saline solution (Triton TBS: 0.3% Triton, 50 mM Trizma buffer, 0.9% saline) containing 2% normal goat

serum (NGS, Vector Laboratories, Burlington, ON, CAN) or using a polyclonal goat primary antibody (concentration 1:4000; Santa Cruz Biotechnology, USA) in Triton TBS containing 2% normal horse serum (NHS, Vector Laboratories, Burlington, ON, Canada) for 40 h at 4° C. We found no differences between these primary antibodies in the pattern or amount of staining for PER2 in a number of different brain regions. Sections stained for PER1 were incubated in a polyclonal primary antibody raised in rabbit (concentration 1: 24 000; generous gift of Dr. S. M. Reppert, University of Massachusetts Medical School, Worcester, MA, USA) in Triton TBS containing 2% NGS and 5% milk buffer for 40 h at 4° C. Staining for DAT was carried out with a monoclonal antibody raised in rat (1:1500, Millipore, USA) in Triton TBS containing 2% NGS for 40 h at 4° C. After incubation in the primary antibody, sections were incubated for 1 h at 4° C in biotinylated secondary antibody (for PER2, anti-rabbit 1:200 or anti-goat 1:400 (both from Vector Laboratories); for PER1, anti-rabbit 1:200; for DAT, anti-rat 1:500 (Millipore)) and then incubated for 2 h at 4° C in an avidin-biotin-peroxidase solution (Vectastain Elite ABC kit; Vector Laboratories). Sections were then rinsed in a 0.5% 3,3-diaminobenzidine (DAB) solution, and immunoreactive (IR) nuclei were visualized with a solution containing 0.5% DAB, 0.1% H_2O_2, and 8% $NiCl_2$. The specificity of the PER2 and PER1 primary antibodies was previously confirmed with blocking experiments, in which addition of the PER2 peptide or the PER1 peptide (1 μg/ml in phosphate-buffered saline) to the primary incubation solution prevented staining of PER2 or PER1 expression, respectively, in the tissue (Hastings et al., 1999; Field et al., 2000).

Sections stained for co-expression of PER2 with glutamic acid decarboxylase (GAD-67) were first incubated in a monoclonal primary antibody against GAD-67 raised in mouse (concentration 1:20,000, Millipore) in TBS containing 2% NHS for 45 h at 4° C. Sections were then transferred to a TBS solution containing 2% NHS and a biotinylated anti-mouse secondary antibody (concentration 1:100; Vector Laboratories) for 1 h at 4° C. After incubation in the secondary antibody, sections were incubated for 2 h at 4° C in an avidin-biotin-peroxidase solution, rinsed in 0.5% DAB, then visualized with a second DAB rinse containing 0.1% H_2O_2. Sections were transferred to a blocking solution of TBS containing 5% NHS for 1 h at 4° C, and then stained for PER2 expression according to the protocol described above. Double-labeling for expression of PER2 and the D1 DA receptor, the D2 DA receptor, enkephalin, or substance P was carried out using 30 µm sections. Sections were first incubated in a Triton-TBS solution containing primary antibody against the first antigen of interest (D1 DA receptor, goat polyclonal 1:200 in 1% NHS; D2 receptor, mouse monoclonal, 1:600 in 1% NHS; enkephalin, rabbit polyclonal, 1:500 in 2% NGS; substance P, mouse monoclonal, 1:800 in 1% NHS; all primary antibodies from Santa Cruz Biotechnology) for 45 h at 4° C, and then transferred to a Triton-TBS solution containing a biotinylated secondary antibody (for D1 DA, anti-goat 1:200 in 1% NHS; for D2, anti-mouse 1:33 in 1% NHS; for enkephalin, anti-rabbit 1:200 in 2% NGS; for substance P, anti-mouse 1:66 in 1% NHS; all secondary antibodies from Vector Laboratories) for 1 h at 4° C. Sections were then incubated in an avidin-biotin-peroxidase solution for 2 h at 4° C, rinsed in a 0.5% DAB solution,

and visualized with a second DAB rinse containing 0.1% H_2O_2. Sections were then incubated in a blocking solution of Triton-TBS containing 5% NHS and were subsequently stained for PER2 expression according to the protocol described above. All sections were mounted on gelatin-coated slides, dehydrated in a series of alcohols, and cleared in Citrisolv. Glass coverslips were secured with Permount.

Microscopy. Striatal sections double-stained for PER2 and GAD-67, PER2 and D1 DA receptors, PER2 and D2 DA receptors, PER2 and enkephalin, or PER2 and substance P were examined under a light microscope at a 100x objective and images were taken using a Leica DFC 480 camera and Leica Firecam 3.1 software. For all other experiments, stained brain sections containing the dorsal striatum and SCN were examined under a light microscope using a 20x objective and 400 x 400 µm images of each region were captured using a Sony XC-77 video camera, a Scion LG-3 frame grabber, and Image SXM software (v1.8, S. D. Barrett, http://www.ImageSXM.org.uk). Images of the right and left dorsal striatum were taken in the upper lateral quadrant from 5-6 sections between approximately +a/p 0.4 mm (from bregma) and the junction of the anterior commissure (approximately –a/p 0.2 mm from bregma). Images of the right and left SCN were taken from 5-6 representative sections. The number of PER2- or PER1-IR nuclei was then counted in each image. The mean number of IR nuclei in the right and left SCN and dorsal striatum was calculated for each subject from the four images having the highest number of labeled nuclei out of all the images taken of that structure in a given hemisphere.

We also examined the pattern of PER2 expression in images taken from the medial part of the striatum in the same sections, as well as the lateral and medial regions of sections that were posterior to the junction of the anterior commissure. We found that the rhythms of PER2 expression were consistent across these regions and that the effects of each experimental manipulation on their expression did not vary within the striatum. As such, we report here only the data from the dorsolateral region of anterior striatal sections.

In vivo microdialysis for extracellular DA levels in the dorsal striatum. In vivo microdialysis sampling was carried out in custom-made Plexiglas chambers (42 x 39 x 33.5 cm^3) with stainless steel rod floors. Each chamber was housed individually in a plywood cabinet. An overhead fluorescent tube in each cabinet provided light on a 12:12 LD schedule that was the same as that maintained in the rats' home cages. Food and water were available ad libitum.

Microdialysis probes. Probes were made from a 4.0 mm length of semipermeable dialysis membrane (Spectra/Por, 240 um OD, 13 000 MW cutoff, Spectrum Laboratories, Rancho Dominguez, CA) and a 21 mm long section of 25 g stainless steel tubing. A 40-50 cm section of polyethylene tubing (PE), flared at one end, was connected to the stainless steel tubing. Three cm from the join between the PE tubing and the stainless steel tubing, an incision was made in the PE tubing to allow the insertion of small diameter fused silica tubing (Polymicro Technologies, Phoenix, AZ). The fused silica tubing extended internally through the PE tubing and into the probe with one end positioned 0.5 mm from the tip of the probe. The incision site was glued with epoxy to prevent

leakage. The opposite end of the silica tubing was attached to the other end of the PE tubing with masking tape and 0.4 ml microcentrifuge tubes (Fisher Scientific Canada, Ottawa, ON) were fastened to the masking tape to collect dialysate samples from the silica tubing. The probe assembly was connected to a liquid swivel, positioned directly above the testing chamber, by the PE tubing. The swivel itself was connected to a variable speed syringe infusion pump (Harvard Apparatus Canada, Montreal, QC) located outside of the sampling chamber. During dialysis testing, the full lengths of the PE tubing and fused silica tubing were covered by a steel spring to protect against chewing by the rat. Dialysis probes were secured to each rat's head by screwing a stainless steel collar at the end of the steel spring onto the guide cannula (HRS Scientific, Montreal, QC).

Sampling procedure. Following stereotaxic surgery, rats remained in their home cages for a one-week recovery period during which they were handled daily. One day before *in vivo* microdialysis sampling began, rats were moved from their home cages to the sampling chambers to habituate them to the sampling environment. The dialysis probes were inserted into the rats' heads approximately 6 h before dialysate sampling began. Artificial cerebrospinal fluid (145 mM Na^{+}, 2.7 mM K^{+}, 1.2 mM Ca^{2+}, 1.0 mM Mg^{2+}+, 150 mM Cl^{-}, 0.2mM ascorbic acid, 2mM $Na^{2}HPO^{4}$, pH 7.4+0.1) was perfused during this period at a rate of 0.5 µl/min to prevent the probes from blocking. Beginning 8 h after the lights turned off, or ZT20, the flow rate was increased to 1 µl/min and dialysate samples were collected from both the right and left hemispheres of each rat

every 30 min for a full 24-h period. Samples were frozen on dry ice and kept at – 80° C until analysis using high-performance liquid chromatography (HPLC).

Analysis of DA content in tissue samples from the dorsal striatum. To quantify the depletion of DA in the dorsal striatum following AMPT treatment, rats were decapitated and brains were flash-frozen using cooled 2-methylbutane. Brains were later sectioned on a cryostat at 300 µm, and 1 mm punches of the right and left anterior dorsal striatum were taken from a single section for each rat (approximately + a/p 0.4 from bregma). Punches were then treated with buffer containing 30 mM citric acid, 60 mM sodium phosphate monobasic, 0.10 mM EDTA, and 0.08 mM sodium dodecyl sulphate (pH 3.35) and were frozen at -80° C for 20 min. Samples were then thawed, centrifuged (10 min at 10 000 rpm, 4° C), and the supernatant was extracted and filtered. Supernatant samples were stored at -80° C until assayed using HPLC. The insoluble material in each pellet was suspended in 0.1 N NaOH overnight, then treated with Bradford reagent and quantified using a spectrophotometer.

HPLC. To assay for DA, samples (10 µl in the case of dialysate, or 5 µl in the case of tissue punch supernatant) were injected into a reverse-phase column (15 cm x 0.46 cm Spherisorb ODS2, 5 µm, Higgins analytical, Mountain View, CA). The mobile phase consisted of 30 mM citric acid, 60 mM sodium phosphate monobasic, 0.10 mM EDTA, 17% acetonitrile, and 0.08 mM sodium dodecyl sulphate (pH 3.35). The mobile phase was pumped through the system at 1.2 ml/min using a Waters 515 HPLC pump. Compounds were detected and quantified with an ESA Coulochem detector (model 5100A) equipped with an

analytical cell (model 5011; E1 = +0.35 V, E2 = -0.3 V, ESA, Inc.). Concentrations of DA were estimated from peak heights by comparison with injections of known amounts of pure DA standard (Sigma) using EZChrom Chromatography Data System (Scientific Software Inc., San Ramon, CA). Extracellular levels of DA measured from *in vivo* microdialysis samples are expressed as pg of DA per µl of dialysate. The amount of DA content measured in striatal tissue punches is expressed as pg of DA per µg of insoluble material.

Statistical analysis of PER2- and PER1-IR nuclei counts. In the experiments involving unilateral injections of 6-OHDA toxin into the MFB, data were analyzed only from those rats with complete unilateral lesions, as confirmed by an absence of DAT staining in the dorsal striatum ipsilateral to the 6-OHDA injection. Differences in PER2- or PER1-IR nuclei were analyzed using mixed-model analyses of variance (ANOVA) with ZT and drug treatment group as between-subjects factors and hemisphere as a within-subjects factor. Differences in PER2 expression in experiments using AMPT, the chronic DA receptor antagonists, and injections of DA receptor agonists in naïve rats were analyzed using two-way ANOVA with ZT and treatment group as between-subjects factors. Alpha level was set at 0.05 for all tests. Significant interactions were analyzed further using one-way ANOVA and Bonferroni-corrected post-hoc comparisons, or t-tests where appropriate.

Analysis of wheel-running activity rhythms. Mean wheel-running activity during the 12-h light and 12-h dark phases was calculated for the last week of the entrainment period, the second week after the 6-OHDA lesion surgery, and the

10-day period of treatment with SKF 81297 or quinpirole (daily injections or chronic treatment via minipump). The effect of the 6-OHDA lesion surgery on the rhythm and amplitude of wheel-running activity was analyzed using separate repeated measures ANOVA with stage of experiment (entrainment and post-lesion surgery) or phase (light and dark) as within-subjects factors. The effects of SKF 81297 and quinpirole treatment on the amount of wheel running in the light and dark phases were analyzed using separate one-way ANOVA, and a repeated measures ANOVA was used to evaluate the rhythm of activity across the light and dark phases. The alpha level was set at 0.05 for all analyses. Significant group differences were followed-up with Bonferroni-corrected post-hoc comparisons.

RESULTS

Phase relation between locomotor activity, PER2 expression and extracellular DA levels in the dorsal striatum

To study the relation between PER2 expression in the dorsal striatum and changes in extracellular DA levels in this region, we first examined the rhythm of PER2 in naïve rats housed in a normal 12:12 LD cycle. Following a 10-day entrainment period, rats were killed at 4-h intervals over 24 h beginning 1 h after lights on, or ZT1, and brain sections containing the dorsal striatum and SCN were stained for PER2 expression using immunohistochemistry. The daily rhythm of PER2 expression in the dorsal striatum and SCN is illustrated in

representative images in Fig. 1, and the mean number of PER2-IR nuclei at each sampling time is shown in Fig. 2a and 2b. As can be seen from Figs. 1 and 2a, the rhythm of PER2 expression in the dorsal striatum peaks in the morning, at the beginning of the light phase (ZT1) and reaches its trough in the evening, at the beginning of the dark phase (ZT13). In the SCN the rhythm of PER2 expression peaks in the evening, in anti-phase to that in the dorsal striatum (Figs.1 and 2b).

Using *in vivo* microdialysis, we then measured extracellular DA levels in the dorsal striatum of three rats over 24 h. As shown in Fig. 2c, extracellular DA levels in the dorsal striatum (expressed as pg DA/μl of dialysate) fluctuate in a circadian rhythm falling during the light phase and peaking in the middle of the dark phase. Thus, this rhythm of extracellular DA levels in the dorsal striatum, while resembling the PER2 rhythm in the SCN, is significantly out of phase with the PER2 rhythm in the dorsal striatum.

To illustrate the temporal relation between the rhythms of PER2 expression, extracellular DA levels in the dorsal striatum, and wheel-running activity, Fig. 2d shows the 24-h pattern of wheel running in those rats killed for PER2 immunohistochemistry. As in the case of striatal levels of DA, wheel-running activity occurs primarily in the dark phase and is absent during the light phase. Thus, the rhythms of PER2 expression in the SCN, extracellular DA levels in the dorsal striatum, and wheel running are in phase with one another, but are in anti-phase to the rhythm of PER2 expression in the dorsal striatum.

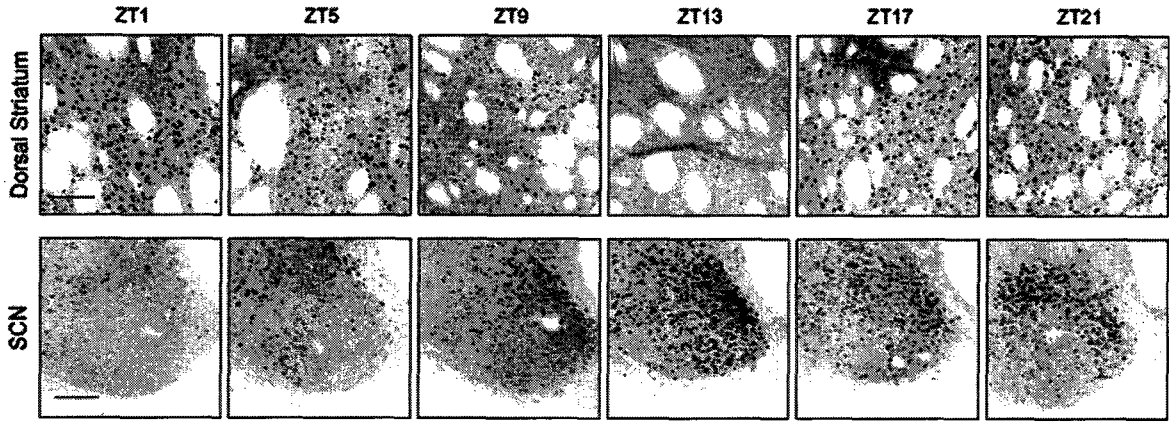

Figure 1.

PER2 immunoreactivity fluctuates in a 24-h rhythm in the dorsal striatum (top row) and the SCN (bottom row). Rats housed in a 12:12 LD cycle were killed at 4-h intervals over 24 h beginning 1 h after lights were turned on (ZT1). In the dorsal striatum, PER2-immunoreactive (IR) nuclei are most abundant around ZT1, whereas in the SCN, PER2-IR nuclei are most abundant around ZT13. Scale bar = 100μm.

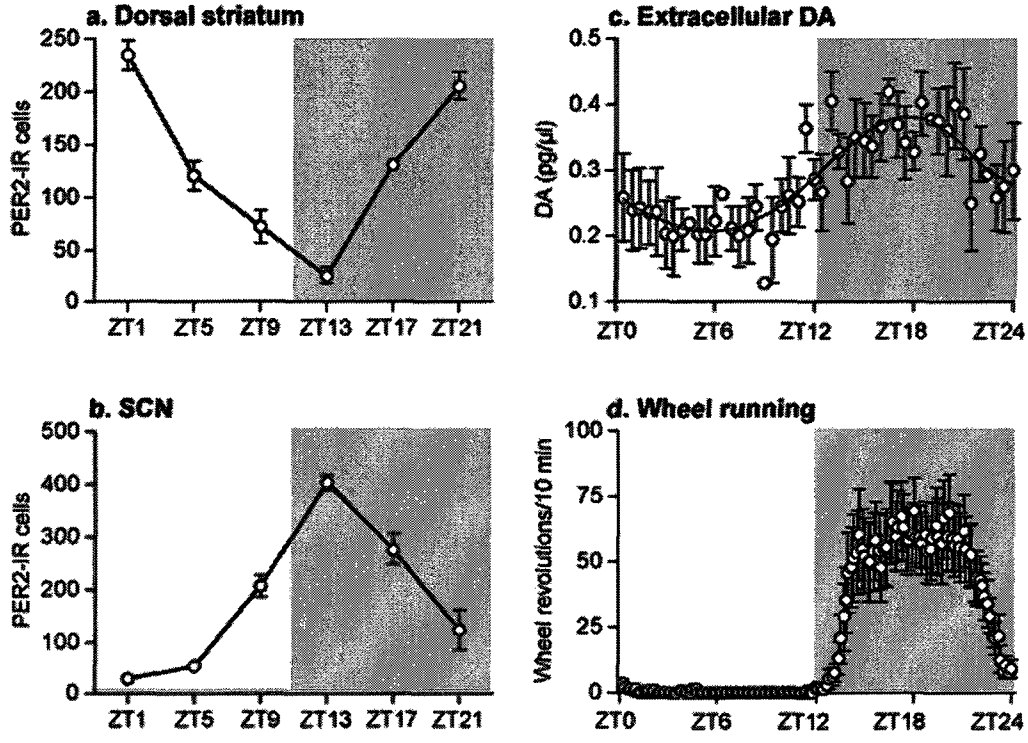

Figure 2.

In rats housed in a 12:12 LD cycle, the 24-h rhythm of PER2 immunoreactivity in the dorsal striatum (a) is opposite in phase to the rhythm of PER2 expression in the SCN (b). The daily fluctuations in extracellular striatal dopamine (DA) levels (c) and wheel-running activity (d) peak at night, preceding the peak of PER2 in the striatum. In each panel, the grey block indicates the time of day when lights were off (between ZT12-24). (a) In the dorsal striatum, PER2 expression is greatest at ZT1, near the beginning of the light phase, and is lowest during the dark phase (expressed as mean number of PER2-IR nuclei ± SEM at each ZT; n = 4 per ZT). (b) In the SCN, PER2 expression is greatest at ZT13, near the beginning of the dark phase, and is lowest during the light phase. (c) *In vivo* microdialysis sampling in the dorsal striatum shows that extracellular DA levels are lowest during the middle of the light phase and highest in the middle of the dark phase (expressed as mean pg DA/µl dialysate ± SEM, samples taken every 30 min; n = 3). (d) Wheel-running activity is also greatest during the dark phase and is virtually absent during the light phase (expressed as mean wheel revolutions/10 min bin over 24 h ± SEM; n = 24).

PER2 expression after unilateral 6-OHDA lesion

To determine whether changes in endogenous levels of DA would affect the normal rhythm of PER2 in the dorsal striatum, we made unilateral injections of 6-OHDA in the right or left MFB to destroy dopaminergic inputs to the dorsal striatum and examined PER2 expression in both the lesioned and intact hemispheres. Immunohistochemical staining of DAT expression confirmed that the unilateral 6-OHDA injection caused a dramatic loss of dopaminergic fibers in the dorsal striatum ipsilateral to the toxin injection (see Fig. 3). Fig. 4 shows examples of PER2 expression and the mean number of PER2-IR nuclei in the dorsal striatum (Fig. 4a, b) and SCN (Fig. 4c, d), on the intact side and lesioned side in rats killed 14 days after 6-OHDA injection. In the dorsal striatum (Fig. 4b), the 6-OHDA injection severely blunted the rhythm of PER2 expression in the lesioned hemisphere, but did not affect the normal PER2 rhythm on the intact side (main effect of ZT, $F(3, 17) = 32.36$, $p < 0.01$; main effect of lesion, $F(1, 17) = 80.33$, $p < 0.01$; ZT x lesion interaction, $F(3, 17) = 92.23$, $p < 0.01$). Specifically, PER2 expression in the 6-OHDA-lesioned striatum was significantly reduced at ZT1 ($t(5) = 13.60$, $p < 0.01$) and ZT19 ($t(3) = 7.41$, $p < 0.01$). In the SCN (Fig. 4d), the 6-OHDA injection had no effect on the normal rhythm of PER2-IR in either the lesioned or the intact hemispheres (main effect of ZT, $F(3, 18) = 44.28$, $p < 0.01$; ZT13 significantly greater than ZT1 and ZT7, $p < 0.05$, post-hoc comparisons with Bonferroni correction; main effect of lesion, $F(1, 18) = 3.70$, n.s.; ZT x lesion interaction, $F(3, 18) = 0.88$, n.s.).

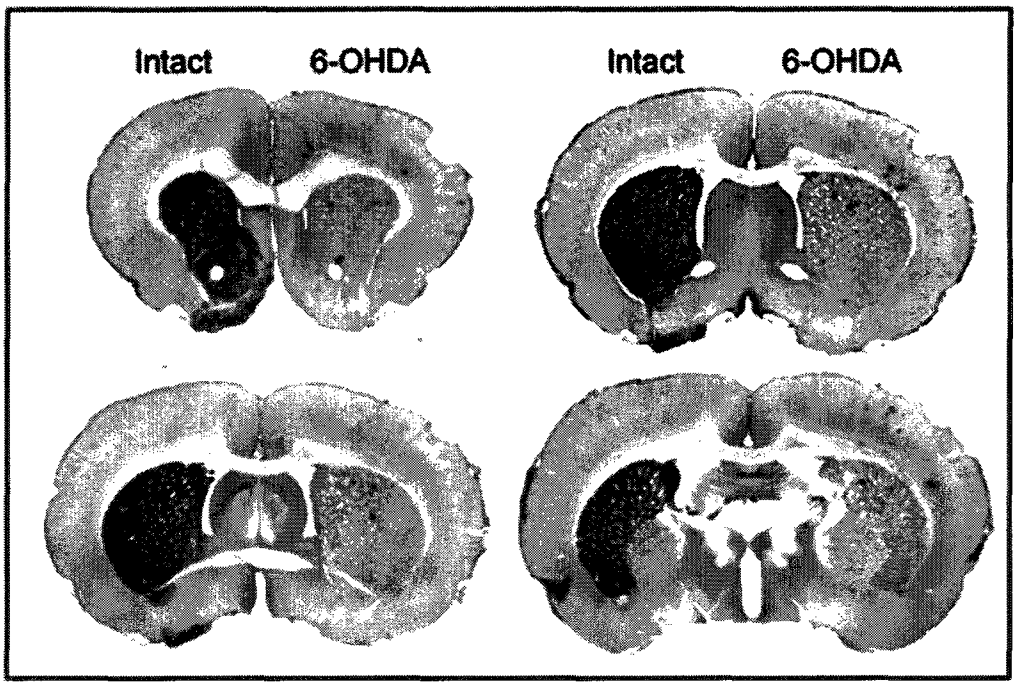

Figure 3.

Photomicrographs showing the effect of unilateral injection of 6-OHDA into the MFB on dopamine transporter (DAT) immunoreactivity (shown in black) in the dorsal striatum ipsilateral to the injection. Lack of stain on the 6-OHDA side indicates a loss of DA input to the striatum in the lesioned hemisphere. Rats were killed 14 days after receiving a unilateral 6-OHDA injection (8 µg).

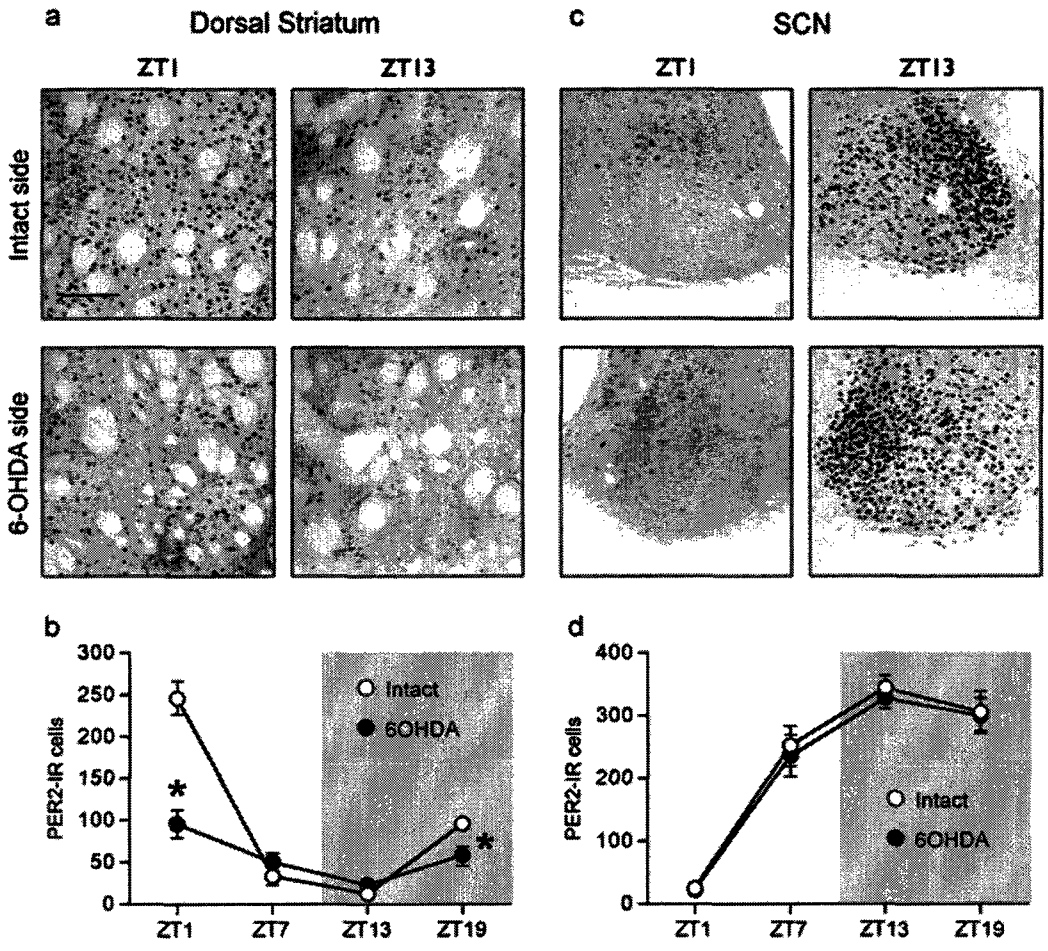

Figure 4.

A unilateral 6-OHDA injection into the MFB selectively reduces the normal morning peak of PER2 expression in the dorsal striatum ipsilateral to the lesion but does not affect the PER2 rhythm in the SCN. (a,c) Photomicrographs showing PER2 expression in the intact and 6-OHDA-treated hemispheres in the dorsal striatum (a) and SCN (c) in rats killed at ZT1 or ZT13. (b, d) Means± SEM of PER2 immunoreactive (IR) nuclei on the intact and lesioned hemispheres in the dorsal striatum (b) and SCN (d) in rats killed at ZT1, 7, 13 and 19, two weeks after 6-OHDA treatment (n = 5-6 per ZT). (* indicates a significant difference between the intact and lesioned hemispheres, $p < 0.05$).

Previous reports have indicated that 6-OHDA-lesioned dopaminergic systems can undergo some degree of recovery over time, particularly when rats engage in exercise after the lesion (Tillerson et al., 2003; Moroz et al., 2004; Anstrom et al., 2007). To determine whether any recovery of PER2 expression would develop in the lesioned striatum with time after the 6-OHDA injection, a group of rats was killed 28 days after receiving a unilateral 6-OHDA injection into the MFB. In the dorsal striatum, PER2 expression at ZT1 was significantly reduced in the lesioned hemisphere compared to the intact hemisphere 28 days after the 6-OHDA injection (data not shown; main effect of ZT, $F(1, 3) = 56.85$, $p < 0.01$; main effect of lesion, $F(1, 3) = 171.94$, $p < 0.01$; ZT x lesion interaction, $F(1, 3) = 194.00$, $p < 0.01$; ZT1 paired samples t-test, $t(1) = 18.42$, $p = 0.04$, ZT13 paired samples t-test, $t(2) = -0.63$, n.s.). In the SCN, there was no effect of the lesion on the daily variation of PER2 expression (data not shown; main effect of ZT, $F(1, 3) = 273.84$, $p < 0.01$; main effect of lesion, $F(1, 3) = 0.62$, n.s.; ZT x lesion interaction, $F(1, 3) = 1.08$, n.s.). This finding indicates that the blunting of peak PER2 expression in the dorsal striatum after a unilateral 6-OHDA lesion does not recover with time, even when rats have access to running wheels in the intervening period.

To confirm that the effect of the 6-OHDA lesion on the PER2 rhythm in the dorsal striatum was attributable to the elimination of DA inputs to this region and not merely to a mechanical lesion of MFB fibers, two additional groups of rats received a unilateral injection of either 6-OHDA or vehicle (0.5% ascorbic acid in saline) into the MFB and were killed 14 days later at either ZT1 or ZT13. As

shown in Fig. 5a, in 6-OHDA-treated rats, PER2 expression in the dorsal striatum was significantly reduced at ZT1 on the lesioned side, whereas there was no effect on PER2 in vehicle-injected rats in either hemisphere (Fig. 5d), indicating that the changes in PER2 in the striatum can be unambiguously attributed to the elimination of the DA innervation. As expected, PER2 expression in the SCN was not affected in either treatment group (Figs. 5b,e).

An additional finding from the 6-OHDA-lesion group in this latter experiment is that although wheel-running activity across the 24-h period remains rhythmic, the amount of nighttime activity in the 6-OHDA lesioned rats is significantly reduced (Fig 5c). This might raise the possibility that the unilateral change in PER2 expression seen after unilateral DA depletion could in some way be related to changes in levels of nighttime activity. Contrary to this idea, however, a similar reduction in activity levels was observed in vehicle-treated rats (Fig. 5f). Thus changes in PER2 expression appear to be related specifically to DA and not to a reduction in locomotor activity.

PER1 expression after unilateral 6-OHDA lesion

In view of evidence that DA receptor agonists modulate the expression of the clock gene *Per1* in the dorsal striatum (Nikaido et al., 2001), we also examined the effect of a unilateral 6-OHDA injection into the MFB on the rhythm of expression of the PER1 protein in the dorsal striatum and SCN using alternate brain sections from rats in our first 6-OHDA lesion experiment. These data are shown in Figure 6. In the dorsal striatum (Fig. 6a), the normal rhythm of PER1

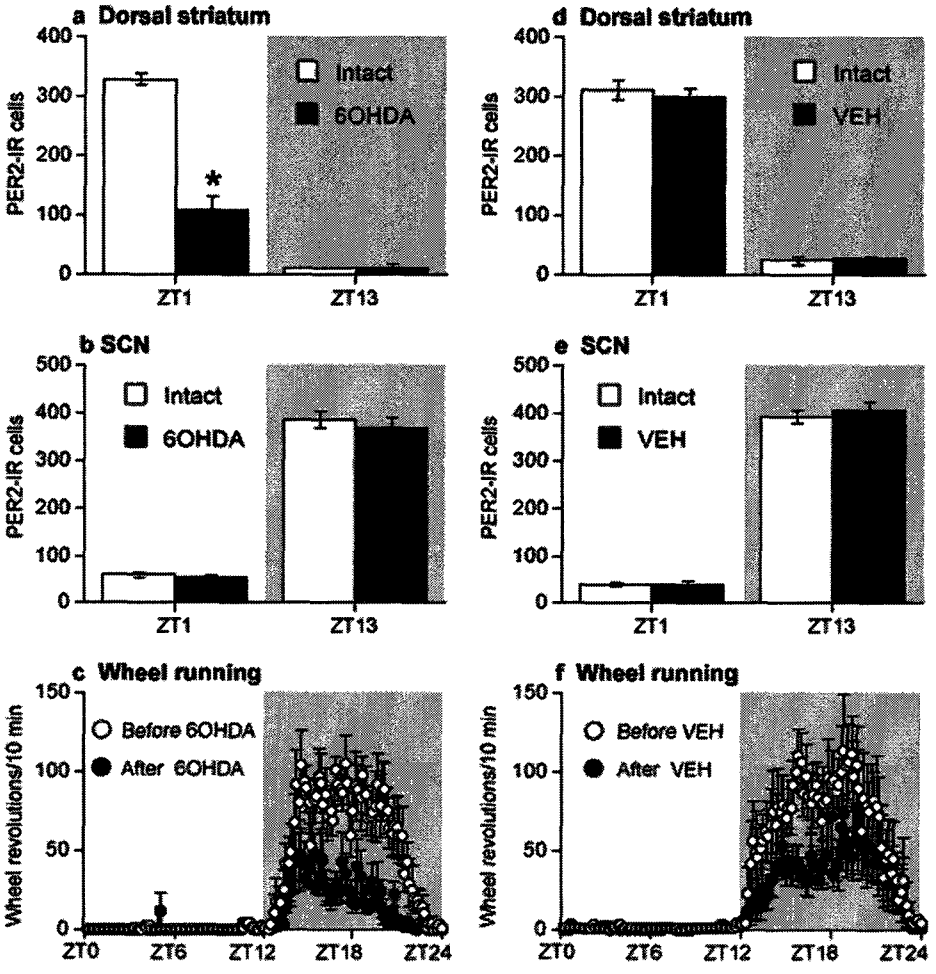

Figure 5.

Intra-MFB injection of 6-OHDA blunts the daytime increase in PER2 expression in the dorsal striatum (a) whereas a vehicle injection into the MFB has no effect (d) (expressed as mean PER2-IR nuclei at each ZT ± SEM; n = 3 per ZT). In the SCN, neither a unilateral 6-OHDA injection (b) nor a vehicle injection (e) into the MFB disrupts the daily fluctuation in PER2 expression. In contrast to the selective effect of 6-OHDA on PER2 in the dorsal striatum, nighttime wheel running activity (expressed as mean wheel revolutions/10 min bin over 24 h ± SEM) is decreased after a unilateral intra-MFB injection of either 6-OHDA (c) or vehicle (f). (* indicates a significant difference between the intact and lesioned hemispheres, $p < 0.05$)

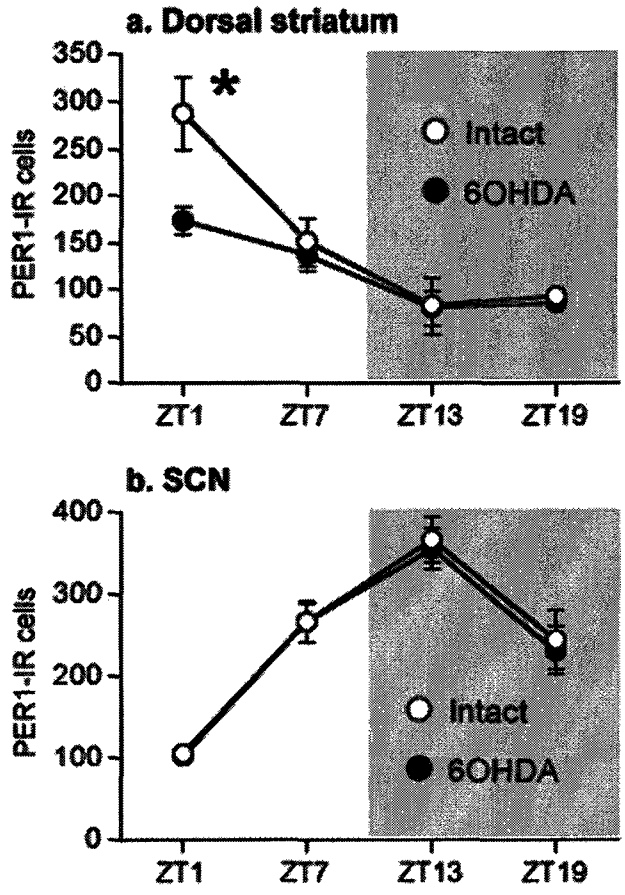

Figure 6.

A unilateral 6-OHDA lesion selectively reduces the normal morning peak of PER1 expression in the dorsal striatum ipsilateral to the lesion but does not affect the PER1 rhythm in the SCN. Means ± SEM of PER1 immunoreactive (IR) nuclei in the intact and lesioned hemispheres in the dorsal striatum (a) and SCN (b) in rats killed at ZT1, 7, 13 and 19, two weeks after 6-OHDA treatment (n = 5-6 per ZT). (* indicates a significant difference between the intact and lesioned hemispheres, $p < 0.05$).

expression approximates the rhythm of PER2 in this region and peaks near ZT1. Following the 6-OHDA lesion, the amplitude of this rhythm in the lesioned hemisphere was significantly blunted compared to the intact hemisphere (main effect of ZT, $F(3, 12) = 10.53$, $p < 0.01$; main effect of lesion, $F(1, 12) = 21.60$, n.s.; ZT x lesion, $F(3, 12) = 11.24$, $p < 0.05$) and PER1 was significantly reduced in the lesioned hemisphere at ZT1 ($p < 0.05$) and ZT19 ($p < 0.05$). In the SCN, the normal rhythm of PER1 expression is similar to the rhythm of PER2 and peaks near ZT13. As shown in Fig. 6b, the 6-OHDA lesion did not affect PER1 in the SCN (main effect of ZT, $F(3, 12) = 11.37$, $p < 0.01$; main effect of lesion, $F(1, 12) = 1.09$, n.s.; ZT x lesion interaction, $F(3, 12) = 0.09$, n.s.).

PER2 expression after inhibition of tyrosine hydroxylase

The results of our 6-OHDA experiments indicate that a unilateral lesion of dopaminergic projections and subsequent depletion of DA in the dorsal striatum significantly blunts the normal rhythm of PER2 expression in this region without affecting the rhythm in the SCN. To determine whether pharmacological depletion of DA without destruction of DA cells would produce the same result, we gave 3 injections of the tyrosine hydroxylase inhibitor, AMPT, over 8 h. The first injection was given at ZT 0 (AMPT 300 mg/kg or vehicle 1 ml/kg), the second injection at ZT4 (AMPT 200 mg/kg or vehicle), and the third injection at ZT8 (AMPT 200 mg/kg or vehicle). Rats were then perfused for immunohistochemistry or decapitated for HPLC quantification of DA at ZT13, 5 h after the last injection, or at ZT1, 17 h after the last injection.

Analysis of striatal tissue punches from both hemispheres using HPLC revealed a 95% reduction in DA content in AMPT-treated rats killed at ZT13 and an 88% reduction in DA content in AMPT-treated rats killed at ZT1 (Fig. 7a), indicating a profound depletion of DA in the dorsal striatum as a result of this treatment (ZT1 independent samples t-test, $t(2) = 13.05$, $p < 0.01$; ZT13 independent samples t-test, $t(2) = 29.02$, $p < 0.01$). Figures 7b and c show the daily fluctuation in PER2 expression in the dorsal striatum and SCN of rats injected with AMPT or vehicle. In the dorsal striatum (Fig. 7b), AMPT significantly reduced PER2 expression at ZT1 (main effect of ZT, $F(1, 11) = 31.45$, $p < 0.01$; main effect of treatment, $F(1, 11) = 10.28$, $p < 0.01$; ZT x treatment interaction, $F(1, 11) = 25.56$, $p < 0.01$; independent samples t test at ZT1, $t(6) = 4.66$, $p < 0.01$) and slightly increased it at ZT13 compared to the vehicle group (independent samples t-test, $t(5) = -3.22$, $p < 0.05$). AMPT treatment had no effect on PER2 expression in the SCN (Fig. 7c; main effect of ZT, $F(1, 12) = 1208.25$, $p < 0.01$; main effect of treatment, $F(1, 12) = 0.17$, n.s.; ZT x treatment interaction, $F(1, 12) = 1.66$, n.s.). These findings corroborate the results of our unilateral 6-OHDA lesion experiments and indicate that depletion of normal DA levels in the dorsal striatum by lesioning or pharmacological means blunts the normal daily peak in PER2 expression in this region. Interestingly, the magnitude of the AMPT effect on striatal PER2 appears larger than the effect of a 6-OHDA lesion. Because inhibition of tyrosine hydroxylase prevents the synthesis of noradrenaline (NA) as well as dopamine, it is possible that the additional

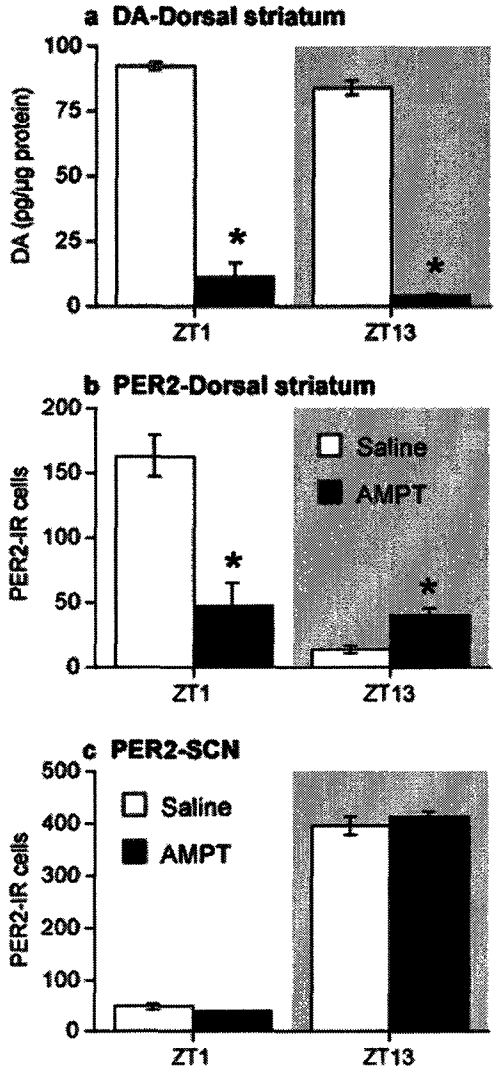

Figure 7.

Inhibition of tyrosine hydroxylase activity suppresses DA levels and daytime PER2 expression in the dorsal striatum. Rats were given 3 injections of AMPT or saline over 8 h and then killed at either ZT13 (5 h after the last injection) or ZT1 (17 h after the last injection). (a) DA content in the dorsal striatum (expressed as pg DA/µg protein) in AMPT-injected rats (black bars) was significantly decreased at both ZT1 and ZT13 compared to saline-injected rats (white bars, n = 2 per treatment group per ZT; * $p < 0.05$). (b) In the dorsal striatum, AMPT treatment decreased daytime PER2 expression at ZT1 and increased PER2 at ZT13 compared to saline controls (expressed as mean PER2-IR nuclei ± SEM at each ZT; n = 4 per treatment group per ZT; * $p < 0.05$). (c) In the SCN, AMPT treatment had no effect on PER2 expression at either time point.

decrease in PER2 following AMPT reflects a role for NA in the regulation of PER2 expression in the dorsal striatum.

PER2 expression in primary motor cortex and hippocampus following 6-OHDA lesion and inhibition of tyrosine hydroxylase

To confirm that the effect of the 6-OHDA lesion on PER2 in the dorsal striatum was attributable to a loss of DA innervation and not to a non-specific lesion of catecholamines fibers in general, we examined the effects of a 6-OHDA lesion and AMPT treatment on PER2 expression in two other regions innervated by NA fibers, namely primary motor cortex (M1) and the dentate gyrus of the hippocampus (DG) (Swanson and Hartman, 1975; Fallon and Moore, 1978; Asan, 1998). In both M1 and DG, the normal daily fluctuation in PER2 expression peaks at ZT1 and falls at ZT13, consistent with the PER2 phase in the dorsal striatum. As shown in Fig. 8, a unilateral 6-OHDA lesion had no effect on PER2 expression in M1 (Fig. 8a) or DG (Fig. 8c) in either hemisphere. In contrast, systemic AMPT treatment dramatically decreased PER2 expression at ZT1 in both M1 (Fig. 8b) and DG (Fig. 8d). Together, these results indicate that catecholamines regulate the daily fluctuation in PER2 expression in M1 and DG. The dissociation between the effects of 6-OHDA and AMPT, however, suggests that the catecholaminergic regulation of PER2 in these regions is primarily attributable to NA. Importantly, the lack of effect of the 6-OHDA lesion on PER2 in these regions indicates that the lesion did not damage NA fibers in addition to DA fibers.

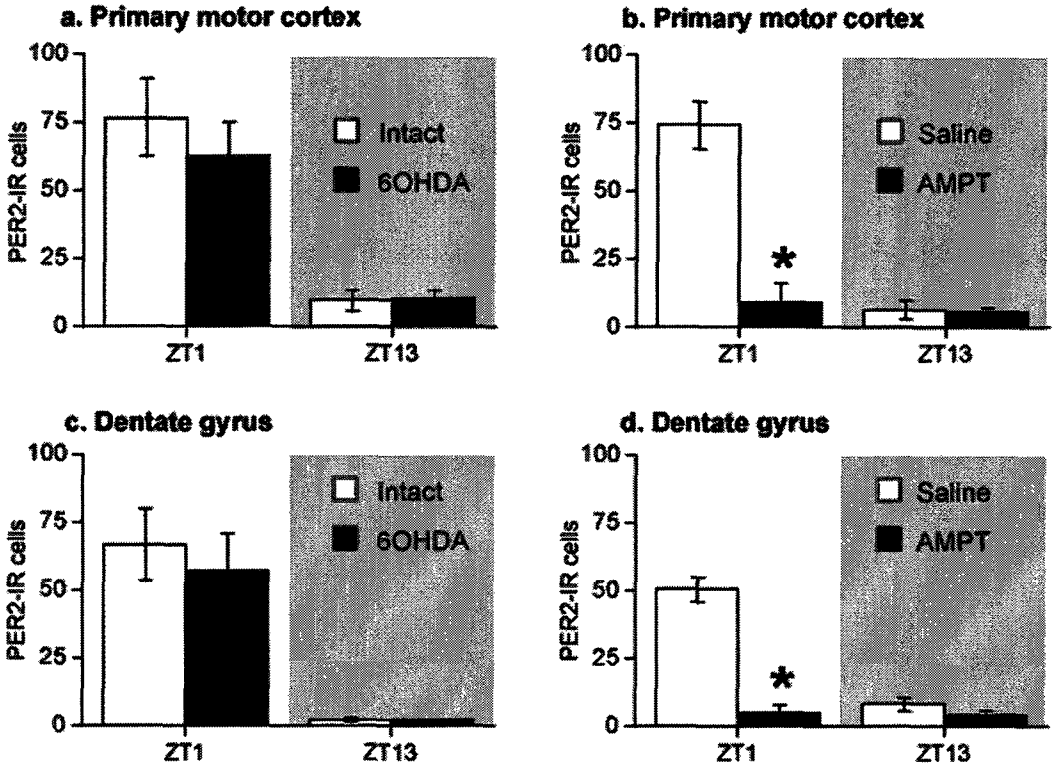

Figure 8.

A unilateral 6-OHDA infusion into the MFB has no effect on PER2 in primary motor cortex (M1) or in the dentate gyrus (DG), whereas treatment with AMPT strongly suppresses the normal peak of PER2 expression in each of these regions. Mean PER2-IR nuclei ± SEM in the intact and 6-OHDA lesioned hemispheres of M1 (a) and DG (c) following a unilateral 6-OHDA lesion (n = 5 per ZT). Mean PER2-IR nuclei ± SEM in primary motor cortex (b) and DG (d) following AMPT treatment (n = 4 per treatment group per ZT; * $p < 0.05$).

PER2 expression during chronic treatment with DA receptor antagonists

Having found that a depletion of DA in the dorsal striatum disrupts the normal rhythm of PER2 expression in this region, we investigated whether blocking either D1 or D2 DA receptors using chronic, 24-h infusion of DA receptor antagonists would affect the normal rhythm of PER2 expression in the dorsal striatum and SCN. Following a 10-day entrainment period, naïve rats underwent surgery to receive osmotic minipumps containing the D1 DA receptor antagonist, SCH 23390 (0.5 mg/kg/24 h), or the D2 DA receptor antagonist, raclopride (2 mg/kg/24 h), or underwent sham surgery (no pump inserted). Rats remained in their home cages for 5 days after pump insertion and were then perfused at either ZT1 or ZT13. The doses of SCH 23390 and raclopride were chosen on the basis of previous studies indicating dose ranges that were effective in occupying approximately 80% of striatal D1 or D2 DA receptors and that did not impede motor function (Creese and Chen, 1985; Neisewander et al., 1998; Wadenberg et al., 2001; Kapur et al., 2003). The duration of drug treatment was limited to 5 days in this experiment because of evidence indicating that longer delivery of DA antagonists is less effective than shorter-term treatment in blocking DA receptors due to increasing sensitivity of DA receptors over time (Samaha et al., 2008).

The results of this experiment are shown in Fig. 9. In the dorsal striatum (Fig. 9a), PER2 expression was unaffected by five days of chronic treatment with the D1 DA antagonist, SCH 23390, but was significantly reduced at ZT1 by

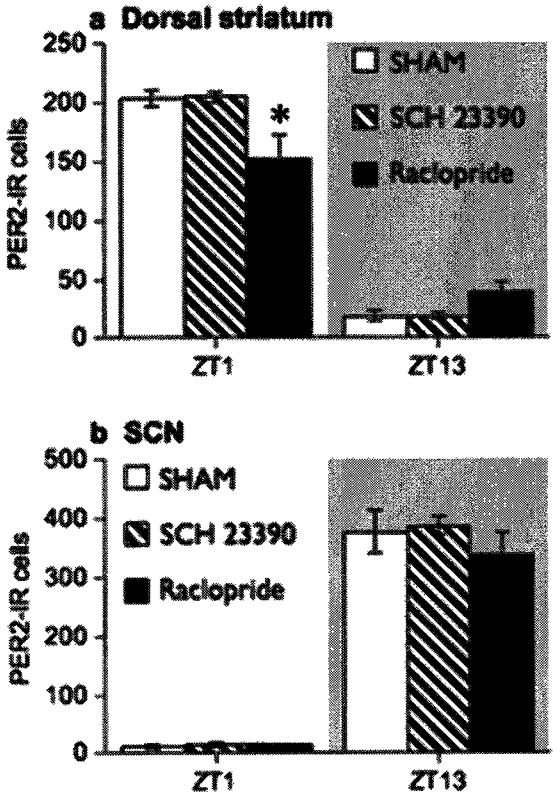

Figure 9.

(a) Continuous infusion of the D2 DA receptor antagonist, raclopride (2 mg/kg/24 h), for 5 days via osmotic minipump reduces daytime expression of PER2 in the dorsal striatum, whereas infusion of the D1 DA antagonist, SCH 23390 (0.5 mg/kg/24 h) has no effect (expressed as mean PER2-IR nuclei ± SEM at each ZT; n = 4 per treatment group per ZT). (b) Neither raclopride nor SCH 23390 infusion affects PER2 in the SCN (* indicates a significant difference between raclopride and sham groups, $p < 0.05$).

chronic infusion of the D2 DA antagonist, raclopride (ZT x treatment interaction, $F(2, 18) = 8.24$, $p < 0.01$; one-way ANOVA at ZT1, $F(2, 9) = 5.53$, $p < 0.05$, raclopride group less than vehicle and SCH 23390 groups, $p < 0.05$, post-hoc comparison with Bonferroni correction). No differences in PER2 expression between groups were found at ZT13 in the dorsal striatum (one-way ANOVA at ZT13, $F(2, 9) = 2.93$, n.s.). In the SCN (Fig. 9b), neither SCH 23390 nor raclopride changed the normal daily variation in PER2 (main effect of ZT, $F(1, 18) = 727.79$, $p < 0.01$; main effect of treatment, $F(2, 18) = 1.07$, n.s.; ZT x treatment interaction, $F(2, 18) = 0.69$, n.s.). These results suggest that the normal PER2 rhythm in the dorsal striatum depends on the availability of D2, but not D1 DA receptors.

Effect of D1 or D2/3DA receptor agonist injections on PER2 expression and wheel running in 6-OHDA-lesioned rats

Previous research indicates that changes in the expression of peptides and receptors in striatal medium spiny neurons that develop following a unilateral 6-OHDA lesion can be reversed with intermittent or chronic DA receptor agonist treatment (Gerfen et al., 1990). To investigate whether such treatments could also restore the normal rhythm of PER2 expression in the 6-OHDA-lesioned dorsal striatum, we gave systemic injections of the D1 DA agonist, SKF 81297 (1 mg/kg), the D2/3 DA agonist, quinpirole (0.5 mg/kg), or vehicle (1 ml/kg) at ZT1 for 10 days, beginning 15 days after surgery. Drug doses were in the range shown previously to enhance expression of immediate early genes, striatal peptides, and electrophysiological responses of striatal neurons in 6OHDA-

lesioned rats (Gerfen et al., 1990; Hu et al., 1992; Gerfen et al., 1995; Zhang et al., 2007; Ballion et al., 2009). We chose to give the daily agonist injection at ZT1, a time at which extracellular DA levels in the striatum are normally low, to determine whether exogenous stimulation of DA receptors would not only restore PER2 expression in the lesioned striatum, but also establish a new rhythm of PER2 expression whose phase was determined by the time of the agonist injection. Rats were then killed on the day after the last injection at either ZT1 or ZT13.

The results of this experiment are shown in Fig. 10. As shown previously, a unilateral 6-OHDA lesion blunted the daily variation of PER2 expression in the dorsal striatum on the lesioned side by strongly reducing the normal peak seen at ZT1 (ZT x lesion interaction, $F(1, 27) = 85.64$, $p < 0.01$) (vehicle-treated rats, Fig. 10a). Daily systemic injection of quinpirole at ZT1 restored and reversed the daily fluctuation of PER2 expression on the lesioned side. Specifically, quinpirole had no effect on PER2 expression at ZT1 (Fig. 10b, one-way ANOVA at ZT1, $F(2, 14) = 0.16$, n.s.), but dramatically increased expression PER2 at ZT13, a time when the normal rhythm of PER2 expression in the striatum would otherwise be at its trough (one-way ANOVA at ZT13, $F(2, 13) = 64.02$, $p < 0.01$). It can also be seen in Fig. 10b that quinpirole injections had no effect on the normal daily variation in PER2 expression in the intact dorsal striatum (main effect of ZT, $F(1, 27) = 174.99$, $p < 0.01$; main effect of treatment, $F(2, 27) = 0.04$, n.s.; ZT x treatment interaction, $F(2, 27) = 2.00$, n.s.). Daily injection of SKF 81297 at ZT1

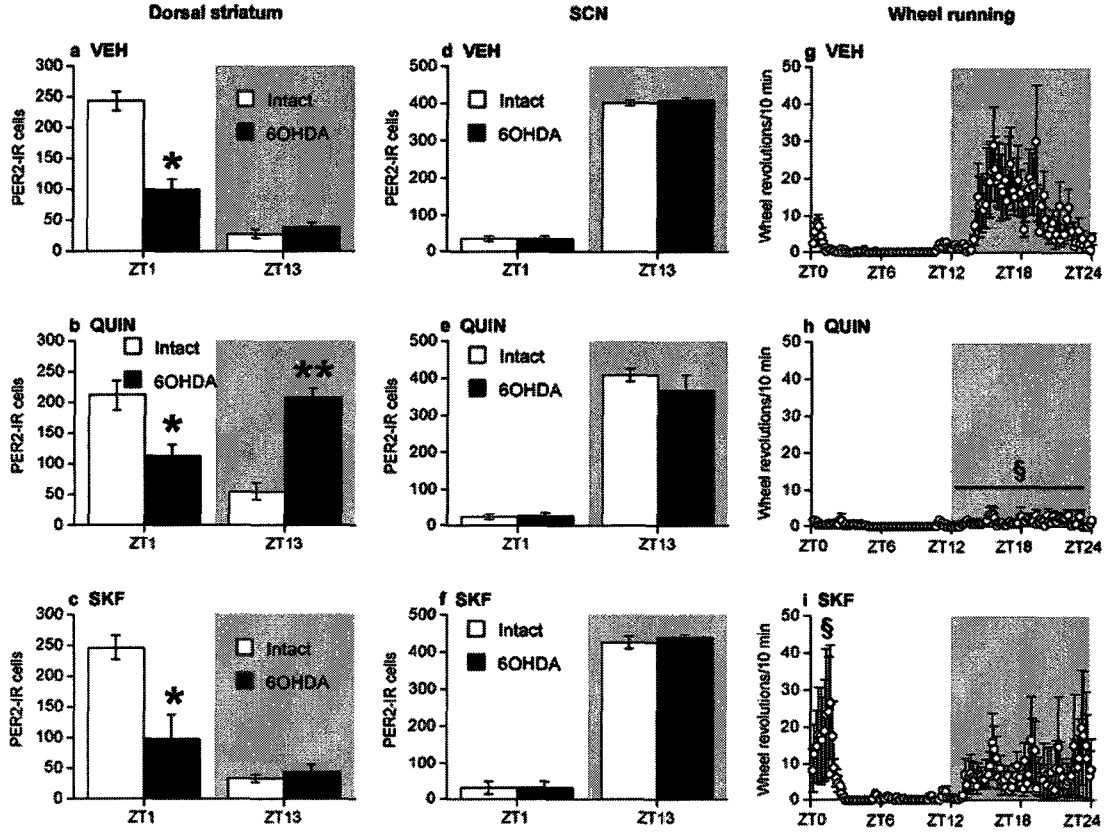

Figure 10.

PER2 expression in the dorsal striatum and SCN and daily wheel running activity in 6-OHDA-lesioned rats injected daily at ZT1 with vehicle, the D2/3 agonist, quinpirole (0.5 mg/kg), or the D1 DA agonist, SKF 81297 (1 mg/kg). In the dorsal striatum (a, b, c), daytime PER2 expression was decreased on the 6-OHDA-lesioned side in vehicle-injected rats (a) (expressed as mean PER2-IR ± SEM at each ZT; n = 7-8 per ZT). Daily injection of quinpirole (b) selectively increased PER2 expression at ZT13 in the dorsal striatum of the 6-OHDA lesioned hemisphere (n = 6 per ZT), whereas daily injection of SKF 81297 (c) had no effect on PER2 in either the lesioned or intact hemisphere (n = 3 per ZT). PER2 expression in the SCN was not affected any of these treatments (d, e, f). The normal 24-h rhythm in wheel running during the 10-day injection period (g, h, i) was not disrupted by daily vehicle injection (g, expressed as mean wheel revolutions/10 min bin over 24 h ± SEM; n = 15). Quinpirole injection (h) suppressed nighttime wheel running (n = 12), whereas SKF 81297 injection (i) increased daytime wheel running (n = 6), particularly in the hour before the injection was given (ZT0-1). (* indicates a significant difference from the intact hemisphere; ** indicates significant difference from vehicle group lesioned hemisphere, $p < 0.05$; § indicates a significant difference from the vehicle group, $p < 0.05$).

(Fig. 10c) had no effect on PER2 expression at ZT1 or ZT13 in either the lesioned or intact dorsal striatum.

Consistent with our earlier results, unilateral injection of 6-OHDA had no effect on the normal daily variation of PER2 expression in the SCN (Fig. 10d). Furthermore, as shown in Figs. 10e, f the daily variation of PER2 expression in the SCN was unaffected by quinpirole or SKF 81297 injection. In contrast, it can be seen from Figs. 10g-i that daily injections of the DA agonists had differential effects on wheel-running activity rhythms. Quinpirole strongly suppressed nocturnal activity (Fig. 10h), whereas SKF 81297 increased activity during the day especially just after the injections (Fig. 10i).

Having found that a daily injection of quinpirole, but not SKF 81297, at ZT1 establishes a new pattern of PER2 expression in the 6-OHDA-lesioned dorsal striatum, though not in the intact striatum, we asked whether similar treatments with quinpirole or SKF 81297 would have any effect on PER2 expression in the dorsal striatum or SCN of naïve, intact rats. No effects of these drug treatments were found in either the dorsal striatum or SCN (data not shown).

Finally, having found that scheduled daily injections of quinpirole restored a rhythm of PER2 expression in the 6-OHDA-lesioned dorsal striatum, whereas SKF 81297 injections did not, we asked whether the induction of a new PER2 rhythm in the lesioned dorsal striatum requires a timed, bolus delivery of a DA receptor agonist or only a general level of DA receptor stimulation that is time-

insensitive. To address this, we prepared rats with unilateral 6-OHDA lesions of the MFB and inserted osmotic minipumps 15 days after the 6-OHDA lesion surgery to deliver a constant 24-h infusion of quinpirole (0.5 mg/kg/24 h) or SKF 81297 (1 mg/kg/24 h). Rats were perfused 10 days later at either ZT1 or ZT13. As shown in Fig. 11a, 6-OHDA had the expected effect on PER2 expression in the dorsal striatum at ZT1. Furthermore it can also be seen that chronic delivery of quinpirole (Fig. 11b) or SKF 81297 (Fig. 11c) at the doses used did not alter the effect of lesions on PER2 expression in the dorsal striatum nor did they alter PER2 expression in the intact hemisphere. No effects were seen in the SCN (Fig. 11d, e, f). Finally, during the 10-day period of chronic infusion of quinpirole or SKF 81297, wheel-running activity remained rhythmic in all groups (Fig. 11g, h, i).

Cell types expressing PER2 in the dorsal striatum

The vast majority of cells in the dorsal striatum are GABAergic medium spiny neurons that project to the output nuclei of the basal ganglia. These medium spiny neurons are subdivided into two populations that are distinguishable on the basis of the regions to which they project and the receptors and peptides expressed by each. Striatonigral neurons, belonging to the 'direct' pathway, project primarily to the substantia nigra pars reticulata and express D1 DA receptors and the peptides, substance P and dynorphin. Striatopallidal neurons, belonging to the 'indirect' pathway, project to the globus pallidus and express D2 DA receptors and enkephalin (for a review, see Gerfen, 2004). On the basis of these known histological characteristics, we used double

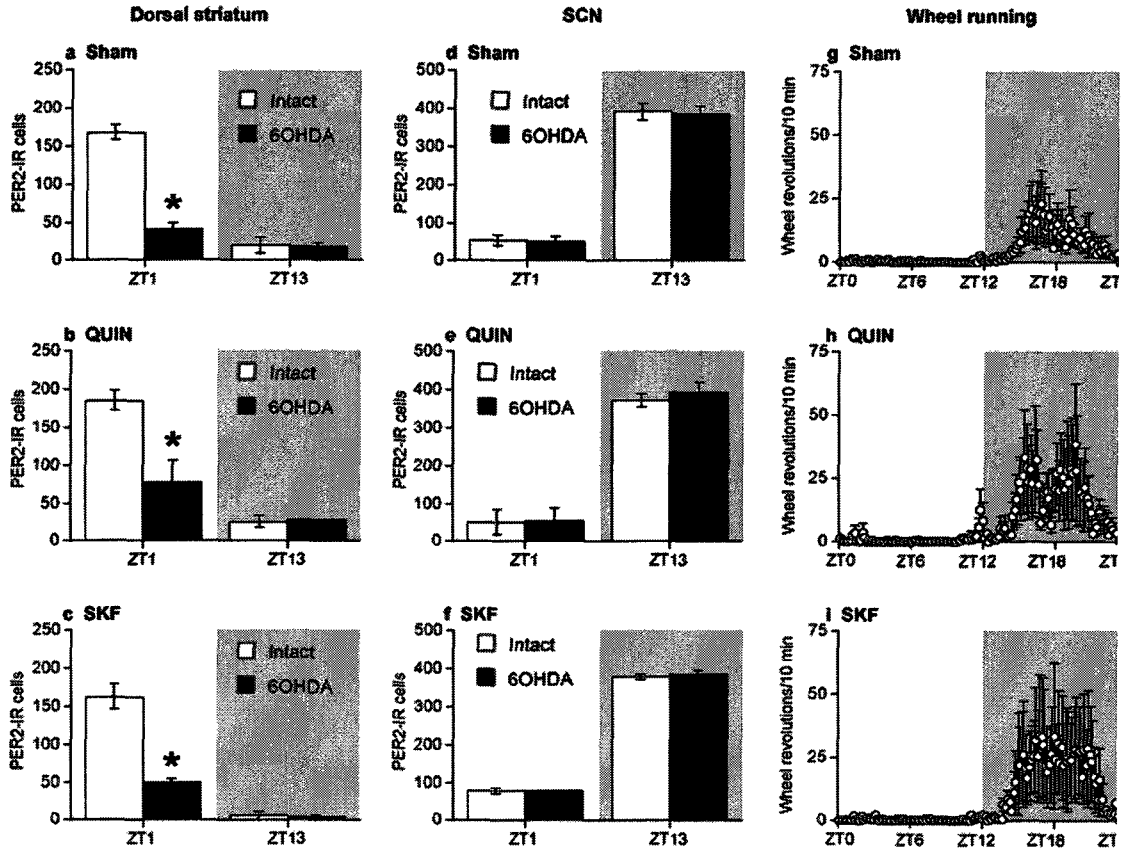

Figure 11.

Continuous infusion of the D2/3 agonist, quinpirole (0.5 mg/kg/24 h; n= 2-3 per ZT), or the D1 DA agonist, SKF 81297 (1 mg/kg/day; n=2-3 per ZT), for 10 days via osmotic minipump has no effect on mean (± SEM) PER2 expression in either the 6-OHDA lesioned or intact dorsal striatum (b, c) or SCN (e, f) compared to sham surgery group (Fig. 10a, d; n=3-5 per ZT). Neither treatment affected daily wheel running activity (expressed as mean wheel revolutions/10 min bin over 24 h ± SEM; Fig. 10g, h, i). (* indicates significant difference from intact hemisphere, $p < 0.05$).

immunohistochemical staining to determine which types of striatal cells express PER2. Representative images of cells in the dorsal striatum double-labeled for PER2 and GAD-67 (the rate-limiting enzyme for the synthesis of GABA), the D2 DA receptor, the D1 DA receptor, enkephalin, or substance P from rats killed at ZT1 are presented in Fig. 12. Double-labeling for GAD-67 and PER2 (Fig. 12a) revealed that many, though not all, GAD-67-IR cells express PER2, demonstrating that PER2 is expressed in medium spiny neurons. Double-labeling for D2 DA receptor and PER2 (Fig. 12b), however, revealed that most D2-IR cells also express PER2. In contrast, only a few D1 DA receptor-IR cells expressed PER2 (Fig. 12c). Double-labeling for PER2 and the peptides enkephalin or substance P revealed that PER2 is frequently co-localized with enkephalin (Fig. 12d), and much less often with substance P (Fig. 12e). These observations demonstrate that PER2 in the dorsal striatum is expressed more frequently in D2 DA receptor- and enkephalin-containing medium spiny neurons belonging to the striatopallidal projection.

DISCUSSION

Daily, timed stimulation of D2 receptors regulates PER2 in the dorsal striatum.

There is evidence that molecular components of the circadian clock, including *Per2*, participate in the regulation of striatal dopaminergic activity in rodents (McClung et al., 2005; Roybal et al., 2007; Hampp et al., 2008). In turn, it has been shown that pharmacological stimulation of DA receptors can affect the

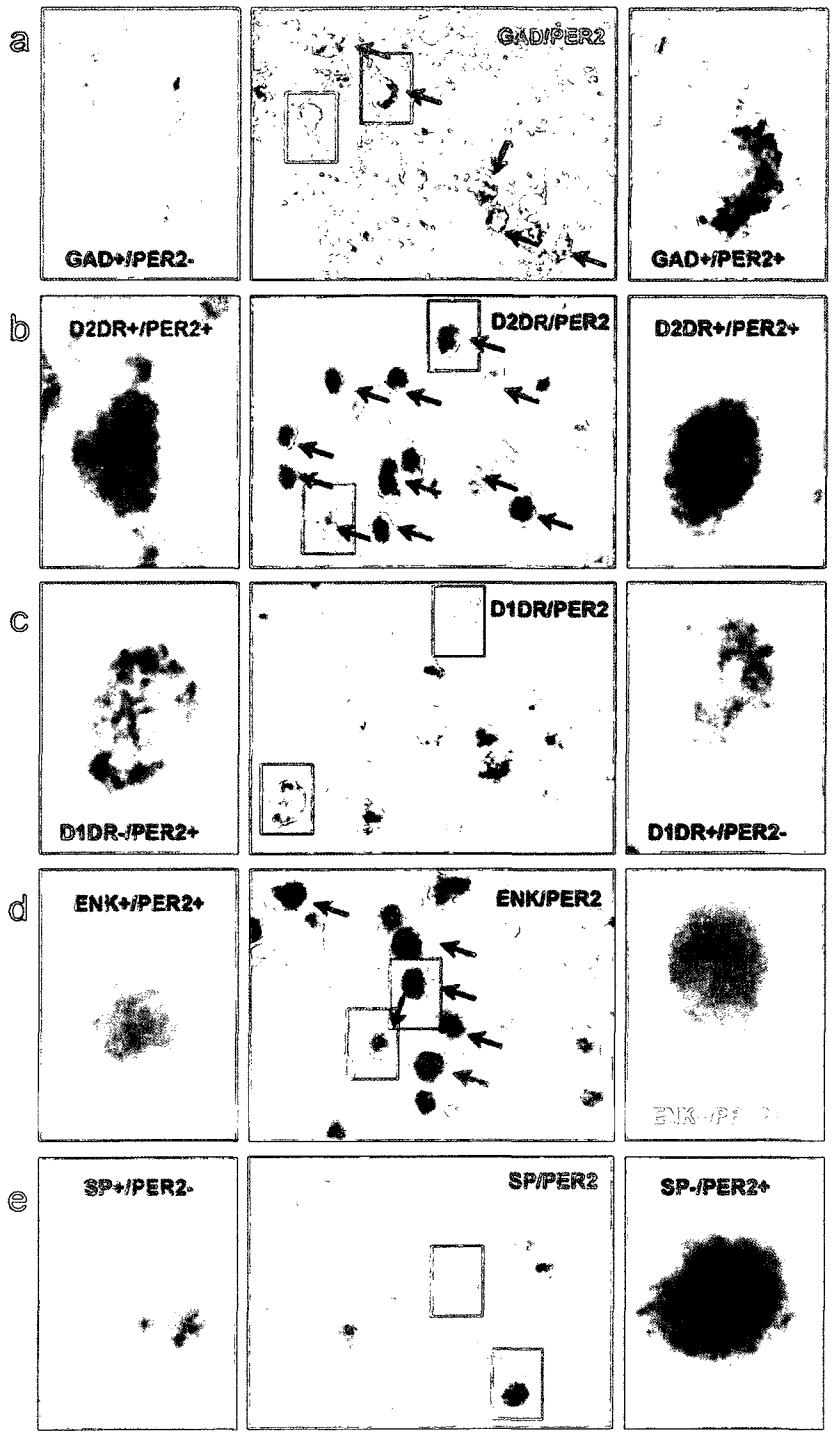

Figure 12.(caption on next page)

Figure 12

Central panel shows high magnification (X100) images depicting dorsal striatal neurons double stained for PER2 and GAD-67, D2 DA receptor, D1 DA receptor, enkephalin (ENK) or substance P (SP). Images in left and right panels are enlargements of framed cells in corresponding central panel. (a) Double immunostaining for GAD-67 (brown cytosolic stain) and PER2 (blue nuclear stain) reveals that many but not all GAD-immunopositive cells co-express PER2 (shown with arrows. (b) Double-immunostaining for D2 DA receptor (brown cytosolic stain) and PER2 (black nuclear stain) revealed frequent co-expression of PER2 in D2 DA receptor-positive cells (marked with arrows). (c) Double immunostaining for D1 DA receptor (brown cytosolic stain) and PER2 (black nuclear stain) reveals segregation between PER2 and D1 DA receptor immunostaining. (d) Double immunostaining for enkephalin (brown cytosolic stain) and PER2 (blue-gray nuclear stain) shows frequent co-expression (shown with arrows). (e) Double immunostaining for substance P (brown cytosolic stain) and PER2 (black nuclear stain) shows segregation between substance P and PER2.

expression of *Per* genes in the striatum (Nikaido et al., 2001; Iijima et al., 2002; Uz et al., 2005; Lynch et al., 2008; Imbesi et al., 2009). Here, we examined the role of *endogenous* DA in the regulation of the expression of the clock protein, PER2, in the dorsal striatum in order to better characterize the relation between DA and the circadian oscillations seen in clock gene expression in this region of the brain. We found that treatments that deplete striatal DA strongly blunt the rhythm of PER2 and, furthermore, that daily stimulation of D2, but not D1 DA receptors, restores and entrains the PER2 rhythm in the DA-depleted striatum. Furthermore, we showed that the peak of the daily rhythm of extracellular DA in the dorsal striatum precedes the peak of PER2. In addition, we observed that PER2 is expressed in striatal cells that co-express D2 DA receptors. Our findings are consistent with the idea that the expression of PER2 within the dorsal striatum is expressed in neurons of the striatopallidal projection and that the rhythm of expression of PER2 in these neurons depends on daily dopaminergic activation of D2 DA receptors.

The daily peak of extracellular DA in the dorsal striatum preceded that of PER2 by approximately 6 h. Specifically, extracellular DA levels peak in the middle of the dark (active) phase and fall during the light (inactive) phase of the daily cycle (see also Paulson and Robinson, 1994; Castaneda et al., 2004), whereas PER2 expression peaks at the beginning of the light phase and reaches its trough near the beginning of the dark phase (see also Amir and Robinson, 2006). When striatal DA was depleted unilaterally using 6-OHDA, the peak expression of PER2 on the lesioned side was strongly suppressed and the daily

rhythm of PER2 in this region was blunted both at 2 and 4 weeks post-surgery. Furthermore, we found that treatment with AMPT, which blocks the synthesis of DA and other catecholamines acutely, suppressed PER2 expression within 24 h. These findings indicate that normal rhythmic expression of PER2 in the dorsal striatum relies on DA availability. We next showed that the rhythm of PER2 in the dorsal striatum relies on phasic activation of D2 DA receptors. Daily injections of a D2 DA receptor agonist, given to induce a peak of activation of DA receptors at a time of day when DA levels are normally low, not only restored, but also shifted the daily peak of PER2 in the lesioned dorsal striatum. Continuous infusion of the D2 DA agonist had no effect, underscoring the role of phasic activation. Importantly, neither daily injections nor continuous infusion of a D1 DA agonist was effective. Together these findings point to a link between the daily rise of endogenous DA, D2 DA receptor activation and the subsequent peak in PER2 expression in the dorsal striatum.

The requirement of D2 DA receptor activation for the maintenance of PER2 rhythm in the dorsal striatum is further demonstrated by our finding that chronic infusion of a selective D2, but not D1, DA receptor antagonist attenuates the daily fluctuation in PER2 expression in the dorsal striatum. Furthermore it is also consistent with our observation that PER2 is found frequently in striatal GABA-containing neurons that express D2 DA receptors and enkephalin, and rarely in those expressing D1 DA and substance P. This apparent anatomical selectivity of PER2 expression within the dorsal striatum, and its modulation by

D2 DA activation may provide a clue to the functional significance of rhythmic PER2 expression in this system.

Mechanisms of PER2 modulation by DA in the dorsal striatum.

The mechanisms whereby DA depletion and D2 DA receptor activation suppress and restore, respectively, PER2 expression in the dorsal striatum remain to be resolved. It is clear that changes in locomotor activity brought about by DA depletion or, indeed, by D2 DA receptor stimulation cannot explain the changes in PER2 expression. For example, unilateral depletion of striatal DA, which strongly blunted the rhythm of PER2 expression on the lesioned side, did not disrupt the daily activity rhythm even though the level of nighttime activity was reduced. Furthermore, comparable changes in level of nighttime activity, but not PER2 expression, were seen after unilateral injection of vehicle into the MFB. A similar lack of relation between PER2 expression in the dorsal striatum and activity emerges from the experiments with the daily morning injections of the D2 DA receptor agonist, quinpirole. Thus, injection of quinpirole given when striatal levels of DA would be low restored the daily variation of PER2 expression in the lesioned striatum while suppressing nighttime activity, but had no effect on PER2 expression in the intact hemisphere; neither did it affect PER2 expression in naïve rats.

The absence of an effect of quinpirole injections on the PER2 rhythm in the intact dorsal striatum and in the striatum of naïve rats is likely due to the time of day when injections were given. The dose of quinpirole used in the present

experiments has been shown to reliably inhibit the activity of DA neurons in naïve rats through activation of D2 DA autoreceptors on DA cell bodies (Lacey et al., 1987; Jeziorski and White, 1989). Indeed, we have found that injecting the same dose of quinpirole in naïve rats at the start of the dark phase, when extracellular DA levels in the dorsal striatum are beginning to rise, strongly reduced the normal peak of PER2 expression in the striatum at ZT1 (unpublished observations). Because quinpirole injections were given at ZT1 in the present experiments, during the trough of the rhythm of extracellular DA in the striatum, a further reduction in dopaminergic cell activity at this time of day would have little impact on the normal PER2 rhythm. Following a 6-OHDA lesion, however, the expression of D2 DA receptors on cell bodies is lost through destruction of DA fibers, whereas post-synaptic expression of D2 DA receptors in medium spiny neurons is upregulated (e.g., Gerfen et al., 1990). As a result, the 6-OHDA-lesioned hemisphere would be highly sensitive to D2 DA receptor stimulation.

The question then remains, how might activation of D2 DA receptors regulate the rhythm of PER2 expression in the dorsal striatum? In addition to the immunohistochemical data we report here, we have preliminary results from an ongoing study using real time PCR (Weigl, 2010) showing a 40-50% reduction in the amplitude of the *Per2* mRNA rhythm in the dorsal striatum ipsilateral to a 6-OHDA injection (unpublished observations). These findings corroborate those of Sahar et al.(2010) who reported a decrease in the amplitude of the *Per2* transcription rhythm in the striatum of D2 knock-out mice, and suggest that D2 signaling modulates PER2 expression in this region at the level of transcription.

This modulation could occur through changes in the cAMP-CREB signaling pathway, which is regulated by DA signaling (Montmayeur and Borrelli, 1991). The *Per2* promoter region contains a CRE-responsive site (Travnickova-Bendova et al., 2002) and previous studies in vitro have demonstrated that D2 receptor stimulation upregulates *Per* expression in the retina through a CREB-dependent mechanism (Besharse et al., 2004; Yujnovsky et al., 2006). It is possible, however, that DA also regulates PER2 expression directly through changes in post-translational phosphorylation mechanisms (Iitaka et al., 2005) or indirectly by modulating other components of the clock, for example through D2 receptor-mediated changes in BMAL1 phosphorylation by GSK3β (Beaulieu et al., 2004; Sahar et al., 2010).

A different possibility is that DA acts through presynaptic D2 DA receptors to affect PER2 expression by modulating the activity of corticostriatal glutamatergic neurons (Bamford et al., 2004) or by modulating the effect of glutamate on D2 DA receptor-expressing striatopallidal neurons (for review, see Surmeier et al., 2007). Indeed, it is well known that glutamate plays a key role in the regulation of clock gene expression in the SCN (Reppert and Weaver, 2001). Importantly, it has been shown that following 6-OHDA-induced lesions of DA inputs to the dorsal striatum there are changes in both glutamate release as well as in responsiveness of D2 DA receptor-bearing striatopallidal neurons to glutamate (see Day et al., 2006). These changes in glutamate transmission within the striatum could potentially be linked in as yet unknown ways to changes in expression of PER2 in these striatal neurons.

Our results also leave open a possible role for NA in the modulation of PER2 expression in the dorsal striatum. AMPT depletion of catecholamines strongly reduced PER2 expression in this region, as well as in M1 and DG, which receive NA inputs. There is mixed evidence supporting NA signaling in the regulation of circadian rhythms, with some findings indicating an important role of forebrain NA for rhythms of sleep-wake, melatonin synthesis, and clock gene expression (Warnecke et al., 2005; Gonzalez and Aston-Jones, 2006, 2008; Wongchitrat et al., 2009) whereas others demonstrate no effect of NA (e.g., Fukuhara et al., 2002; Reilly et al., 2008). Taken together, it would seem that an involvement of NA in clock gene expression is probably region-specific, similar to the role proposed for DA here.

DA does not regulate PER2 in the SCN.

It should be noted that in all our experiments we assessed PER2 expression in the SCN. Without exception, however, no changes in PER2 expression were found either after DA depletion or activation/blockade of DA receptors. This insensitivity of the SCN is consistent with previous reports that the adult SCN, unlike the fetal SCN (Weaver et al., 1992; Schurov et al., 2002), is impervious to dopaminergic manipulations (Duffield et al., 1998; Masubuchi et al., 2000) and is also consistent with our finding that dopaminergic manipulations did not affect the daily rhythm of locomotor activity.

Functional significance of PER2 regulation by DA.

Finally, it is interesting to speculate about the role of clock genes in functions mediated by the striatum. One idea is that clock genes, including *Per2*, play a role in the modulation of reward processes known to involve dopaminergic activity in the striatum (Abarca et al., 2002; McClung et al., 2005; Roybal et al., 2007). This is supported by the evidence that *Per2* mutant mice exhibit greater responsivity to cocaine (Abarca et al., 2002), drink more alcohol and, interestingly, have higher levels extracellular glutamate in the brain secondary to a reduction in glutamate uptake (Spanagel et al., 2005). Another idea, more directly related to the present findings, is that the loss of circadian rhythms in clock gene expression in the striatum after DA depletion could be linked to disruptions of daily behavioral and physiological rhythms frequently reported in Parkinson's disease (Bruguerolle and Simon, 2002; Cai et al., 2010). Current models posit that the symptoms of Parkinson's disease result from changes in excitability of striatopallidal neurons secondary to depletion of striatal DA (Day et al., 2006). Our findings showing that loss of striatal DA can disrupt the rhythm of PER2, an essential component of the circadian clock, and that PER2 is expressed in D2 DA receptor-containing striatal neurons may provide a clue to understanding the origins of the circadian symptoms of the disease.

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CHAPTER 2

The effects of modulating endogenous dopamine levels on the daily fluctuation of PER2 expression in the limbic forebrain.

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Abstract

The rhythms of expression of the clock protein, PER2, in several regions of the rat limbic forebrain are normally entrained by signals from the master clock in the SCN; however, they are also sensitive to a number of non-photic zeitgebers, which can uncouple the timing of these rhythms from the SCN. We have shown in the dorsal striatum that daily stimulation of D2 dopamine (DA) receptors is required for the normal daily fluctuation in PER2 expression in this region, but not in the SCN. Here, we examined the effect of manipulating endogenous levels of DA on the daily fluctuation in PER2 expression in several forebrain regions – specifically, the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), the central nucleus of the amygdala (CEA), the basolateral amygdala (BLA), and dentate gyrus (DG) – using additional brain sections collected in the experiments described in chapter 1. Depletion of DA using a unilateral 6-OHDA lesion blunted the normal daily peak in PER2 expression selectively in the BNSTov, but had no effect in the other regions examined. In contrast, inhibition of catecholamine synthesis in general using AMPT had no effect on the BNSTov or on the CEA, yet strongly suppressed the daily peak in PER2 expression in the BLA and DG. Daily stimulation of D2 receptors, but not D1 receptors, inverted the normal PER2 pattern in the BNSTov and CEA, but had no effect on the BLA or DG. Taken together, these findings indicate that DA signaling at D2 receptors modulates PER2 expression in the BNSTov and CEA, but likely via mechanisms that are different from those involved in the dorsal

striatum. In contrast, endogenous DA signaling does not play a role in rhythmic PER2 expression in the BLA or DG.

INTRODUCTION

In the preceding experiments, it was found that DA acting at D2 receptors is necessary for rhythmic PER2 expression in the dorsal striatum, but has no effect on the PER2 rhythm in the SCN of rats housed in a 12:12 LD cycle. In addition to the dorsal striatum, PER2 is expressed rhythmically in several other regions of the limbic forebrain that also receive DA projections from the midbrain. These regions include the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), the central amygdala (CEA), the basolateral amygdala (BLA), and the dentate gyrus (DG) (Fallon et al., 1978; Fallon and Moore, 1978; Freedman and Cassell, 1994; Shieh, 2003; Amir et al., 2004; Lamont et al., 2005a). As in the dorsal striatum, PER2 rhythms in these limbic forebrain regions are entrained by the SCN, but are also influenced by non-photic factors that do not otherwise affect the rhythm of the master clock. Notably, the PER2 rhythms in these regions do not respond uniformly to all non-photic factors. For example, the PER2 rhythms in the BNSTov and CEA, which are in phase with each other and with the rhythm in the SCN, are modulated by fluctuations in steroid hormone levels, such as corticosterone (Amir et al., 2004; Lamont et al., 2005a; Segall et al., 2006). Removal of the corticosterone rhythm in rats through adrenalectomy significantly blunts PER2 expression in the BNSTov and CEA, but has no effect on PER2 in the BLA, DG, dorsal striatum, or in the SCN.

Using indirect DA agonists such as cocaine or methamphetamine, a limited number of studies has implicated DA receptor activation in the modulation of clock gene expression in areas outside of the dorsal striatum, such as the

hippocampus and parietal cortex (e.g., Masubuchi et al., 2000; Uz et al., 2005). To determine whether changes in levels of endogenous DA *in vivo* contribute to rhythmic clock gene expression in the BNSTov, CEA, BLA, or DG and to follow-up on some preliminary work from this laboratory (Verwey et al., 2006), we studied brain sections from animals in each of the experiments described in the previous chapter. A portion of these results, specifically those demonstrating the effects of a unilateral 6-OHDA lesion and AMPT treatment on PER2 expression in the DG, were already presented in chapter 1, but are reviewed here.

METHODS

All of the methods and experimental procedures used were described in full in the previous chapter.

Microscopy. The BNSTov, CEA, BLA, and DG were visualized with a light microscope using a 20x objective and images were taken using a Sony XC-77 video camera, a Scion LG-3 frame grabber, and Image SXM software. 400 μm x 400 μm images were taken of each region on the right and left hemispheres of 5-6 representative sections. PER2-IR nuclei were counted in each image of a given structure. The mean number of IR nuclei in the right and left hemisphere was then calculated for each subject from the four images having the highest number of labeled nuclei out of all the images taken of that structure in a given hemisphere.

Statistical analysis of PER2-IR nuclei counts. In the experiments involving unilateral injections of 6-OHDA toxin into the MFB, data were analyzed only from

those rats with complete unilateral lesions, as confirmed by an absence of DAT staining in the dorsal striatum ipsilateral to the 6-OHDA injection (for an example of DAT staining following a unilateral injection of 6-OHDA toxin into the MFB, see Fig. 3 in chapter 1). Differences in PER2-IR nuclei counts were analyzed using mixed-model ANOVA with ZT and drug treatment group as between-subjects factors and hemisphere as a within-subjects factor. Differences in PER2 expression in the experiments involving injections of AMPT, chronic infusion of DA receptor antagonists, and injections of DA receptor agonists in naïve rats were analyzed using two-way ANOVA with ZT and treatment group as between-subjects factors. Alpha level was set at 0.05 for all tests. Significant interactions were analyzed further using one-way ANOVA and Bonferroni-corrected post-hoc comparisons, or t-tests where appropriate.

RESULTS

Table 1 summarizes the effects of each experimental manipulation on PER2 expression at ZT1 and ZT13 in the BNSTov, CEA, BLA, and DG. For comparison purposes, the effects of these manipulations on PER2 in the dorsal striatum that were described in chapter 1 are also reported in Table 1. The results of each experiment are described in brief below.

Table 1.

manipulation \ *region*	SCN	dorsal striatum	BNSTov	CEA	BLA	DG
unilateral 6-OHDA lesion	no effect	§ lesion blunts PER2 peak	§ lesion blunts PER2 peak	no effect	no effect	no effect
AMPT	no effect	§ AMPT blunts PER2 peak	no effect	§ AMPT decreases PER2 at trough (ZT1)	§ AMPT blunts PER2 peak	§ AMPT blunts PER2 peak
chronic infusion of raclopride or SCH 23390	no effect	§ raclopride blunts PER2 peak	no effect	no effect	§ raclopride increases PER2 at trough (ZT13)	no effect
6-OHDA lesion + daily quinpirole or SKF 81297 injection (injection at ZT1)	no effect of 6-OHDA lesion; no effect of quinpirole or SKF 81297 injection	§ lesion blunts PER2 peak; § quinpirole inverts daily PER2 pattern on the lesioned side	§ lesion blunts PER2 peak; § quinpirole inverts daily PER2 pattern on both lesioned and intact sides	no effect of 6-OHDA lesion; § quinpirole inverts daily PER2 pattern on both lesioned and intact sides	no effect of 6-OHDA lesion; § quinpirole decreases PER2 peak on intact side; § SKF 81297 increases PER2 at trough (ZT13) on lesioned side	no effect of 6-OHDA lesion; no effect of quinpirole or SKF 81297 injection
naive + daily quinpirole or SKF 81297 injection (ZT1)	no effect	no effect	§ quinpirole increases PER2 at trough (ZT1)	§ quinpirole increases PER2 at trough (ZT1)	no effect	no effect
6-OHDA lesion + chronic *quinpirole or* SKF 81297 infusion	no effect of 6-OHDA lesion; no effect of quinpirole or SKF 81297 infusion	§ lesion blunts PER2 peak; no effect of quinpirole or SKF 81297 infusion	§ lesion blunts PER2 peak; no effect of quinpirole or SKF 81297 infusion	no effect of 6-OHDA lesion; no effect of quinpirole or SKF 81297 infusion	no effect of 6-OHDA lesion; no effect of quinpirole or SKF 81297 infusion	no effect of 6-OHDA lesion; no effect of quinpirole or SKF 81297 infusion

Table 1. Summary of the effects of each experimental manipulation described in Chapters 1 and 2 on the daily fluctuation of PER2 expression in the limbic forebrain and SCN. § indicates a statistically significant ($p < 0.05$) effect.

PER2 expression after a unilateral 6-OHDA lesion

As mentioned above, in the BNSTov and CEA, PER2 rhythms are synchronized and in phase with the rhythm of PER2 in the SCN, which peaks at the beginning of the dark phase (Amir et al., 2004; Lamont et al., 2005a). PER2 rhythms in the BLA and DG are synchronized with each other and with the rhythm in the dorsal striatum, and peak at the beginning of the light phase (approximately 12 h out-of-phase with the peak of the SCN PER2 rhythm) (Lamont et al., 2005a).

Figure 1 shows the mean number of PER2-IR nuclei in the BNSTov, CEA, BLA, and DG at four time points in rats killed 14 days after a unilateral 6-OHDA injection into the MFB. The effect of the lesion on PER2 expression in each of these regions is summarized in row 1 of Table 1. In the BNSTov (Fig. 1a; column 1 of Table 1), a unilateral 6-OHDA lesion significantly blunted the peak of the PER2 rhythm in the hemisphere ipsilateral to the lesion but did not affect the rhythm in the BNSTov of the intact hemisphere (ZT x lesion interaction, $F(3, 18) = 8.62$, $p < 0.01$); specifically, PER2 expression in the lesioned hemisphere was significantly decreased at ZT7 ($t(4) = 4.26$, $p < 0.01$) and ZT13 ($t(5) = 4.10$, $p < 0.01$).

In the CEA (Fig. 1b; column 2 of Table 1), PER2 expression was slightly reduced in the lesioned hemisphere compared to the intact hemisphere. Although statistical analysis indicated a significant main effect of lesion ($F(3, 18) = 11.01$, $p < 0.01$), there was no interaction of the lesion effect with ZT (ZT x

lesion, F (3, 18) = 0.52, n.s.), and this reduction in PER2 expression on the lesioned side was not significant when comparisons were made at individual time points. In the BLA (Fig. 1c; Table 1, column 3) and in the DG (Fig. 1d; Table 1, column 4), no effect of a unilateral 6-OHDA lesion on PER2 was observed in either hemisphere.

Effect of tyrosine hydroxylase inhibition on PER2

Having found that a unilateral 6-OHDA injection into the MFB blunts PER2 expression in the BNSTov and CEA, but not in the BLA or DG, we next examined whether inhibition of catecholamine synthesis in general using AMPT affects PER2 expression in these forebrain regions. As described in the previous chapter, three i.p. injections of AMPT or saline were given over the course of one day (at 4-h intervals) in this experiment and rats were killed at either ZT13, 5 h after the last injection, or ZT1, 17 h after the last injection. This treatment with AMPT profoundly decreased DA content in the dorsal striatum to approximately 10% of saline-injected levels (see Fig. 7 in chapter 1 for striatal DA content in saline- and AMPT-treated rats; DA levels in other regions were not assessed).

The results of this experiment are summarized in row 2 of Table 1. Fig. 2 shows the mean number of PER2-IR nuclei in the BNSTov, CEA, BLA, and DG in AMPT- and saline-treated rats. In the BNSTov (Fig. 2a), the PER2 rhythm was not affected by AMPT treatment. In the CEA (Fig. 2b), AMPT reduced PER2 expression at ZT1 ($t(6) = 2.81$, $p < 0.05$), but had no effect on the normal increase in PER2 expression at ZT13.

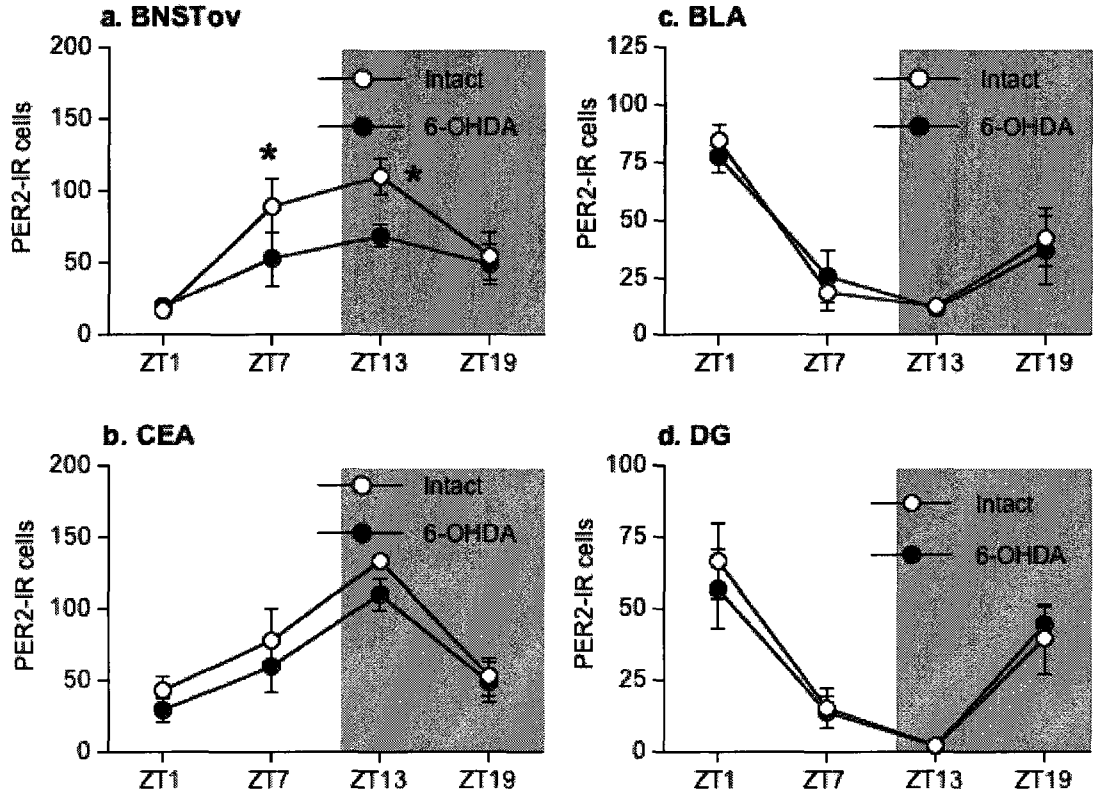

Figure 1.

A unilateral 6-OHDA injection into the medial forebrain bundle decreases the normal peak of PER2 expression in the BNSTov (a) ipsilateral to the 6-OHDA injection, but has no effect on the normal daily fluctuation in PER2 expression in the CEA (b), the BLA (c), or the DG (d). For each brain region, data are shown as mean PER2-immunoreactive (IR) nuclei ± SEM per ZT in the intact hemisphere (open circles) and lesioned hemisphere (black circles); n = 5-6 per ZT; * indicates $p < 0.05$ compared to intact hemisphere.

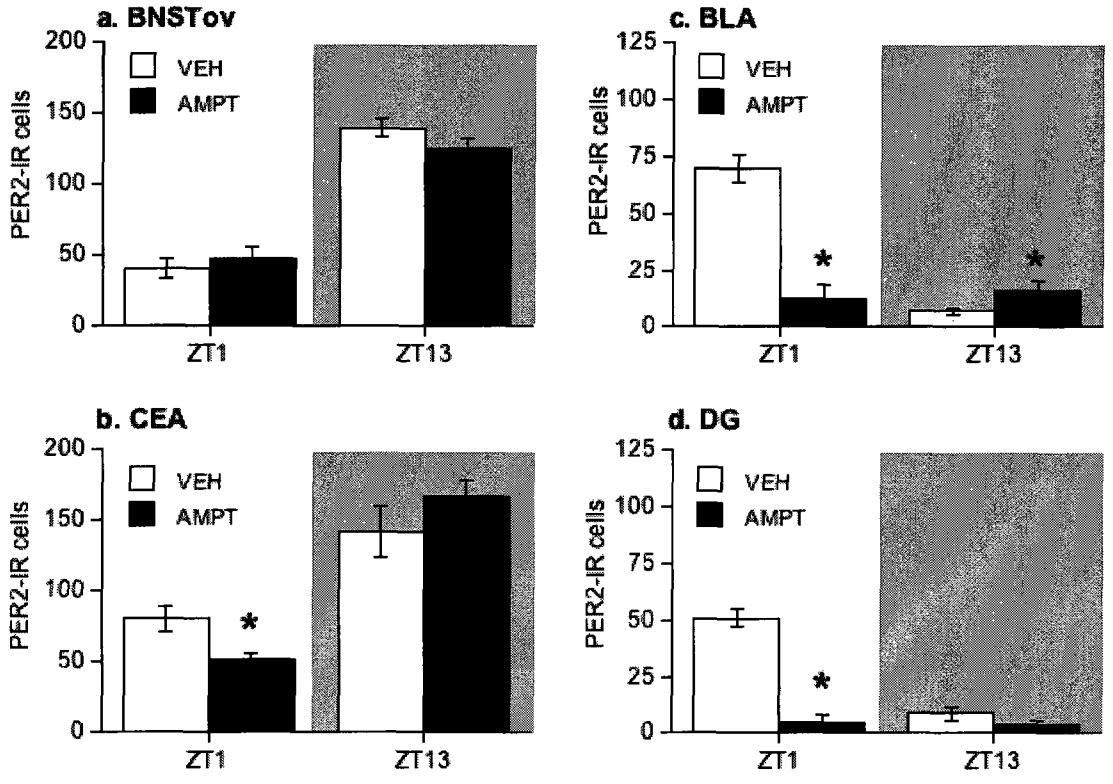

Figure 2.

Inhibition of catecholamine synthesis using the tyrosine hydroxylase inhibitor, AMPT, does not affect the normal daily pattern of PER2 in the BNSTov (a) or in the CEA (b), but strongly suppresses the daily peak in PER2 expression at ZT1 in the BLA (c) and DG (d). For each brain region, data are shown as mean PER2-immunoreactive (IR) nuclei ± SEM per ZT in the vehicle-injected groups (open bars) and AMPT-injected groups (black bars); n = 4 per treatment group per ZT; * indicates $p < 0.05$ compared to vehicle.

In the BLA and DG, AMPT treatment had a marked effect. As shown in Fig. 2c, PER2 expression in the BLA was dramatically decreased at ZT1 in AMPT-treated rats ($t(6) = 6.56$, $p < 0.01$) and was significantly increased at ZT13 compared to saline-treated rats ($t(6) = -2.57$, $p < 0.05$). In the DG (Fig. 2d), PER2 expression was strongly reduced at ZT1 in AMPT-treated rats ($t(6) = 8.70$, $p < 0.01$) but there was no difference between groups at ZT13. Thus, in contrast to the selective effect of a unilateral 6-OHDA lesion on PER2 expression in the BNSTov, AMPT treatment did not affect the normal daily fluctuation in PER2 expression in the BNSTov or in the CEA but strongly suppressed the daily increase in PER2 expression at ZT1 in the BLA and DG.

Effect of chronic infusion of DA receptor antagonists on PER2

We examined PER2 expression in the forebrain of naïve rats given chronic, 24-h infusion of the D2 DA receptor antagonist, raclopride (2 mg/kg/day), or the D1 DA receptor antagonist, SCH 23390 (0.5 mg/kg/day), via osmotic minipump for 5 days. These results are summarized in row 3 of Table 1 and the number of PER2-IR nuclei in each region is shown in Fig. 3. In the BNSTov (Fig. 3a) and CEA (Fig. 3b), neither raclopride nor SCH 23390 infusion significantly affected the daily fluctuation of PER2. In the BLA (Fig. 3c), chronic infusion of raclopride significantly increased PER2 expression at ZT13 in comparison to the SCH 23390 and sham groups ($F(2, 9) = 13.39$, $p < 0.01$). In the DG (Fig. 3d), no effect of either SCH 23390 or raclopride was observed.

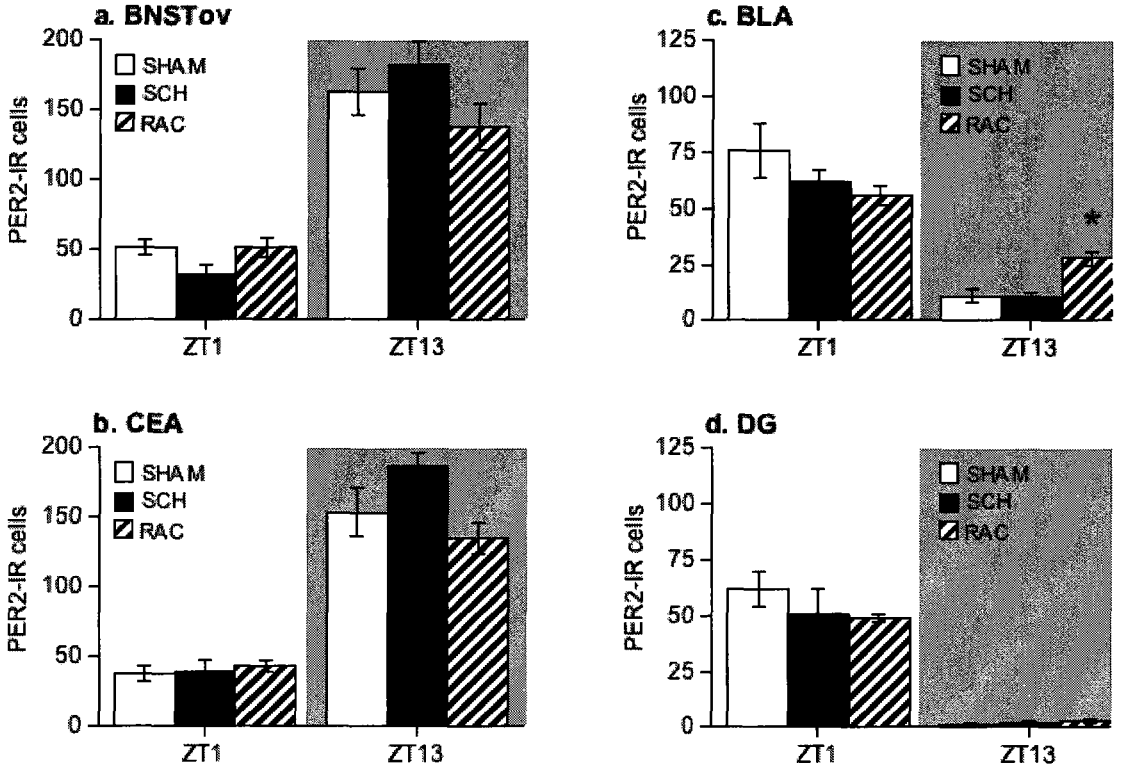

Figure 3.

In the BNSTov (a) and the CEA (b), chronic infusion of the D1 receptor-selective antagonist, SCH 23390 (0.5 mg/kg/24 h; black bars), or the D2 receptor-selective antagonist, raclopride (2 mg/kg/24 h; hatched bars), via osmotic minipump for 5 days does not affect the normal daily fluctuation in PER2 expression. In the BLA (c), raclopride increases PER2 expression at ZT13 but does not affect the normal peak of PER2 at ZT1. In the DG (d), there is no effect of either raclopride or SCH 23390 infusion. For each brain region, data are shown as mean PER2-immunoreactive (IR) nuclei ± SEM per ZT in each treatment group; n = 4 per treatment group per ZT; * indicates $p < 0.05$ compared to sham.

Effect of daily quinpirole or SKF 81297 injections on PER2

Unilateral 6-OHDA lesioned rats

We then examined the effect of daily injections of the D2/3 DA receptor agonist, quinpirole (0.5 mg/kg), the D1 agonist, SKF 81297 (1 mg/kg), or saline (1 ml/kg), given at ZT1 for 10 days, on PER2 expression in the BNSTov, CEA, BLA, and DG of rats with a unilateral 6-OHDA lesion. In the previous chapter, it was reported that in 6-OHDA-lesioned rats, daily injections of quinpirole at ZT1 restored and entrained a daily fluctuation in PER2 expression in the dorsal striatum ipsilateral to a 6-OHDA lesion, such that PER2 expression was increased at ZT13 on the lesioned side, whereas PER2 expression in the intact striatum and in the SCN was unaffected.

The effects of these daily injections on PER2 expression in the forebrain are summarized in row 4 of Table 1 and are depicted in Fig. 4. In the BNSTov (Fig. 4a), a unilateral 6-OHDA lesion significantly reduced the daily peak in PER2 expression at ZT13 in the lesioned hemisphere, consistent with the findings of the first 6-OHDA experiment described above, and daily injections of saline at ZT1 did not affect this reduction in PER2. Daily injection of quinpirole given at ZT1 (Fig. 4b) increased PER2 expression at ZT1 in the BNSTov in both the lesioned ($F(2, 14) = 3.92$, $p < 0.05$) and intact hemispheres ($F(2, 14) = 11.55$, $p < 0.01$) and decreased PER2 at ZT13 in both hemispheres (lesioned side, $F(2, 13) = 6.20$, $p < 0.05$; intact side, $F(2, 13) = 17.87$, $p < 0.01$) in comparison to the SKF 81297- and saline injected-groups. Overall, quinpirole injections inverted the

normal daily fluctuation in PER2 expression in the BNSTov, such that PER2 expression was greater at ZT1, the time of the daily quinpirole injection, than at ZT13 ($p < 0.05$), on both the 6-OHDA-lesioned and intact sides. In contrast, daily injection of SKF 81297 (Fig. 4c) had no effect on PER2 in the BNSTov in either the 6-OHDA-lesioned or intact hemisphere, indicating that PER2 expression in this region is not modulated by D1 receptor stimulation.

In the CEA (Fig. 4d), a unilateral 6-OHDA lesion did not affect the normal daily fluctuation in PER2 expression nor did daily saline injections at ZT1 affect this normal pattern. Quinpirole injections at ZT1 (e) did not affect the level of PER2 expression at ZT1 in either the 6-OHDA-lesioned or intact hemisphere but significantly reduced the normal evening peak of PER2 expression at ZT13 in both hemispheres in comparison to the SKF 81297 and saline groups (lesioned side, $F(2, 13) = 9.16$, $p < 0.01$; intact side, $F(2, 13) = 8.30$, $p < 0.01$). Daily injection of SKF 81297 at ZT1 (f) had no effect on PER2 expression in the CEA of either hemisphere. Taken together, these findings suggest that PER2 expression in the CEA is modulated by D2 receptor stimulation and not by D1 receptors. However, because quinpirole injections did not affect PER2 expression in this region at ZT1, the time of the daily injection, the magnitude of this influence of D2 receptor stimulation on PER2 in the CEA would appear to be less than the effect in the BNSTov, in which quinpirole actually inverted the daily pattern of expression.

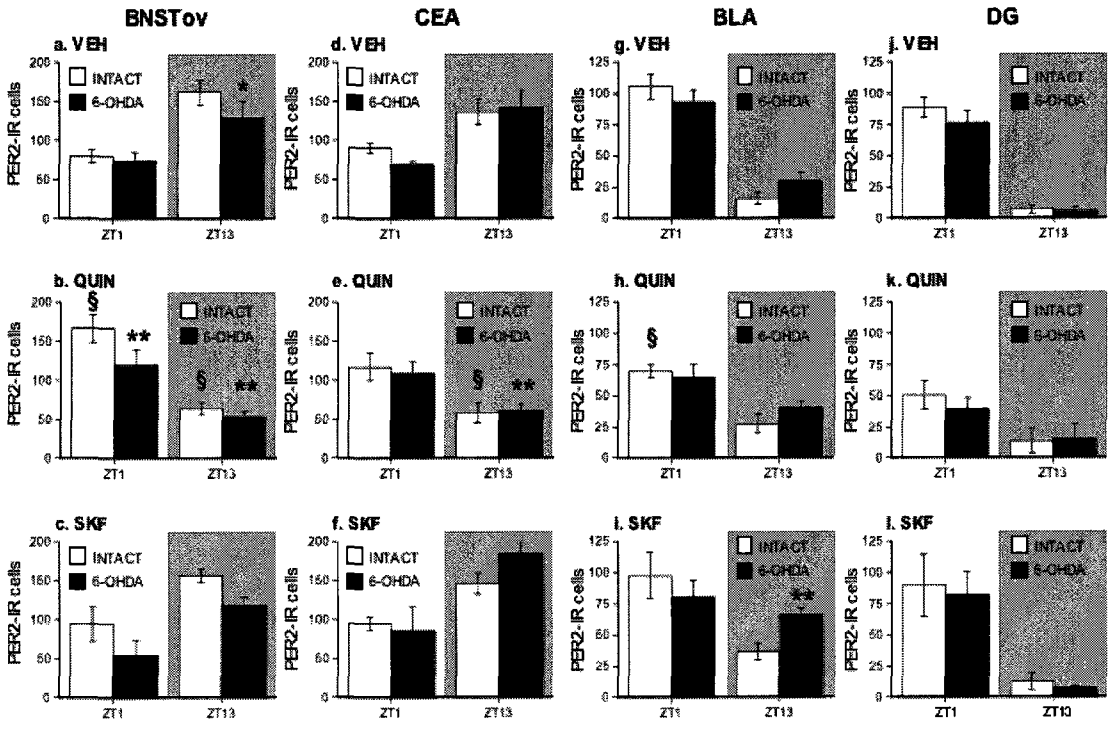

Figure 4.

(a) In the BNSTov, a 6-OHDA lesion significantly decreases the normal peak of PER2 at ZT13; (b) daily injection of quinpirole (0.5 mg/kg) at ZT1 increases PER2 at ZT1 in both the lesioned and intact hemispheres, and decreases PER2 at ZT13; (c) daily injection of SKF 81297 (1 mg/kg) at ZT1 has no effect. (d) In the CEA, PER2 expression is unaffected by a unilateral 6-OHDA lesion; (e) daily injection of quinpirole at ZT1 significantly decreases PER2 at ZT13 in both the lesioned and intact hemispheres, whereas daily injections of SKF 81297 (f) have no effect. (g) In the BLA, PER2 expression is unaffected by a unilateral 6-OHDA lesion; (h) daily injection of quinpirole at ZT1 decreases PER2 at ZT1 in the intact hemisphere compared to saline control, whereas daily injections of SKF 81297 (i) increase PER2 at ZT13 in the 6-OHDA-lesioned hemisphere compared to saline control. (j) In the DG, PER2 expression is unaffected by a unilateral 6-OHDA lesion, (k) by daily injections of quinpirole, (l) or by daily injections of SKF 81297. For each region and treatment group, data are shown as mean PER2-immunoreactive (IR) nuclei ± SEM per ZT in the intact hemisphere (open bars) and lesioned hemisphere (black bars); saline n = 7-8 per ZT; quinpirole n = 6 per ZT; SKF n = 3 per ZT; * $p < 0.05$ compared to intact hemisphere; ** $p < 0.05$ compared to 6-OHDA hemisphere in saline condition; § $p < 0.05$ compared to intact hemisphere in saline condition.

In the BLA (Fig. 4g), the normal daily fluctuation in PER2 expression was unaffected by a unilateral 6-OHDA lesion and by daily saline injection at ZT1. Daily injections of quinpirole at ZT1 (h) did not affect PER2 expression in the 6-OHDA-lesioned hemisphere, but decreased the normal peak of PER2 expression at ZT1 in the intact hemisphere compared to the saline group ($F(2, 14) = 3.91$, $p < 0.05$). Daily injection of SKF 81297 at ZT1 (i) increased PER2 expression at ZT13 in the 6-OHDA-lesioned hemisphere in comparison to the saline group ($F(2, 13) = 5.30$, $p < 0.05$).

In the DG (Fig. 4j), a unilateral 6-OHDA lesion did not affect the normal PER2 fluctuation nor did daily saline injections at ZT1 affect this pattern. Daily injection of quinpirole at ZT1 (k) appeared to reduce PER2 expression at ZT1 in both the 6-OHDA-lesioned and intact hemispheres in comparison to the saline group, but this effect was not significant. Daily injection of SKF 81297 at ZT1 (l) had no effect on PER2 in either the 6-OHDA-lesioned or the intact hemispheres.

Naïve rats

It was shown in chapter1 that in naïve rats, the normal PER2 rhythms in the dorsal striatum and SCN were unaffected by daily injections of quinpirole or SKF 81297 at ZT1. We examined in these same rats the effect of daily quinpirole and SKF 81297 injections, given at ZT1 for 10 days, on the PER2 rhythms in the BNSTov, CEA, BLA, and DG. These data are summarized in row 5 of Table 1 and shown in Figure 5. In the BNSTov (Fig. 5a), daily quinpirole injections at ZT1 significantly increased PER2 expression at ZT1 ($F(2, 6) =$

43.95, $p < 0.01$), the time of the daily injection, but did not affect PER2 levels at ZT13, whereas SKF 81297 injections had no effect at either time point. Similarly, in the CEA (b), quinpirole injections at ZT1 significantly increased PER2 expression at ZT1 ($F(2, 6) = 8.27$, $p < 0.05$), the time of the daily injection, but had no effect at ZT13. Daily injection of SKF 81297 at ZT1 did not affect the normal daily fluctuation in PER2 expression in the CEA. In the BLA (c) and the DG (d), the normal PER2 pattern was unaffected by daily injections of quinpirole or SKF 81297 at ZT1. Thus, quinpirole injections at ZT1 in naïve rats increased PER2 expression in the BNSTov and CEA at ZT1, when PER2 levels normally reach their trough in these regions, and thereby blunted the normal daily fluctuation in PER2. In contrast, neither daily quinpirole injection nor SKF 81297 at ZT1 affected the daily fluctuation in PER2 expression in the BLA or DG.

Effect of chronic infusion of DA receptor agonists on PER2

We next examined whether chronic infusion of quinpirole (0.5 mg/kg/day) or SKF 81297 (1 mg/kg/day) via osmotic minipump for 10 days would affect the rhythms of PER2 expression in the forebrain of rats with unilateral 6-OHDA lesions. The effects of these treatments are summarized in row 6 of Table 1 and shown in Figure 6. In the BNSTov of rats with a unilateral 6-OHDA lesion (Fig. 6a), PER2 expression was significantly reduced in the 6-OHDA-lesioned hemisphere and this pattern was unaffected by sham minipump surgery. Chronic infusion of quinpirole (b) or SKF 81927 (c) for 10 days did not affect the daily fluctuation in PER2 in the BNSTov of either the 6-OHDA-lesioned or intact

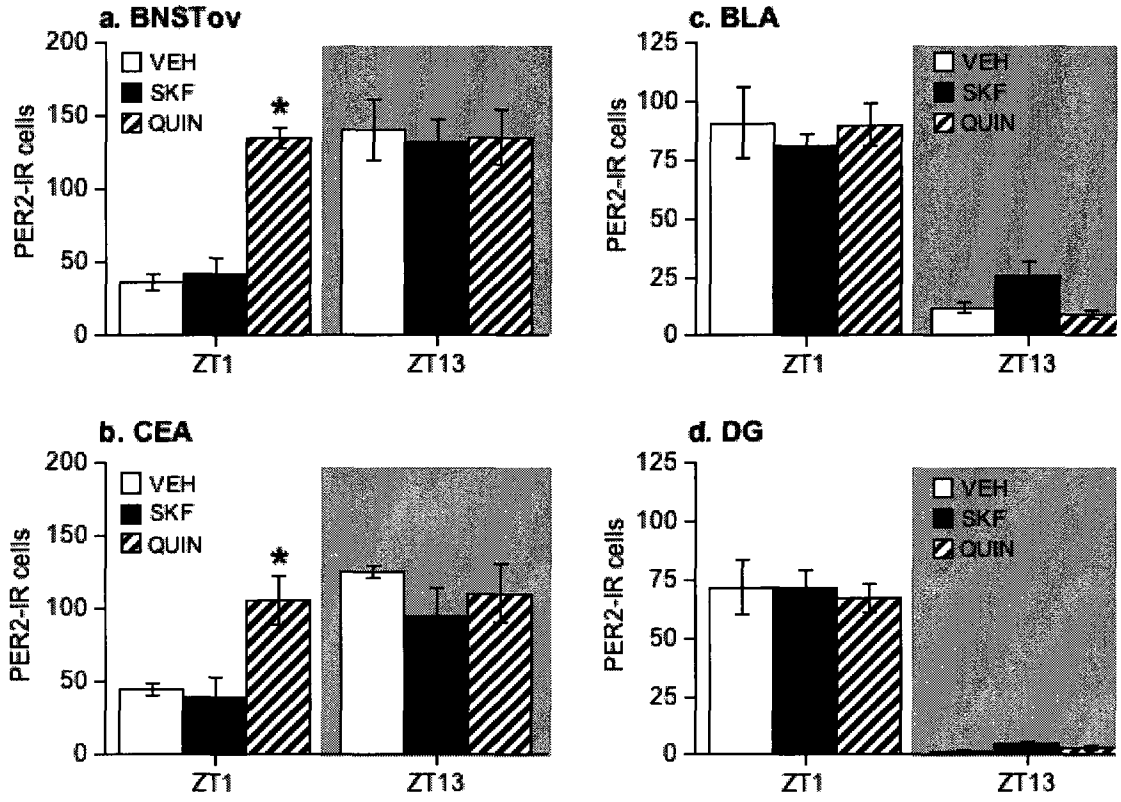

Figure 5.

In naïve rats, the normal daily fluctuation in PER2 in the BNSTov (a) and CEA (b) is blunted by daily injections at ZT1 of quinpirole (0.5 mg/kg; hatched bars), but not by SKF 81297 (1 mg/kg; black bars) or saline (1 mL/kg; open bars): daily quinpirole injections significantly increased PER2 expression at ZT1, the normal trough of the PER2 fluctuation in the BNSTov and CEA. In the BLA (c) and DG (d), daily injections of quinpirole or SKF 81297 have no effect on the normal PER2 fluctuations. For each region, data are shown as mean PER2-immunoreactive (IR) nuclei ± SEM per ZT; n = 3 per treatment group per ZT; * indicates $p < 0.05$ compared to saline.

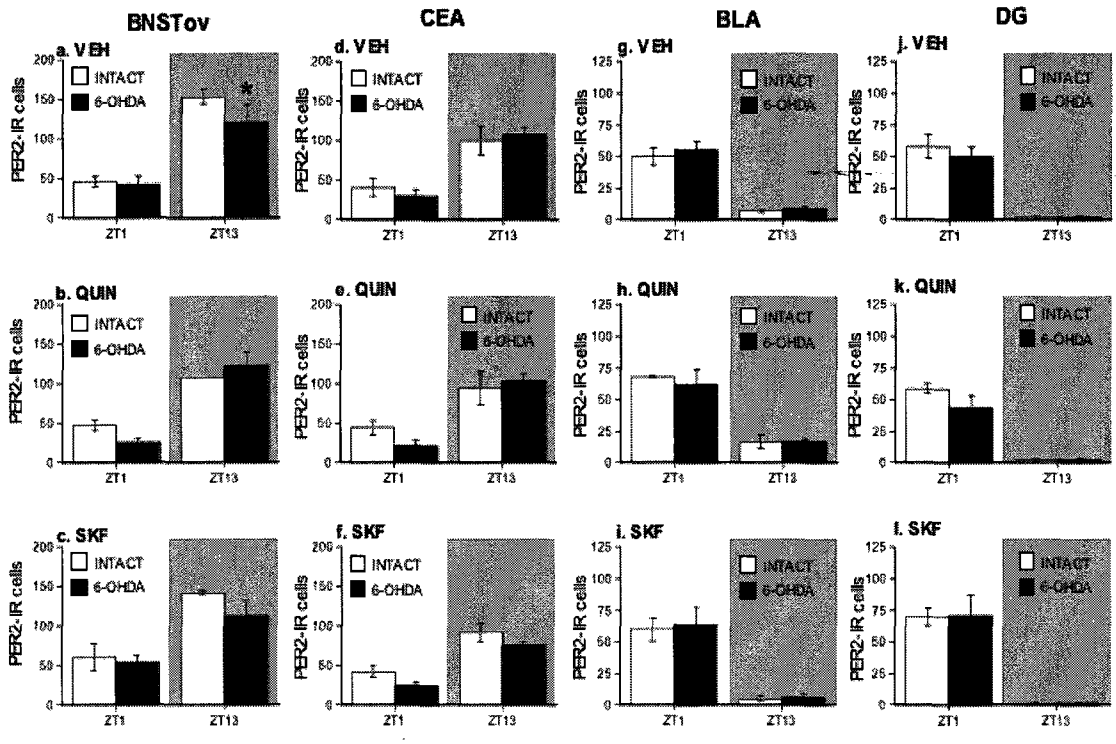

Figure 6.

(a) In the BNSTov, a unilateral 6-OHDA lesion decreases the daily peak of PER2 at ZT13; (b) chronic infusion via osmotic minipump of quinpirole (0.5 mg/kg/24 h) or (c) SKF 81297 (1 mg/kg/24 h) has no effect on the daily fluctuation in PER2 expression in the BNSTov. In the CEA (d, e, f), the BLA (g, h, i), and the DG (j, k, l), the daily fluctuation in PER2 expression is unaffected by a unilateral 6-OHDA lesion or by chronic infusion of quinpirole or SKF 81297. For each treatment group, data are shown as mean PER2-immunoreactive (IR) nuclei ± SEM per ZT in the intact hemisphere (open bars) and lesioned hemisphere (black bars). Sham n = 3-5 per ZT; quinpirole pump n = 2-3 per ZT; SKF pump n = 2-3 per ZT; * indicates $p < 0.05$ compared to intact hemisphere.

hemispheres. In the CEA (d, e, f), BLA (g, h, i), and DG (j, k, l), PER2 expression was unaffected by a unilateral 6-OHDA lesion, or by chronic infusion of quinpirole or SKF 81297.

DISCUSSION

Only a limited number of studies have previously shown an effect of changes in catecholamine activity on the expression of clock genes in areas of the brain outside of the SCN. In the present experiments, the effect of manipulating endogenous DA signaling on rhythmic PER2 expression was specifically examined in regions of the limbic forebrain that are innervated by fibers from midbrain DA cell groups and that have been previously demonstrated to be sensitive to several types of non-photic entraining stimuli. Unlike the consistent and marked impact in the dorsal striatum of DA depletion and restoration of activity at D2 DA receptors on PER2 expression, these same manipulations produced varying effects on PER2 in the BNSTov, CEA, BLA, and DG. Unilateral depletion of DA using a 6-OHDA lesion blunted the daily peak of PER2 expression in the BNSTov, whereas it had no effect in the CEA, BLA, or DG. Furthermore, injections of the D2/3 DA receptor agonist, quinpirole, blunted the normal daily fluctuation in PER2 expression in the BNSTov and in the CEA, but did not affect the BLA or DG. In contrast, global inhibition of catecholamine synthesis using AMPT injections suppressed PER2 in the BLA and DG without affecting the normal fluctuation of PER2 in the BNSTov or CEA. Overall, the effects in the BNSTov and CEA resemble some of those observed in the dorsal striatum and suggest that D2/3 receptor activity modulates the PER2 rhythm in

these regions whereas the results in the BLA and DG indicate that DA is not the primary neurotransmitter regulating PER2 rhythms in these nuclei.

Because AMPT blocks the synthesis of not only DA but also noradrenaline (NA), the reduction in PER2 in the BLA and DG could be attributable to changes in NA levels following AMPT treatment. A potential role for NA signaling in modulating PER2 expression in the dorsal striatum was also identified in chapter 1. As previously noted, there is evidence indicating that NA innervation of the forebrain is required for normal circadian rhythms of activity and clock gene expression in mice (Warnecke et al., 2005; Gonzalez and Aston-Jones, 2006, 2008). Both the BLA and DG are innervated by NA fibers arising in part from locus coeruleus (Fallon et al., 1978; Loy et al., 1980; Asan, 1998; Buffalari and Grace, 2007), whereas the BNSTov and lateral CEA receive only sparse NA projections. Thus, a reduction in NA levels and not DA could perhaps account for these region-specific decreases in PER2 as a result of AMPT treatment. The lack of any clear effect of DA receptor agonist injections or infusion of DA receptor antagonists on PER2 expression is also consistent with the idea that DA is not a critical signal in the regulation of PER2 in these regions.

In the BNSTov, only a 6-OHDA lesion and not AMPT injections or infusion of DA receptor antagonists reduced PER2 expression, which suggests that this reduction in PER2 is not directly attributable to a depletion of DA (which, at least in the dorsal striatum, is reported to occur within 2-3 days of an intra-MFB 6-OHDA injection (Neve et al., 1982; Altar et al., 1987)). Instead, this effect might involve changes in a second system that develop as a consequence of DA fiber

loss. One candidate system is corticotrophin-releasing hormone (CRH), which is expressed in both the BNSTov and CEA. A 6-OHDA injection into the MFB decreases CRH mRNA in the BNSTov and central amygdala of the lesioned hemisphere (Day et al., 2002); this decrease in CRH mRNA is specific to the BNSTov and CEA, as no changes were found in other CRH-containing regions, including the ventrolateral BNST. Preliminary studies from our lab have shown that PER2 expression in the BNSTov is sensitive to CRH levels, and suppression of CRH protein synthesis using injections of double stranded ribonucleic acid (dsRNA) into this region blunts the normal peak of PER2 expression in the BNSTov at ZT13 (Bhargava et al., 2006). DA afferents to the BNSTov form synapses with cell populations known to express CRH (Shimada et al., 1989; Bayer et al., 1991; Phelix et al., 1994) and these inputs are thought to positively regulate CRH tone in this region (Day et al., 2002). Thus, a reduction in CRH expression resulting from the 6-OHDA-induced decrease in DA input could underlie the reduction in PER2 expression selectively in the BNSTov.

One difficulty with this interpretation is that a unilateral 6-OHDA lesion had little effect on PER2 in the CEA, even though the lateral CEA also contains CRH-positive cells and expression of this peptide in the CEA is decreased following a 6-OHDA lesion of the MFB. This finding, together with the minimal reduction of PER2 at ZT1 in the CEA after AMPT treatment, implies that rhythmic PER2 expression in this region does not depend on catecholamine activity or CRH levels to the same extent as does PER2 in the BNSTov. This difference in the effect of 6-OHDA lesioning on PER2 between the BNSTov and CEA is unusual,

because several studies concerning the impact of non-photic stimuli on PER2 rhythms in these regions have shown that the BNSTov and CEA typically respond in a similar way to a given stimulus, such as manipulations of steroid hormones or restricted feeding schedules.

The finding that quinpirole injections blunted the daily fluctuation in PER2 expression in both the BNSTov and CEA, however, clearly indicates that stimulation of D2 DA receptors can modulate PER2 in these regions. Interestingly, PER2 expression in the BNSTov and CEA was increased in both lesioned and intact hemispheres of 6-OHDA-lesioned rats and in naïve rats around the time of the quinpirole injection (ZT1), which is the normal trough of the PER2 rhythm in these regions. The consistency of this effect indicates that the action of quinpirole in these regions occurs independently of DA depletion, unlike the dorsal striatum in which PER2 was affected only on the lesioned side. This finding might indicate that the specific mechanisms by which quinpirole modulates PER2 in the BNSTov and CEA are not identical to those in the dorsal striatum. D2 receptors are found in both the BNSTov and CEA and, within the CEA, these receptors are segregated primarily to the lateral region where PER2-, CRH-, and ENK-expressing cells are also found (Day et al., 2002; Eliava et al., 2003; Lamont et al., 2005a). It is known that DA afferents in both of these regions regulate glutamatergic activity (Liu et al., 2004; Kash et al., 2008); thus, quinpirole could act at DA receptors to modulate PER2 expression via glutamatergic mechanisms. In addition, a role for CRH in mediating the excitatory actions of DA on glutamatergic activity in both the BNSTov and CEA has been

described, which could further support the relation between DA, CRH, and PER2 in these regions suggested above (Liu et al., 2004; Kash et al., 2008).

Taken together, these results indicate that changes in endogenous DA levels affect the daily fluctuation in PER2 in the BNSTov, and do so to a lesser extent in the CEA, but have minimal impact on PER2 expression in the BLA and DG. Although D1 and D2 DA receptors are found within the BLA and DG, changes in DA levels did not consistently affect rhythmic PER2 expression in these structures. In the BNSTov and CEA, the mechanisms by which DA modulates PER2 expression are unlikely to be the same as those operating in the dorsal striatum, given that acute DA depletion using AMPT did not affect the normal PER2 rhythm in these regions. Furthermore, although stimulation of D2 receptors and not D1 receptors reversed the normal rhythm of PER2 expression in these regions, this effect was not restricted to only the DA-depleted hemisphere as it was in the dorsal striatum. More generally, these results also suggest that, in addition to the dorsal striatum, the normal rhythms of clock gene expression in the BNSTov and CEA are potentially vulnerable to certain pathologies affecting catecholamine systems, such as substance abuse and Parkinson's disease (PD). Given the involvement of the BNSTov and CEA in the regulation of a variety of physiological and behavioral functions, such as activity of the hypothalamic-pituitary-adrenal axis, it is possible that, as a result of changes in DA tone, a disruption in the circadian activity of these areas could contribute to some of the circadian symptoms reported to occur in PD, such as

an abnormal rhythm of cortisol secretion (Bruguerolle and Simon, 2002; Willis, 2008).

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SECTION 2: Daily injections of morphine and withdrawal of morphine disrupt the daily pattern of wheel running and expression of the clock protein, PERIOD2, in regions of the limbic forebrain but not in the suprachiasmatic nucleus.

In Chapter 3, timed, daily injections of morphine were used to examine the effect of manipulating DA and other catecholamines on PER2 expression in the suprachiasmatic nucleus (SCN) and regions of the limbic forebrain. PER2 expression in the SCN and the dorsal striatum, BNSTov, CEA, BLA, and DG was examined in the morning and the evening at three different intervals following three weeks of morphine injections given daily during the mid-light phase: (1) within 24 h after the last morphine injection, (2) within 46-58 h after the last morphine injection, (3) and 7 days after the last morphine injection. Furthermore, to determine if treatments that alleviate some symptoms associated with opiate withdrawal also modify the expression of PER2 in the forebrain (Harris and Aston-Jones, 1994), an additional group was given a daily injection of the D2/3 agonist, quinpirole, for two days beginning the evening after the last morphine injection.

CONTRIBUTION OF AUTHORS – CHAPTER 3

Dr. Shimon Amir, a supervisor and a principle investigator, contributed to the design of the experiments in chapter 3. He also contributed to the writing and editing of this manuscript.

Dr. Jane Stewart, a supervisor and a principle investigator, contributed to the design of the experiments in chapter 3. She also contributed to the writing and editing of this manuscript.

Pamela Cassidy is an undergraduate student who assisted with the execution of the experiments described in chapter 3.

Sarah Matthewson was an undergraduate student who assisted with the execution of the experiments described in Chapter 3.

Barry Robinson is the laboratory manager for Dr. Amir and developed the immunohistochemical protocols used in chapter 3.

CHAPTER 3

Daily morphine injections and withdrawal disrupt the circadian rhythm of wheel running and expression of the clock protein PERIOD2 in the rat limbic forebrain.

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ABSTRACT

Symptoms of opiate withdrawal include disturbances in circadian rhythms. Other motivationally salient events, such as restricted feeding schedules, have been shown to affect rhythms of locomotor activity and the expression of clock genes in several forebrain regions without affecting the master clock in the suprachiasmatic nucleus (SCN). Here, we examined the effect of daily, mid-morning morphine injections (5-40mg/kg) and a 1-week withdrawal period on rhythms of wheel running and expression of the clock protein PERIOD2 (PER2) in the SCN, dorsolateral striatum, and limbic forebrain in rats housed in a 12:12 light-dark cycle. Morphine injections suppressed wheel running in the dark phase, and elicited a modest amount of anticipatory running 3 h before the daily injection. Morphine withdrawal blunted the normal nighttime peak of PER2 expression in the oval nucleus of the bed nucleus of the stria terminalis (BNSTov) and central nucleus of the amygdala (CEA), without affecting PER2 expression in the SCN. In dorsolateral striatum and basolateral nucleus of the amygdala (BLA), the normal pattern of PER2 expression was inverted within the first 24 h of withdrawal, but recovered after 1 week in withdrawal. Injections of the dopamine D2/D3 agonist, quinpirole (0.5 mg/kg), given after the last morphine injection, restored the normal pattern of PER2 expression in BNSTov and CEA, but not in dorsolateral striatum or BLA. These findings demonstrate that daily morphine injections and withdrawal disrupt normal rhythms of wheel running and PER2 expression in forebrain regions outside of the SCN, and that changes in dopaminergic signaling contribute to these effects.

INTRODUCTION

Opiate withdrawal is characterized by profound physiological and motivational deficits that include disturbances in circadian rhythms of activity, hormone secretion, and expression of clock genes (Li et al., 2009a; Li et al., 2009b). Disruptions in sleeping and diurnal activity patterns are common symptoms reported in humans undergoing withdrawal and are thought to contribute to increased risk of relapse (Howe et al., 1980; Stinus et al., 1998; Shi et al., 2007). Recent findings suggest that opiate exposure and withdrawal profoundly affect rhythms of adrenocorticotropic hormone (ACTH) and cortisol levels, as well as the transcription of the *Period1* (*Per1*) and *Period2* (*Per2*) clock genes for at least one month in recently abstinent opiate users (Li et al., 2009b).

Circadian timing is generated at the molecular level by a core group of genes that interact in a transcriptional feedback loop (for a review, see Reppert and Weaver, 2002). Two basic helix-loop-helix-containing transcription factors, CLOCK and BMAL, form heterodimers that positively regulate the transcription of the *Period* genes and *Cryptochrome* genes (Hogenesch et al., 1998; Bunger et al., 2000), the protein products of which interact with CLOCK and BMAL to inhibit their transcription in a cycle that is completed once every 24 h (Kume et al., 1999; Vitaterna et al., 1999; Shearman et al., 2000). Mutations of the *Period* genes and disruptions of translation of the PERIOD2 protein (PER2) result in behavioral arrhythmicity demonstrating a critical role for *Period* genes in maintaining circadian clockwork (Bae et al., 2001; Zheng et al., 2001; Gavrila et al., 2008).

There are parallels between the effects of drugs of abuse and those of other motivationally salient stimuli on circadian locomotor patterns and clock gene expression. For example, restricted mid-morning feeding schedules in rats elicit intense bouts of wheel running in advance of food delivery and entrain the rhythm of PER2 expression in several brain regions that are otherwise subordinate to signals from the master clock in the suprachiasmatic nucleus (Verwey et al., 2007; Waddington Lamont et al., 2007). Similarly, daily injections of methamphetamine, nicotine, and other stimulant drugs in rats housed in constant dim light can entrain locomotor activity rhythms and elicit anticipatory bouts of wheel running in advance of each daily injection by 1-2 h (Shibata et al., 1994; Kosobud et al., 1998; Iijima et al., 2002; Kosobud et al., 2007). Interestingly, this anticipatory activity does not develop when drugs without positive motivational effects or abuse potential, such as haloperidol, are administered (Kosobud et al., 2007).

Although anticipatory locomotor activity is reported to develop in response to several drugs of abuse, it has not yet been determined whether repeated drug injections also entrain rhythms of clock protein expression in brain regions that are responsive to other motivationally relevant manipulations such as restricted feeding schedules. Studies to date have used a variety of drug administration modes and schedules and have limited their interest in clock gene activity primarily to the striatum (Iijima et al., 2002; Uz et al., 2005; Wang et al., 2006). In the present study, we examined the effect of daily, mid-morning injections of morphine and subsequent morphine withdrawal in rats housed in 12:12 light-dark

(LD) conditions on rhythms of wheel running and PER2 expression in SCN, dorsolateral striatum, and regions we have previously shown to be responsive to restricted feeding schedules; namely, oval nucleus of the bed nucleus of the stria terminalis (BNSTov), central nucleus of the amygdala (CEA), basolateral amygdala (BLA), and dentate gyrus of the hippocampus (DG) (Waddington Lamont et al., 2007). We report here that although morphine withdrawal does not alter the rhythm of PER2 expression in the SCN, it profoundly affects the normal rhythm of PER2 expression in all other regions examined.

The mechanisms whereby drugs of abuse modulate clock gene expression are unknown, although evidence suggests that changes in dopaminergic signaling contribute to their effects. Opiate and stimulant drugs increase extracellular concentrations of dopamine within terminal regions of the mesolimbic system, including dorsal and ventral striatum (Di Chiara and Imperato, 1988), prefrontal cortex (Moghaddam and Bunney, 1989), BNST (Carboni et al., 2000), and amygdala (Harmer et al., 1997), whereas early withdrawal from repeated drug use is associated with a reduction in the activity of mesolimbic dopamine neurons and in extracellular dopamine levels (Acquas et al., 1991; Rossetti et al., 1992; Tran-Nguyen et al., 1998; Diana et al., 1999). In support of a role for dopamine in drug-induced modulation of clock gene expression, it has been found that methamphetamine-induced shifts in locomotor activity rhythms can be antagonized by pre-treatment with dopamine receptor antagonists and mimicked with injections of dopamine receptor agonists (Honma et al., 1987; Shibata et al., 1994; but see Nikaido et al., 2001) and that induction

of Per1 mRNA in mouse striatum by an acute injection of methamphetamine is blocked by pre-treatment with a D1 antagonist (SCH 23390) (Nikaido et al., 2001). More recently, dopaminergic modulation of clock gene expression has been demonstrated directly both in vitro and in vivo (Yujnovsky et al., 2006; Amir et al., 2008; Imbesi et al., 2009).

We sought to address the role of dopaminergic signaling by administering the dopamine D2/3 agonist, quinpirole, to morphine-dependent rats immediately following the last morphine injection. Previous studies have demonstrated that injections of quinpirole alleviate some somatic symptoms of opiate withdrawal in rats (e.g., Harris and Aston-Jones, 1994) and we have found in rats with unilateral 6OHDA lesions of the medial forebrain bundle that daily intraperitoneal injections of quinpirole increase PER2 expression in dorsal striatum 12 h after the injection (Amir et al., 2008).

MATERIALS AND METHODS

Subjects

A total of 57 male Wistar rats, weighing 300-350 g at the start of each experiment, were used (Charles River, St. Constant, QC). For the running-wheel activity experiment (experiment 1), rats were housed singly in shoebox cages with running wheels and kept in individual lightproof and sound-attenuated cabinets. Within each cabinet, an overhead fluorescent tube provided light on a 12:12 LD cycle (light intensity within the cage was approximately 300 lux) and wheel-running activity was monitored continuously by computer (VitalView,

Minimitter, Sunview, OR, USA). For the studies examining the effect of morphine on PER2 protein immunoreactivity in brain (experiments 2 and 3), rats were housed individually in plastic shoebox cages without running wheels in a light- and temperature-controlled room (temperature 21 ° C) on a 12:12 LD cycle (lights on at 0900 h). Throughout all experiments, rats had ad libitum access to laboratory chow (Purina Foods) and tap water. All experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of Concordia University.

Drugs

Morphine hydrochloride (Sigma, Oakville, Ont.) was dissolved in saline at a concentration of 40 mg/ml. Quinpirole hydrochloride (Sigma) was dissolved in distilled water at a concentration of 1 mg/ml.

Immunohistochemistry

At the end of experiments 2 and 3, rats were given an overdose of sodium pentobarbital (Somnotol, 100 mg/kg) at their assigned zeitgeber time (ZT) (ZT1, 1 h after lights on, or ZT13, 1 h after lights off) and perfused intracardially with 300 ml of cold 0.09% saline, followed by 300 ml of cold 4 % paraformaldehyde in 0.1 M phosphate buffer. Brains were extracted and kept in 4 % paraformaldehyde solution overnight at 4 ° C, then sectioned at 50 µm using a vibratome. Sections were stored in Watson's cryoprotectant (Watson et al., 1986) at –20 ° C until immunohistochemistry was performed. Immunohistochemical staining for PER2 protein was carried out according to a

protocol described previously (Amir et al., 2004) using an affinity-purified polyclonal antibody raised in rabbit against PER2, 1:1000 (ADI, San Antonio, TX).

Microscopy

Stained brain sections were examined under a light microscope using a 20x objective and 400 x 400 μm images of unilateral SCN, BNSTov, CEA, BLA, DG, and dorsolateral striatum were captured from each hemisphere using a Sony XC-77 video camera, a Scion LG-3 frame grabber, and Image SXM software (v1.8, S. D. Barrett, http://www.ImageSXM.org.uk). The number of cells immunoreactive for PER2 was then counted in each image captured. For each brain region of interest, the mean number of PER2-immunoreactive cells was calculated for each group by finding for each rat the mean cell count from six images having the highest number of labeled nuclei.

PROCEDURES

Experiment 1: Running-wheel activity.

Rats were randomly assigned to morphine or saline conditions and allowed to entrain to the LD cycle for 6 days before injections began. Following the entrainment period, morphine or saline was injected subcutaneously every day, between ZT3-4. Initially, a dose of 5 mg/kg morphine was given for 6 days. The dose was increased to 20 mg/kg for the next 6 days, and then increased to 40 mg/kg for another 6-day period. Following the sixth injection of 40 mg/kg of

morphine or saline, injections were discontinued and rats were left unhandled in their cages for another 6 days (referred to as the withdrawal period).

Experiment 2: Effect of morphine withdrawal on PER2 expression.

Before the start of injections, rats were allowed to entrain to the LD cycle for 10 days. Rats were randomly assigned to one of 8 groups in a 4 (treatment: saline control, day 1 morphine withdrawal (MWD-1), day 2 morphine withdrawal (MWD-2), or day 7 morphine withdrawal (MWD-7)) x 2 (ZT1 or ZT13) design. Morphine or saline was injected intraperitoneally (IP) between ZT3-4 every day. Rats in MWD-1 were killed either in the evening following their last morphine injection (ZT13, 10 h after the last morphine injection) or the next morning (ZT1, 22 h after the last morphine injection). Rats assigned to MWD-2 were injected with saline 24 h after their last morphine injection, then killed during the morning (ZT1, 46 h after the last morphine injection) or the evening (ZT13, 58 h after the last morphine injection) of the following day. Those in MWD-7 were given saline injections every day for 6 days beginning 24 h after the last morphine injection, then killed at either ZT1 or ZT13 of the following day. Saline controls were killed at times matched with the MWD-1 group.

The dosing of morphine began at 5 mg/kg and increased by 5 mg/kg every second day up to a maximum dose of 40 mg/kg. This 40 mg/kg dose was then maintained for 7 days. The rate at which the drug dose was increased in this experiment was slower than that of experiment 1 because of a higher incidence of mortality due to overdose in rats housed without running wheels.

Experiment 3: Effect of quinpirole injection on PER2 expression on day 2 of withdrawal of morphine (MWD-2).

Rats were randomly assigned to one of 4 conditions in a 2 (drug treatment: quinpirole or vehicle) x 2 (ZT1 or ZT13) design, and were given 10 days to entrain to the LD cycle before injections began. All rats were injected with morphine between ZT3-4 using a dosing schedule similar to that described for experiment 2. On day 1 of injections, morphine was given at a dose of 5 mg/kg. The dose of morphine was increased by an additional 5 mg/kg every second day over 15 days until a maximum dose of 40 mg/kg was reached. On day 15, all rats received an injection of 40 mg/kg between ZT3-4 and were then given an injection of either quinpirole (0.5 mg/kg) or vehicle (distilled water, 0.5 ml/kg) that evening at ZT13. The next day, no injection of morphine was given between ZT3-4, but as on the previous day, an injection of either quinpirole or vehicle was given at ZT13. Rats were killed the following day at either ZT1 (46 h after last morphine injection) or ZT13 (58 h after last morphine injection).

Statistical analyses

Wheel-running activity during the 12-h light and 12-h dark periods was analyzed using separate 2 (treatment: saline and morphine) x 4 (drug dose: 0 (entrainment period), 5, 20, and 40 mg/kg) x 6 (number of days per dose) mixed model analyses of variance (ANOVAs). Significant interactions were examined further using ANOVA to compare group activity within a particular drug dose. Separate 2 (treatment) x 6 (number of days in withdrawal) ANOVAs were

conducted for wheel running activity in light and dark periods during withdrawal. The development of anticipatory activity to daily morphine injection was analyzed with a 2 (treatment) x 3 (drug dose: 5, 20, and 40 mg/kg) x 6 (number of days per dose) ANOVA to compare mean wheel running in each group during the 3-h period before each injection (ZT0-3). The presence of anticipatory activity during this same 3-h period in withdrawal was also examined with a separate 2 (treatment) x 6 (number of days in withdrawal) ANOVA. Alpha level for all tests was set at 0.05.

PER2-immunoreactive cell counts were analyzed using two-way ANOVAs for treatment group x ZT. Significant interactions were analyzed further using one-way ANOVAs and Fisher LSD post-hoc tests, or independent samples t-tests where appropriate. Alpha level for all tests was set at 0.05.

RESULTS

Experiment 1: Wheel-Running Activity.

Figure 1 shows the mean daily wheel-running activity in morphine- and saline-treated groups during the 6-day stages of entrainment (Fig. 1a), of morphine treatment; 5, 20, 40 mg/kg (Fig. 1b-d), and withdrawal (Fig. 1e). It can been seen that during the 12-h light period morphine and saline groups did not differ significantly in the total amount of wheel running in any stage of the experiment (main effect of drug treatment: $F(1, 10) = 1.24$, n.s.; main effect of drug dose: $F(3, 30) = 1.31$, n.s.; drug treatment x drug dose interaction: $F(3, 30) = 0.85$, n.s.). Although the morphine group tended to run more in the light period

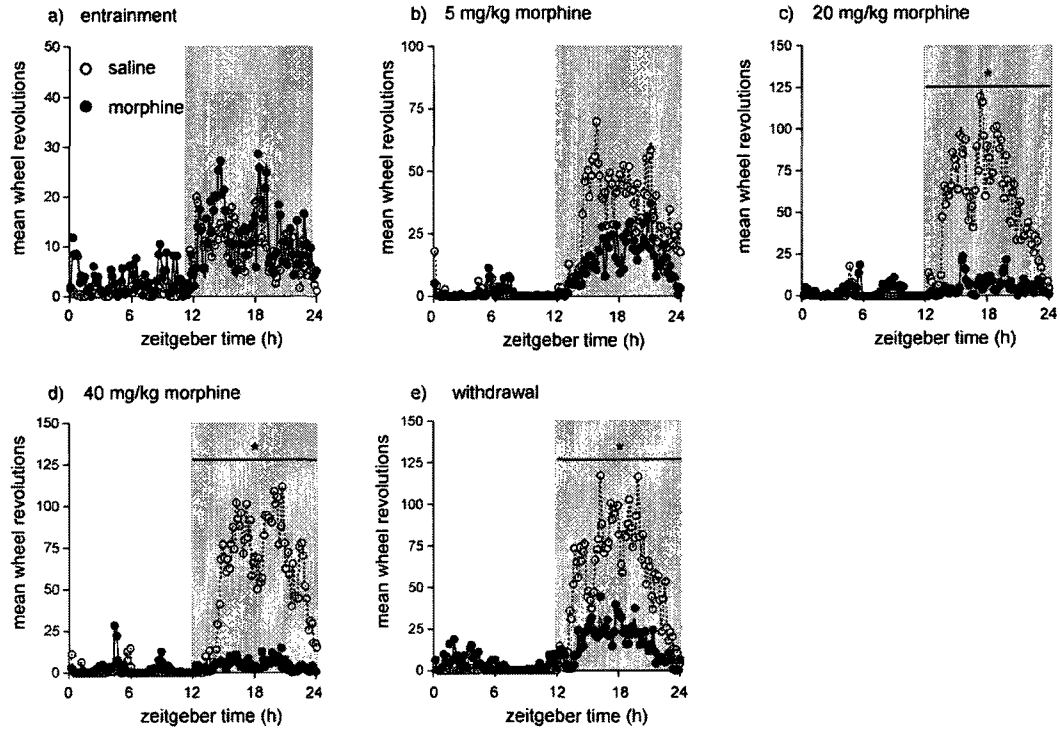

Figure 1.

Mean daily wheel-running activity in saline-treated (open circles) and morphine-treated (filled circles) rats during each 6-day stage of Experiment 1. Rats were housed in a 12:12 light-dark (LD) cycle; the gray panel indicates the 12-h period when lights were off. Daily injections of morphine or saline were given between zeitgeber time 3-4. (a) Entrainment period. (b) Morphine injections, 5 mg/kg. (c) Morphine injections, 20 mg/kg. (d) Morphine injections, 40 mg/kg. (e) Withdrawal period (no injections given). *Significant difference in mean wheel running during the 12-h dark period between morphine and saline groups. Morphine group (n = 6), saline group (n = 6).

than did the saline group during withdrawal, this difference was not significant (main effect of drug treatment: $F(1, 10) = 2.83$, n.s.).

In the 12-h dark period, morphine-treated rats ran less than saline controls rats; this difference was significant at the 20 mg/kg and 40 mg/kg doses of morphine (drug treatment x drug dose interaction: $F(3, 30) = 8.42$, $p < 0.01$; ANOVA at 5 mg/kg, main effect of drug treatment: $F(1, 10) = 1.43$, n.s.; ANOVA at 20 mg/kg, main effect of drug treatment: $F(1, 10) = 5.03$, $p = 0.05$; ANOVA at 40 mg/kg, main effect of drug treatment: $F(1, 10) = 8.00$, $p = 0.02$). This suppression of wheel-running activity in the dark was also observed during the withdrawal period (main effect of drug treatment, $F(1, 10) = 5.39$, $p = 0.04$). Although not shown in this figure, this suppression of wheel-running activity gradually abated over days of withdrawal towards levels seen in the saline group; the interaction term, however, did not reach statistical significance (drug treatment x days in withdrawal interaction: $F(5, 50) = 2.21$, $p = 0.07$).

We also investigated whether the morphine injections affected the pattern of wheel-running activity in the dark by comparing the activity of morphine- and saline-treated rats in 3-h intervals over the 12-h dark period. Although these data are not shown, an analysis of activity in 3-h intervals at each dose of morphine revealed that both morphine and saline groups were active during each of the 3-h intervals of the dark period but showed the greatest amount of running during the second and third 3-h intervals (main effect of interval, 5mg/kg: $F(3, 30) = 3.08$, $p = 0.04$; 20 mg/kg: $F(3, 30) = 3.52$, $p = 0.03$; 40 mg/kg: $F(1, 10) = 8.00$, $p = 0.02$). As reported above, morphine injections decreased wheel running overall, but did

not significantly change this pattern of wheel running over the 3-h intervals as compared to saline control (drug treatment x interval, 5mg/kg: $F(3, 30) = 0.32$, n.s.; 20 mg/kg: $F(3, 30) = 2.24$, n.s.; 40 mg/kg: $F(3, 30) = 1.96$, n.s.).

To determine whether rats developed anticipatory activity to the daily scheduled morphine injection, we examined wheel-running activity in the 3-h period before the injection across the three doses of morphine, as well as in the corresponding time window in withdrawal. These data are shown in Figure 2. Overall, during the morphine treatment stage, morphine-treated rats ran significantly more than did saline controls in the 3-h period before their injections (main effect of drug treatment: $F(1, 10) = 6.21$, $p = 0.03$). Although there was little difference between groups during the initial injection period at the 5 mg/kg dose, the group x dose interaction was not significant ($F(2, 20) = 2.14$, n.s.). In the withdrawal stage, wheel-running activity in the morphine group appears from Figure 2 to be considerably more than in the saline group, but this was highly variable across both rats and days during the 3-h 'pre-injection' window and therefore the difference was not statistically significant ($F(1, 10) = 2.67$, n.s) (morphine group mean (± SEM) = 129.5 (± 41.1), saline group mean (± SEM) = 6.8 (± 3.7)). This variability was attributable to a few rats that became very active in this 3-h period, but only after several days into withdrawal.

Though not shown, in both morphine and saline groups there were slight increases in wheel-running activity in the 3-h period after the disturbance of the injections, but the groups did not differ ($F(1, 10) = 0.26$, n.s.).

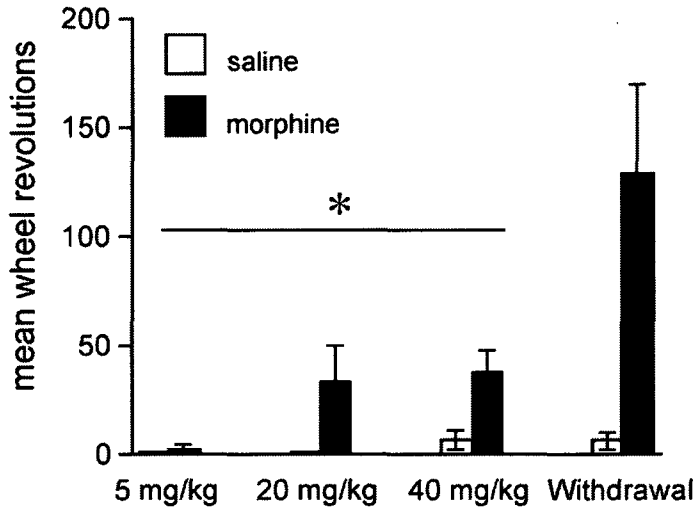

Figure 2.

Mean anticipatory wheel-running activity in saline (open bars) and morphine groups (filled bars) during the 3-h period before each daily injection, shown for each dose of morphine (5 mg/kg, 20 mg/kg, and 40 mg/kg) and for the corresponding time period in withdrawal when no injections were given. Each bar represents the mean (± SEM) of 6 days' activity. *Significant main effect of drug treatment. Morphine group (n = 6), saline group (n = 6).

Experiment 2: Effect of morphine withdrawal on PER2 expression

Figure 3 shows the mean PER2-immunoreactive cell counts in each brain region examined as a function of treatment (saline control, MWD-1, MWD-2 and MWD-7) and ZT (1, 13). In the saline control group, PER2 expression in SCN, BNSTov, and CEA, as expected, was high at ZT13 and low at ZT1 (Amir et al., 2004; Lamont et al., 2005a). As shown in Figure 3a, in the SCN this pattern was unaffected by morphine withdrawal, although overall PER2 counts in MWD-2 and MWD-7 groups were reduced compared to the saline control group (main effect of treatment group: $F(3, 24) = 3.23$, $p = 0.04$; main effect of ZT: $F(1, 24) = 1619.57$, $p < 0.01$; treatment group x ZT interaction: $F(3, 24) = 0.62$, n.s.). In contrast, in BNSTov (Fig. 3 b) and CEA (Fig. 3 c), withdrawal from morphine significantly affected the pattern of PER2 expression such that in MWD-1, MWD-2, and MWD-7 groups the number of cells expressing PER2 was significantly reduced at ZT13 compared to that in the saline control group (BNSTov treatment group x ZT interaction: $F(3, 24) = 9.91$, $p < 0.01$; one-way ANOVA at ZT13: $F(3, 12) = 7.16$, $p < 0.01$; Fisher's LSD post-hoc comparisons of saline control and each MWD group at ZT13, $p < 0.05$; CEA treatment group x ZT interaction: $F(3, 24) = 3.58$, $p = 0.03$; one-way ANOVA at ZT13: $F(3, 12) = 4.07$, $p = 0.03$; Fisher's LSD post-hoc comparisons of saline control and each MWD group at ZT13, $p < 0.05$). Furthermore, early in withdrawal (MWD-1) PER2 expression at ZT1 in the BNSTov was greater than in saline control (BNSTov, one-way ANOVA at ZT1: $F(3, 12) = 4.27$, $p = 0.03$; Fisher's LSD comparison of saline control and MWD-1 at ZT1, $p < 0.05$).

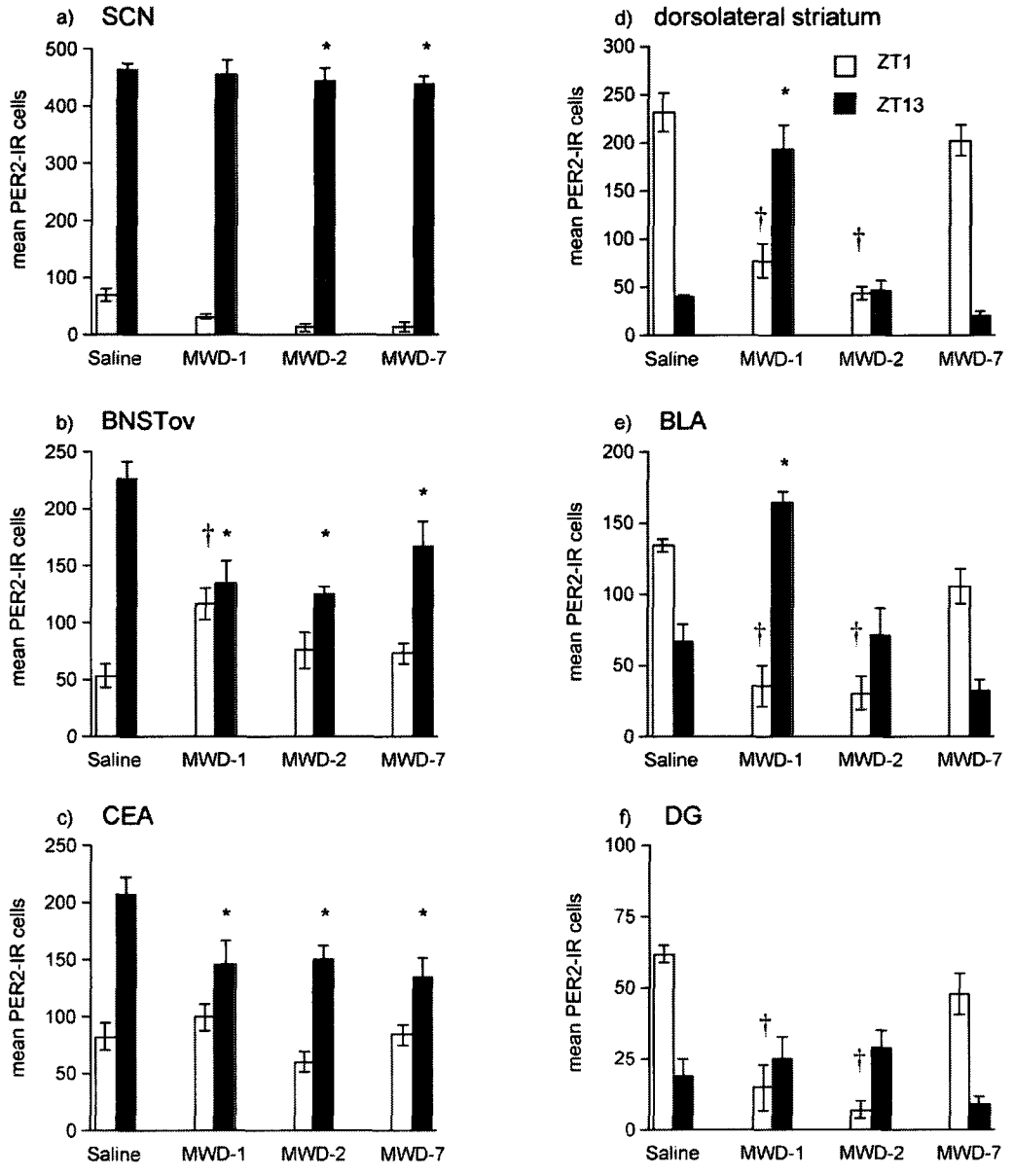

Figure 3.

Mean number of PER2-immunoreactive (PER2-IR) cells (± SEM) at zeitgeber time (ZT) 1 (open bars) and ZT13 (filled bars) in the SCN and regions of the limbic forebrain and striatum in saline-treated rats and morphine-treated rats in 1 (MWD-1), 2 (MWD-2), or 7 (MWD-7) days of withdrawal. (a) SCN. (b) BNSTov. (c) CEA. (d) Dorsolateral striatum. (e) BLA. (f) DG. *Significant difference from saline ZT13 group; †significant difference from saline ZT1 group. Saline group ZT1 (n = 4), ZT13 (n = 4); MWD-1 ZT1 (n = 4), ZT13 (n = 4); MWD-2 ZT1 (n = 4), ZT13 (n = 4); MWD-7 ZT1 (n = 4), ZT13 (n = 4).

It can be seen in Figure 3 that in saline-treated rats PER2 expression in dorsolateral striatum, BLA, and DG was high at ZT1 and low at ZT13 in antiphase with the rhythm of PER2 expression in SCN (Lamont et al., 2005a). This normal pattern was markedly changed in each of these three regions in early withdrawal from morphine. In dorsolateral striatum (Fig. 3d), the pattern of PER2 expression in group MWD-1 was reversed from that seen in the saline control group, such that it was significantly reduced at ZT1 and elevated at ZT13 compared to that in saline control (drug treatment x ZT interaction: $F(3, 24) = 48.55$, $p < 0.01$; one-way ANOVA at ZT 1: $F(3, 12) = 32.57$, $p < 0.01$; Fisher's LSD comparison of saline control and MWD-1 at ZT1, $p < 0.05$; one-way ANOVA at ZT13: $F(3, 12) = 31.59$, $p < 0.01$; Fisher's LSD post-hoc comparison of saline control and MWD-1 ZT13, $p < 0.05$). In the MWD-2 group, PER2 expression at ZT1 was also significantly diminished compared to that in saline control (Fisher's LSD comparison, $p < 0.05$), but did not differ from saline at ZT13. In the MWD-7 group, the pattern of PER2 expression recovered and did not differ significantly from that in the saline control groups at either ZT1 or ZT13.

Similar results were found for PER2 expression in BLA (Fig. 3e) in both MWD-1 and MWD-2 groups (drug treatment x ZT interaction: $F(3, 24) = 32.96$, $p < 0.01$; one-way ANOVA at ZT1: $F(3, 12) = 20.42$, $p < 0.01$; one-way ANOVA at ZT13: $F(3, 12) = 21.23$, $p < 0.01$). In MWD-1, the normal pattern of PER2 expression was inverted compared to that in the saline group (Fisher's LSD comparison of saline control and MWD-1 at ZT1, $p < 0.05$; comparison of saline control and MWD-1 at ZT13, $p < 0.05$). In MWD-2, as in dorsolateral striatum,

PER2 expression at ZT1 was significantly reduced relative to that in the saline group ($p < 0.05$). No differences were found between the MWD-7 groups and saline control groups at either ZT.

In DG (Fig. 3f), the normally elevated level of PER2 expression at ZT1 was significantly decreased in both MWD-1 and MWD-2 groups compared to saline control (drug treatment x ZT interaction: $F(3, 24) = 16.85$, $p < 0.01$; one-way ANOVA at ZT1: $F(3, 12) = 21.07$, $p < 0.01$; Fisher's LSD comparison of saline control and MWD-1 at ZT1, $p < 0.05$; comparison of saline control and MWD-2 at ZT1, $p < 0.05$). At ZT1 no differences were found MWD-7 and the saline control group. At ZT13 no differences in PER2 expression were found between groups (one-way ANOVA at ZT13: $F(3, 12) = 2.34$, n.s.).

Experiment 3: Effect of quinpirole on PER2 expression in early morphine withdrawal.

In experiment 3, following repeated daily injections of morphine, rats were given IP injections of either vehicle or 0.5 mg/kg quinpirole during the early withdrawal period from morphine in an attempt to prevent or reverse the effects of withdrawal on the rhythm of PER2 expression. Figure 4 shows the effects of quinpirole treatment on PER2 expression in MWD-2 groups in SCN, BNST, CEA, dorsolateral striatum, BLA, and DG at both ZT1 and ZT13. As expected, in the SCN the rhythm of PER2 expression was unaffected by morphine withdrawal (Fig. 4a), although there was small but significant increase in PER2 expression at ZT13 in the quinpirole-treated group (main effect of treatment group: $F(1, 9) =$

7.08, $p = 0.03$; main effect of ZT: $F(1, 9) = 1940.19$, $p < 0.01$; treatment group x ZT interaction ($F(1, 9) = 10.67$, $p = 0.01$; ZT13 independent samples t-test: $t(4) = -2.67$, $p = 0.05$).

In BNSTov (Fig. 4b) and CEA (Fig. 4c), quinpirole treatment reversed the effect of morphine withdrawal on the rhythm of PER2 expression in that it significantly increased PER2 expression at ZT13 over that in the vehicle-treated group (BNSTov treatment group x ZT interaction: $F(1, 9) = 12.30$, $p < 0.01$; ZT13 independent samples t-test: $t(4) = -10.94$, $p < 0.01$; CEA treatment group x ZT interaction: $F(1, 9) = 8.25$, $p = 0.02$; ZT13 independent samples t-test: $t(4) = -4.63$, $p = 0.01$). The quinpirole treatment did not significantly alter PER2 expression either in dorsolateral striatum (Fig. 4d; main effect of treatment: $F(1, 9) = 1.36$, n.s.; treatment group x ZT interaction: $F(1, 9) = 3.62$, n.s.) or in DG (Fig. 4f; main effect of treatment: $F(1, 9) = 0.78$, n.s.; treatment group x ZT interaction: $F(1, 9) = 1.19$, n.s.) whereas in BLA (Fig. 4e), quinpirole treatment significantly increased PER2 counts at both ZT1 and ZT13 compared to vehicle controls (main effect of treatment group: $F(1, 9) = 5.45$, $p = 0.04$).

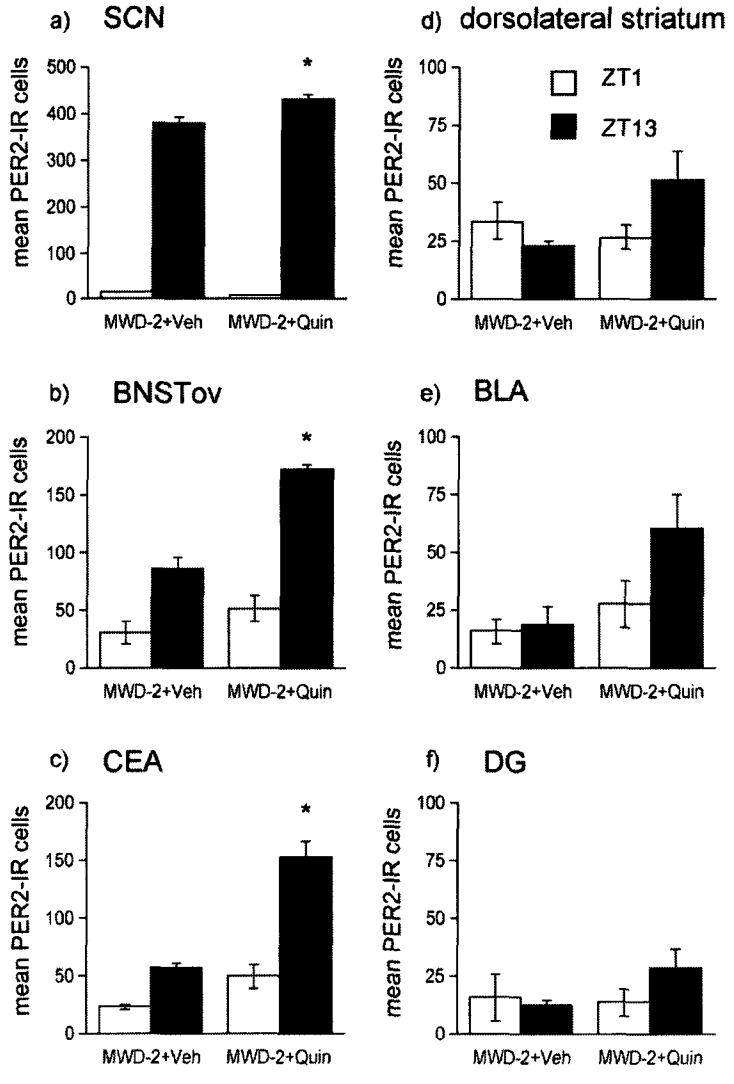

Figure 4.

Mean number of PER2-immunoreactive (PER2-IR) cells (± SEM) at zeitgeber time (ZT) 1 (open bars) and ZT13 (filled bars) in the SCN and regions of the limbic forebrain and striatum in morphine-treated rats given an injection of quinpirole (MWD-2+Q; 0.5 mg/kg, IP) or vehicle (MWD-2+V) at ZT13 for 2 days beginning the evening after the last morphine injection. (a) SCN. (b) BNSTov. (c) CEA. (d) Dorsolateral striatum. (e) BLA. (f) DG. *Significant difference from MWD-2+V ZT13 group. MWD-2+Q ZT1 (n = 4), ZT13 (n = 4); MWD-2+V ZT1 (n = 3), ZT13 (n = 2).

DISCUSSION

The findings of primary interest from the present study are that, in rats housed in 12:12 LD, scheduled daily injections of morphine given early in the light phase disrupted the normal circadian pattern of wheel-running activity, such that running in the dark phase (beginning 8 h after the daily injections) was completely suppressed at the higher doses of morphine. In addition there was evidence for a modest increase in wheel running in the 3-h period before the daily injections of morphine. This same schedule of morphine injections led to profound changes in the rhythm of PER2 expression in several regions of the limbic forebrain including BNSTov, CEA, dorsolateral striatum, BLA, and DG without affecting the PER2 rhythm in the SCN, itself.

Nighttime wheel-running activity

To our knowledge, this dramatic and prolonged effect of daily morphine injections on wheel-running activity during the dark phase has not been reported previously. Studies using different methods of drug delivery and different light schedules, however, have shown that opiate agonists can change circadian activity rhythms. For example, in mice housed in 12:12 LD, chronic morphine given via subcutaneous pellet decreased spontaneous locomotor activity in the light phase (Navarro et al., 1992). Using conditions of constant dim light, others have shown that an acute injection of an opiate agonist in mice and hamsters induces phase shifts in the rhythm of wheel running (Marchant and Mistlberger, 1995; Vansteensel et al., 2005). This effect was found to be contingent upon a

period of drug-induced hyperactivity, as immobilization immediately after the injection prevented the phase shift from occurring (Marchant and Mistlberger, 1995). In these latter studies, however, it was not reported whether the injection also affected the amplitude of wheel running in the active phase in addition to shifting the onset of daily activity. Interestingly, we have given daily injections of morphine (5 – 40 mg/kg) in the middle of the inactive phase to rats housed in constant darkness (DD) and have not observed any shift in the phase of the wheel-running rhythm (unpublished observations). Consistent with the present findings under 12:12 LD conditions, however, in these rats housed in DD the amplitude of wheel running during the active phase was reduced.

One possible explanation for the reduction in nighttime wheel-running activity seen the present experiment could be the development of withdrawal symptoms over the 24 h following the morning morphine injection. Spontaneous withdrawal from chronic morphine treatment, delivered via subcutaneous pellet or twice-daily injections, has been reported to diminish nighttime locomotor activity in rats (Stinus et al., 1998; Georges et al., 1999). Symptoms of withdrawal from morphine have been observed within 14 h after the last morphine injection in rats given twice-daily injections over one week (Li et al., 2009a). Given that our injections were made eight hours before lights were turned off, the emergence of some withdrawal symptoms over the course of the dark period could have interfered with the ability or motivation to run. Although, as can been seen in Figure 1, nighttime wheel running was reduced even at the lowest dose of morphine, it was profoundly and significantly decreased only when the higher

doses of morphine were given. Furthermore, the suppression of nighttime wheel running persisted for the first few days after morphine injections were discontinued, and then began to increase towards saline control levels over the remainder of the withdrawal period.

The disruption of normal nighttime wheel running could also be associated with the changes in the expression of PER2 in the dorsolateral striatum that were found to occur after repeated morphine treatment. The results of experiment 2 show that the pattern of PER2 expression in the dorsolateral striatum was reversed within the first 24 h after the last morphine injection; that is, PER2 expression was strongly increased 10 h after the morphine injection, at the onset of the dark phase, and decreased 22 h after morphine, at the onset of the light phase. Although our observations of wheel-running activity and PER2 expression were made in different groups of rats, these findings suggest that changes in the rhythm of striatal clock gene expression could be functionally related to the circadian regulation of locomotor activity. In support of this argument, Masubuchi and colleagues (2000) have shown that in rats housed on a 12:12 LD cycle and given ad libitum access to methamphetamine (in the drinking water), rhythms of wheel-running activity and clock gene expression in the striatum gradually desynchronize from the LD schedule and from clock gene expression in the SCN, but remain in phase with each other. We have also found in rats with unilateral 6OHDA lesions of the medial forebrain bundle that daily mid-morning quinpirole injections, to which dependence does not develop, decrease normal nighttime wheel-running activity and strongly increase PER2

expression at ZT13 in the dopamine-depleted dorsal striatum (Amir et al., 2008), similar to the pattern of results we report here. A link between the rhythms of wheel-running activity and striatal PER2 expression is further supported by our observation in the present experiments that both wheel running in the dark phase and the rhythm of PER2 expression in the dorsolateral striatum normalized after 6-7 days in withdrawal.

Effects of morphine on daytime wheel-running activity

Although there was no effect of daily morphine injections on overall daytime wheel-running activity, we did find modest, but significant increases in wheel running in advance of the morphine injections, a finding consistent with other reports in the literature that rats show anticipatory locomotor activity when given predictable daily injections of drugs of abuse, such as methamphetamine and nicotine (Shibata et al., 1994; Iijima et al., 2002; Kosobud et al., 2007). Interestingly, anticipatory activity also develops when rats are given daily restricted access to food, although the level of such anticipatory activity is much greater compared to that seen with daily drug injections. Because the development and timing of drug-associated anticipatory behavior resembles that of food anticipation, similar mechanisms could be mediating and controlling the timing of these anticipatory behaviors for both of these motivationally salient stimuli. In the present study, unlike what has been reported in previous drug studies (Shibata et al., 1994; Iijima et al., 2002), this anticipatory activity was not evident during the first two days of withdrawal. Some morphine-treated rats did

run more than saline-treated rats during the 'pre-injection' 3-h window but only after several days into the period of withdrawal.

It is possible that morphine-treated rats demonstrated signs of anticipation of the daily morphine injection other than wheel running. For example, Eikelboom and Stewart (1979) reported that in rats given injections of morphine at the same time of day over many days, body temperature decreased in a 1-h period in advance of the injection. This hypothermia was found to occur whether or not rats were tested in the same environment that predicted drug injections, suggesting that the decrease in temperature was mediated via a circadian mechanism.

Effect of morphine withdrawal on PER2 protein expression

Our findings reveal profound changes in the rhythm of PER2 expression in several limbic forebrain regions in rats killed after withdrawal from daily morphine injections, although no effects were seen on the rhythm of PER2 expression in the SCN. In BNSTov and CEA, PER2 expression at ZT13 was significantly decreased in rats killed one, two, and seven days after termination of morphine injections. This result is consistent with that of Li and colleagues (Li et al., 2009a) showing a flattening of the rhythm of *Per1* and *Per2* mRNA transcription in CEA, 24-48 h after withdrawal from twice-daily injections of morphine. Our finding of a similar effect of morphine withdrawal on the pattern of PER2 expression in both CEA and BNSTov was expected given the high degree of

anatomical and neurochemical similarity between these two structures (Alheid and Heimer, 1988; Dong et al., 2001a).

That the reduction in PER2 immunoreactivity at ZT13 in these regions was present after seven days of withdrawal suggests that the disruptive effects of opiates on circadian rhythms, including those of clock genes, can be long lasting. In humans undergoing withdrawal from heroin, a blunting of the rhythms of *Per1* and *Per2* mRNA transcription in blood mononuclear cells, as well as ACTH and beta-endorphin secretion, have been observed to persist over 30 days of abstinence (Li et al., 2009b). We have previously shown that the rhythm of PER2 expression in BNSTov and CEA is dampened under conditions of altered glucocorticoid secretion, as well as after a reduction in corticotropin releasing factor (CRF) protein levels in BNSTov (Amir et al., 2004; Bhargava et al., 2006; Segall et al., 2006). Given that withdrawal from drugs of abuse is associated with changes in extracellular levels of CRF (Merlo Pich et al., 1995; Richter and Weiss, 1999; Olive et al., 2002) and CRF mRNA transcription in BNSTov and CEA (Maj et al., 2003; Houshyar et al., 2004), it is possible that changes in these systems over the course of withdrawal contribute to the prolonged blunting of PER2 expression in these regions.

Perhaps the most dramatic finding from the present experiments was the reversal of the normal rhythm of PER2 expression in dorsolateral striatum, BLA, and DG observed within 10 h after the last morphine injection (MWD-1). Under normal conditions, the rhythm of expression of PER2 protein in dorsolateral striatum, BLA, and DG is 180 degrees out of phase with that in BNSTov and CEA

(Amir et al., 2004; Lamont et al., 2005a). Here we see that daily morphine injections actually reversed the pattern of PER2 expression in dorsolateral striatum, BLA, and DG, an effect that was evident early in withdrawal after which the rhythm gradually re-entrained to the LD cycle. Reversal of the rhythm of PER2 expression in BLA and DG has also been found to occur under daytime restricted feeding schedules. Peak PER2 expression in these regions synchronizes with those in BNSTov and CEA and occurs 12 h after food presentation (Waddington Lamont et al., 2007).

In a preliminary attempt to investigate the mechanisms underlying these changes in PER2 expression, we examined the role of dopamine signaling. Because mesolimbic dopaminergic activity is reduced during the acute period of opiate withdrawal (Diana et al., 1999), and because injections of D2 dopamine receptor agonists reduce some symptoms of withdrawal (Harris and Aston-Jones, 1994), we gave injections of the D2/D3 receptor agonist, quinpirole, to morphine-treated rats beginning the evening of the last morphine injection. Quinpirole significantly reversed the loss of PER2 immunoreactivity at ZT13 in BNSTov and CEA (58 h following the last morphine injection), and tended to increase PER2 expression in dorsolateral striatum, BLA, and DG at this time without restoring the normal peak of PER2 expression in these regions at ZT1. These results indicate that stimulation of dopamine D2/D3 receptors in opiate withdrawal at the beginning of the dark phase, when dopamine levels would normally increase in terminal regions of the mesolimbic dopamine system (e.g., Castaneda et al., 2004), can increase the expression of PER2, but that the

strength of this effect varies between forebrain regions. This variation in the effect of quinpirole could be attributable to regional differences in requirements for dopaminergic stimulation and possibly to differences in the rhythm of dopamine receptor sensitivity. A circadian rhythm in the locomotor activating effects of quinpirole in drosophila and in mice (Andretic and Hirsh, 2000; Akhisaroglu et al., 2005) and in the induction of PER1 in mouse striatum by quinpirole (Imbesi et al., 2009) has been reported.

The effect of morphine treatment and withdrawal on wheel running rhythms and PER2 expression in the limbic forebrain could be also mediated through other mechanisms, such as the activation of opiate receptors. There is some evidence for a role of mu receptors in the development of anticipatory activity to food presentation under conditions of restricted feeding (Kas et al., 2004) and previous studies have demonstrated that the mu receptor agonist, fentanyl, can modulate the effects of light on circadian rhythms of locomotor activity and on the induction of *Per1* mRNA in SCN (Vansteensel et al., 2005). Mu opiate receptors are expressed in all of the regions examined for PER2 immunoreactivity in the present experiments (Ding et al., 1996).

Changes in glutamatergic signaling as a result of morphine treatment and withdrawal might also be involved in the disruption of normal PER2 rhythms observed in the present experiments. An increasing number of studies have demonstrated that repeated drug exposure leads to alterations in the responsiveness of cells to glutamatergic excitation in regions including striatum, BNSTov, and amygdala (Boudreau and Wolf, 2005; Kauer and Malenka, 2007;

Kourrich et al., 2007; Dumont et al., 2008). Notably, the *Per2* gene has been implicated in the normal functioning of glutamate systems and in glutamatergic-mediated responses to drugs of abuse, such as cocaine and alcohol (Abarca et al., 2002; Spanagel et al., 2005). Given that glutamate is one of the primary neurotransmitters regulating the effects of light on the entrainment of clock gene activity in the SCN (for review, see Reppert and Weaver, 2001), it seems likely that opiate modulation of glutamatergic activity could have important effects on the rhythmic expression of clock genes in the forebrain regions examined in the present study. Other manipulations that alter normal glutamatergic signaling in dorsal striatum, such as depletion of dopamine via 6OHDA lesions (Day et al., 2006), have been found to strongly reduce the normal rhythm of PER2 expression (Amir et al., 2008).

CONCLUSION

Taken together, our results show dramatic effects of daily morphine injections, given in the light phase, on circadian rhythms of wheel running and expression of PER2 in the limbic forebrain. Morphine-treated rats exhibited profound suppression of the normal nighttime wheel-running activity and modest levels of anticipatory wheel running in the 3-h period before the daily morphine injection. In addition, we found major changes in the rhythms of PER2 expression in BNSTov, CEA, BLA, DG and dorsolateral striatum in rats killed after withdrawal from daily morphine injections, although no effects were seen on the rhythm of PER2 expression in the SCN. The finding that repeated morphine injections suppress wheel-running activity during the dark phase and reverse the

normal pattern of PER2 expression in the dorsolateral striatum suggests that striatal clock gene activity is closely associated with the circadian regulation of this behavior (see also Masubuchi et al., 2000). Future studies will investigate further the role of dopamine and other neurotransmitters in the modulation of PER2 expression resulting from morphine treatment.

Acknowledgements

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General Discussion

Rhythmic clock gene expression in tissues outside of the SCN can be influenced independently of entraining signals from the master clock by timed, daily non-photic events. Previous evidence has suggested that catecholamine signaling serves as a non-photic zeitgeber in specific tissues, such as the retina. These findings are supported by an increasing body of research indicating an effect of drugs of abuse and withdrawal states in shifting clock gene expression in several brain regions and a number of physiological and behavioral rhythms. However, a role for daily, endogenous activity of midbrain DA systems in the normal rhythms of clock gene expression in the brain has not been previously demonstrated in vivo.

The experiments described in this thesis were designed to directly examine whether endogenous DA signaling contributes to the daily pattern of expression of the clock protein, PER2, in the SCN and several forebrain regions in which PER2 rhythms have been found to entrain to a variety of non-photic stimuli. Several methods were used to address this question. In one approach, activity at DA receptors was directly manipulated using 6-OHDA lesions of DA fibers, D1 or D2 receptor-selective antagonists, and D1 or D2/3 receptor-selective agonists. A second approach involved manipulating DA activity, but non-selectively, using acute injections of the tyrosine hydroxylase inhibitor, AMPT, and by using daily injections of morphine and subsequently withdrawing the drug. The results of these combined approaches to this question demonstrate an important and region-specific role for DA in the daily fluctuation

of PER2 expression in the rat forebrain. These findings offer new insight into the mechanisms that regulate circadian clockwork at the cellular level as well as the ways in which disturbances of DA and catecholamine systems can disrupt normal circadian functions. The findings of these experiments are summarized in Table 2[1] and are reviewed in the sections below.

Dorsal Striatum

The most salient finding of these experiments is that endogenous dopaminergic activity at D2 receptors is necessary for the normal daily fluctuation in PER2 in the dorsal striatum, but not in any other region examined. Targeted disruption of normal DA activity using a unilateral 6-OHDA lesion or blockade of D2 receptors with raclopride decreased the normal peak of PER2 in the dorsal striatum, whereas daily stimulation of D2/3 receptors using quinpirole injections restored an increase in PER2 expression in this region. Importantly, the timing of this increase in PER2 appeared to be entrained by the timing of the quinpirole injection, given that this increase occurred when the PER2 rhythm in the striatum normally reaches its trough. This result indicates that the phase and amplitude of the PER2 rhythm in the dorsal striatum are influenced by a daily fluctuation in D2 receptor stimulation, which is presumably associated with the 24-h rhythm of extracellular DA levels in this region.

[1] Table 2 (next page). Summary of the effects of each experimental manipulation described in Chapters 1, 2, and 3 on the daily fluctuation of PER2 expression in the limbic forebrain and SCN. § indicates a statistically significant ($p < 0.05$) effect.

Table 2.

manipulation	region: SCN	dorsal striatum	BNSTov	CEA	BLA	DG
unilateral 6-OHDA lesion	no effect	§ lesion blunts PER2 peak;	§ lesion blunts PER2 peak;	no effect	no effect	no effect
AMPT	no effect	§ PER2 blunted at ZT1 and increased at ZT13	no effect	§ PER2 blunted at ZT1	§ PER2 blunted at ZT1 and increased at ZT13	§ PER2 blunted at ZT1
chronic infusion of raclopride or SCH 23390	no effect	§ PER2 blunted at ZT1	no effect	no effect	§ raclopride increases PER2 at ZT13	no effect
6-OHDA lesion + daily quinpirole or SKF 81297 injection (injection at ZT1)	no effect of 6-OHDA lesion no effect of quinpirole or SKF 81297 infusion	§ lesion blunts PER2 peak; § quinpirole increases PER2 at ZT13 on the lesioned side	§ lesion blunts PER2 peak; § quinpirole increases PER2 at ZT1 and decreases it at ZT13 on both lesioned and intact sides	no effect of 6-OHDA lesion § quinpirole decreases PER2 at ZT13 on both lesioned and intact sides	no effect of 6-OHDA lesion § quinpirole decreases PER2 at ZT1 on intact side § SKF 81297 increases PER2 at ZT13 on lesioned side	no effect of 6-OHDA lesion no effect of quinpirole or SKF 81297 injections
naive + daily quinpirole or SKF 81297 injection (ZT1)	no effect	no effect	§ quinpirole increases PER2 at ZT1	§ quinpirole increases PER2 at ZT1	no effect	no effect
6-OHDA lesion + chronic quinpirole or SKF 81297 infusion	no effect of 6-OHDA lesion no effect of quinpirole or SKF 81297 infusion	§ lesion blunts PER2; no effect of quinpirole or SKF 81297 infusion	§ lesion blunts PER2; no effect of quinpirole or SKF 81297 infusion	no effect of 6-OHDA lesion no effect of quinpirole or SKF 81297 infusion	no effect of 6-OHDA lesion no effect of quinpirole or SKF 81297 infusion	no effect of 6-OHDA lesion no effect of quinpirole or SKF 81297 infusion
10-22 h after last morphine injection (injection at ZT3-4)	no effect	§ morphine decreases PER2 at ZT1 and increases it at ZT13	§ morphine increases PER2 at ZT1 and decreases it at ZT13	§ morphine increases PER2 at ZT1 and decreases it at ZT13	§ morphine decreases PER2 at ZT1 and increases it at ZT13	§ morphine decreases PER2 at ZT1 and increases it at ZT13
46-58 h after last morphine injection	no effect	§ PER2 blunted at ZT1	§ morphine increases PER2 at ZT1 and decreases it at ZT13	§ morphine increases PER2 at ZT1 and decreases it at ZT13	§ PER2 blunted at ZT1	§ PER2 blunted at ZT1
46-58 h after morphine + quinpirole (ZT13)	*no effect*	*no effect*	*quinpirole* increases PER2 at ZT13	*quinpirole* increases PER2 at ZT13	*no effect*	*no effect*
7 days after last morphine injection	no effect	no effect	no effect	no effect	no effect	no effect

The effects of the experiments in which not only DA but also NA and adrenaline levels were manipulated further demonstrate the necessity of normal dopaminergic activity for PER2 expression in the striatum. The normal peak of PER2 was strongly suppressed following one day of AMPT treatment and approximately 48 h after withdrawing daily morphine injections, two separate manipulations that are associated with a profound reduction in DA levels in the striatum. Importantly, a daily mid-morning morphine injection also changed the normal daily pattern of PER2 expression in this region, such that the normal peak at ZT1 was suppressed and an increase was observed at ZT13, 8-9 h after the last morphine injection. The similarity of this pattern of PER2 expression to that produced by daily quinpirole injections in 6-OHDA lesioned rats implies that the effect of the daily morphine injection was mediated at least in part through a stimulation of D2/3 receptors. It seems reasonable to speculate that the schedule of daily morphine injections used in this experiment affected the normal circadian rhythm of extracellular DA release in the dorsal striatum, and additional experiments using *in vivo* microdialysis would be useful to test whether this is indeed the case.

BNSTov and CEA

The results of both DA-specific and catecholamine-general manipulations indicate that PER2 expression in the BNSTov and CEA is responsive to changes in DA tone, but that dopaminergic activity is not directly, or solely, responsible for the normal daily fluctuation in PER2 in these regions. This conclusion is supported by the observations that, although a 6-OHDA lesion decreased peak

PER2 expression in the BNSTov, neither chronic infusion of DA antagonists nor an acute suppression of catecholamine synthesis following AMPT treatment affected the normal daily PER2 fluctuation in these regions. As discussed in chapter 2, this pattern of results would suggest that the reduction in PER2 in the BNSTov ipsilateral to the 6-OHDA lesion is attributable to changes in another system that arise in response to a prolonged reduction in DA content, such as the CRH system.

Interestingly, daily stimulation of D2/3 receptors using quinpirole injections was found to disrupt the normal PER2 fluctuation. Specifically, in 6-OHDA-lesioned rats daily quinpirole injections given at ZT1 increased PER2 expression at its normal trough at ZT1, whereas the normal peak of PER2 at ZT13 was reduced. Notably, these effects occurred in both the intact and 6-OHDA-lesioned hemispheres. This pattern of results stands in contrast to the effect of quinpirole in the dorsal striatum, in which daily quinpirole injections at ZT1 increased PER2 at ZT13, and this increase occurred only in the 6-OHDA-lesioned hemisphere and not in the intact hemisphere. On the basis of the results observed in the BNSTov and CEA, it would appear that the effect of quinpirole injections on PER2 is not mediated by DA afferents to these regions because the effect was the same irrespective of whether dopaminergic fibers had been destroyed with 6-OHDA toxin. Although the specific mechanisms underlying this effect remain to be determined, these findings suggest that the influence of quinpirole on PER2 expression in these regions occurs through D2 receptor-mediated effects on other systems, perhaps CRH and glutamate as discussed in chapter 2.

In the morphine experiments, the finding that the normal PER2 fluctuation in the BNSTov and CEA was disrupted both immediately after the last morphine injection and for several days following withdrawal of daily morphine injections suggests that changes in catecholamine systems, in addition to DA alone, influence the expression of PER2 in these regions. Furthermore, the persistence of this disruption in PER2 for up to 7 days after morphine withdrawal suggests that the effect of daily morphine injections and withdrawal on PER2 expression involves a prolonged change in DA tone and/or subsequent changes in other neurotransmitter or peptide systems. As noted above, the necessity of a prolonged change in DA and other catecholamine levels to produce an effect on the daily fluctuation of PER2 in the BNSTov and CEA is reinforced by the finding that an acute suppression of catecholamine synthesis using one day of AMPT injections had no effect on PER2.

BLA and DG

The daily fluctuations of PER2 expression in the BLA and DG were affected by manipulations of catecholamine levels in general, although the lack of effect of DA-selective manipulations on PER2 expression in these regions indicates that the changes in PER2 were not attributable to DA per se. In particular, a unilateral 6-OHDA lesion did not affect the normal pattern of PER2 expression and chronic infusion of DA receptor antagonists did not clearly change the daily PER2 fluctuation in either region. Furthermore, treatment with DA receptor agonists had minimal effect on the normal PER2 pattern in the BLA

and DG. Together, these findings suggest that dopaminergic activity does not regulate PER2 expression in either of these regions.

Manipulation of catecholamine levels using AMPT treatment and daily morphine injection and withdrawal, however, had a profound effect on the daily pattern of PER2 in the BLA and DG. These findings highlight an important role for NA in the regulation of PER2 expression in these regions. Following the last morphine injection, PER2 expression in both the BLA and DG was reduced at the normal peak time of ZT1, but was strongly elevated at ZT13, 8-9 h after the final injection. The normal peak of PER2 expression in these regions was also strongly suppressed within 48 h after the last morphine injection and following AMPT treatment. Taken together, this pattern of results suggests that NA signaling specifically contributes to the daily fluctuation in PER2 expression in these regions, and opens an interesting new avenue for research into this relation.

SCN

None of the experimental manipulations disrupted the normal daily fluctuation in PER2 expression in the SCN. This finding was not unexpected, given that the LD schedule is the primary zeitgeber to entrain clock gene rhythms in this region and a normal 12:12 LD cycle was maintained in all of these experiments. Furthermore, other studies examining the effects of non-photic cues in the entrainment of clock gene expression, such as restricted feeding schedules, have shown that the rhythm of PER2 expression in this region

remains entrained to normal LD conditions (Waddington Lamont et al., 2007). As previously noted, DA plays a critical role during early development of the rat in the entrainment of the rhythm of *fos* expression in the foetal SCN, as well as the subsequent locomotor activity rhythm in the neonate. However, studies of the adult rat indicate that changes in catecholamine signaling have no effect on clock gene expression in the master clock and the results of this thesis support this conclusion.

Mechanisms of DA modulation of PER2 expression

In the following sections, the possible mechanisms by which DA signaling via D2 receptors modulates PER2 are considered. The potential contributions of other, non-dopaminergic signaling mechanisms are also reviewed, given that the effects of a 6-OHDA lesion, AMPT treatment, and daily morphine injections and withdrawal are not limited to DA but also extend to NA, CRH, opiate, and glutamate signaling. Previous evidence suggests that changes in these systems, as well as another, melatonin, could play a role in modulating PER2 expression.

DA stimulation of D2 receptors

As discussed in chapter 1, several different mechanisms could underlie the effect of DA signaling on PER2 expression, including changes at the level of *Per2* mRNA transcription, changes at the level of the PER2 protein itself through post-translational modifications, and changes in the transcription and/or translation of other components of the molecular clockwork that in turn affect the expression of *Per2*. Preliminary findings from Weigl and colleagues (2010) using

real time PCR suggest that at least part of the effect of DA on PER2 expression in the dorsal striatum that we observed with 6-OHDA lesioning is mediated at the level of *Per2* transcription. This finding is consistent with other reports of a role for D2 receptors in regulating rhythmic *Per2* expression in the striatum (Sahar et al., 2010). These results do not exclude the possibility that stimulation of D2 receptors could also directly modify the expression of the PER2 protein, however. For example, converging evidence suggests a possible mechanism whereby D2 receptor stimulation increases phosphorylation of PER2 by activation of the Akt-GSK3ß pathway. In the dorsal striatum, binding of D2 receptors increases the phosphorylation activity of GSK3ß (Beaulieu et al., 2004; Beaulieu et al., 2007), a kinase thought to play a critical role in regulating the 'speed' of the circadian clock through phosphorylation of clock proteins, including PER2 (Iitaka et al., 2005). Specifically, phosphorylation of PER2 by GSK3ß is reported to affect the rate of nuclear translocation of PER2 from the cytoplasm by modulating its interaction with other clock proteins (Martinek et al., 2001). Importantly, GSK3ß phosphorylates other components of the clock, such as BMAL1 (Sahar et al., 2010), suggesting that D2 receptor-mediated changes in GSK3ß activity could affect PER2 protein expression indirectly as well as directly.

An additional route of action of DA receptor signaling on PER expression has been proposed in studies of the retina. As noted in the general introduction and chapter 1, *in vitro* studies of the African clawed frog (*xenopus laevi*) and mouse retina have demonstrated that stimulation of D2 receptors using quinpirole upregulates expression of *Per1* and *Per2* through a CREB-dependent

mechanism (Besharse et al., 2004; Yujnovsky et al., 2006). Activation of the D2 receptor regulates the cAMP-CREB pathway (Montmayeur and Borrelli, 1991) and a CRE-responsive element has been identified on the *Per2* promoter (Travnickova-Bendova et al., 2002). Interestingly, Yujnovsky and colleagues (2006) proposed a model in which D2 receptor signaling increases levels of phosphorylated CREB, which interacts with the CLOCK:BMAL1 dimer to enhance its transcriptional activity at E box sites on the *Per1* promoter. Thus, in this model, the enhancement of PER expression by DA in the retina is mediated through actions on other components of the clock. It is important to note, however, that findings from a study of explants of retina from PER2::Luc mice suggest that the phase of the PER2 rhythm in this tissue is regulated by an action of DA at D1 receptors and not D2 (Ruan et al., 2008). This finding suggests that the mechanisms by which DA signaling affects PER2 expression in this tissue remain unclear and caution against extrapolating results from one tissue to another.

Noradrenaline (NA)

Given that AMPT treatment and daily morphine injection and withdrawal are not DA-selective manipulations, but influence noradrenergic activity as well, changes in NA signaling are a likely contributor to the effects on PER2 expression observed in these experiments. A possible role for NA in regulating PER2 expression is best illustrated by the results of the AMPT experiment, in which a profound decrease in PER2 was observed not only in the dorsal striatum but also in the BLA and DG (and primary motor cortex, reported in chapter 1).

Although the amount of noradrenergic innervation to the dorsal striatum is modest relative to DA innervation, the greater reduction in PER2 expression following inhibition of catecholamine synthesis with AMPT compared to the effect of a 6-OHDA lesion suggests that NA can influence PER2 in the striatum. An effect of NA signaling in clock gene expression is also evidenced by the dramatic reduction in the daily peak of PER2 expression in the BLA and DG following AMPT treatment but not after a 6-OHDA lesion. This outcome is particularly interesting, because it suggests that the effect of different catecholamines on the regulation of PER2 expression is specific to different regions of the forebrain.

The effects of daily morphine injections and withdrawal of morphine also support a tissue-specific involvement of NA in regulation of PER2 expression. What is interesting to note in the context of the morphine experiment, however, is that previous evidence shows that the activity of noradrenergic cell groups increases following cessation of chronic treatment with opiates (Maldonado, 1997), an event that is associated with the somatic symptoms of opiate withdrawal (Delfs et al., 2000). However, PER2 expression in the dorsal striatum, BLA, and DG was significantly decreased at 46-58 h after morphine withdrawal as well as following the NA-depleting effects of AMPT treatment. To better characterize the contribution of NA to the changes in PER2 observed in this early period after the last morphine injection, we are conducting additional experiments in which clonidine, an inhibitor of NA release, is administered for two days beginning immediately after the final morphine injection.

As reviewed in chapters 1 and 2, there is already some evidence in the literature suggesting a role for NA signaling in the regulation of circadian rhythms. In particular, efferents from the SCN project indirectly to noradrenergic neurons in the locus coeruleus (Aston-Jones et al., 2001) and levels of NA in the forebrain are modulated by light exposure (Gonzalez and Aston-Jones, 2006). SCN-mediated activation of NA inputs to the pineal gland in rats and hamsters regulates the circadian rhythm of melatonin synthesis and the expression of *Per1* and *Cry1* in this region (Simonneaux et al., 2004). Furthermore, depletion of NA levels in the forebrain of the mouse, as a result of a mutation in the development of noradrenergic projections, is associated with a loss of normal clock gene expression in cortex and impaired phase-shifting of locomotor activity following a light pulse (Warnecke et al., 2005). Other findings, however, indicate no effect of NA on clock gene expression in peripheral tissues (Reilly et al., 2008). Taken together, the evidence to date suggests that the role of NA in regulating clock gene expression might be region-specific, similar to the action of DA.

The specific intracellular mechanisms by which NA might regulate clock gene expression have not, to my knowledge, been investigated. It would be of interest to determine whether the actions of NA and DA are similar; for example, involving changes in the cAMP-CREB pathway. Future studies using 6-OHDA lesions to target NA fibers specifically, such as through direct injection of the toxin into the LC or using pre-treatment with a DAT-selective blocker, like GBR 12909, to protect DA fibers could address these questions.

CRH

As previously noted, research from the Amir laboratory has demonstrated that CRH levels affect PER2 expression in the BNSTov (Bhargava et al., 2006). CRH levels, specifically in the BNSTov and CEA, were very likely affected by some of the experimental manipulations performed, such as 6-OHDA lesioning and daily morphine injections. These effects have been discussed in the previous chapters and future experiments in which CRH levels are directly measured or manipulated, using central infusions of CRH receptor-selective drugs or double-stranded RNA (ds-RNA) against CRH for example, might prove useful in determining the contribution of this system to the effect of DA on PER2 in the BNSTov and CEA. Furthermore, in view of evidence that CRH levels can modulate the effect of other signals, such as glutamate, in regulating the activity of cells in regions such as the BNSTov (e.g., Kash et al., 2008), CRH signaling might act as an intermediary between DA and other systems in the modulation of clock gene expression. In support of this idea, Bhargava et al. (2006) showed in the BNSTov that PER2 and CRH are expressed in separate cell populations.

Related to the effects of CRH on clock gene expression in these regions is the influence of corticosterone levels on PER2. As previously noted, rhythmic corticosterone secretion is regulated by signals from the SCN and exogenous delivery of corticosterone to adrenalectomized rats can entrain the PER2 rhythm in the BNSTov and CEA (Segall et al., 2006; Segall and Amir, 2010). Previous evidence has shown that basal corticosterone levels significantly increase in rodents following the cessation of chronic opiate exposure (Pechnick, 1993). We

have also observed an increase in basal corticosterone levels in rats following the last daily morphine injection (unpublished observations). Similarly, in humans withdrawing from long-term heroin use, the normal circadian rhythm of adrenocorticotropin releasing hormone secretion, a critical signal in the regulation of the HPA axis, is blunted for up to one month after the last drug use (Li et al., 2009b). As such, it is possible that changes in corticosterone secretion as a result of the daily morphine injections and withdrawal of morphine are implicated in the changes in clock gene expression in the BNSTov and CEA.

Opiates

As reviewed in chapter 3, opiates have been shown previously to affect aspects of circadian functioning, including locomotor activity rhythms (Marchant and Mistlberger, 1995), the firing rate of SCN neurons, and modulating the effects of light in inducing *Per1* expression in the hamster SCN (Vansteensel et al., 2005). Other reports indicate that repeated injections of morphine upregulate expression of *Per2* in frontal cortex (Ammon et al., 2003), and that the rhythms of *Per1* and *Per2* transcription in multiple brain regions, including CEA, hippocampus, nucleus accumbens, and ventral tegmental area, are disrupted following cessation of repeated morphine injections (Li et al., 2009a). As previously noted, acute injection of opiates including morphine results in increased extracellular levels of DA in terminal regions of the mesolimbic DA system through a disinhibition of DA cell bodies in the ventral tegmental area. However, opiate receptors are also present in each forebrain region examined in these experiments (Ding et al., 1996), which allows for the possibility of a local

action of morphine on clock gene expression. The specific mechanisms by which opiates influence clock gene expression have not been investigated. Within the dorsal striatum, however, the observation that μ opiate receptors are primarily expressed by D1 receptor-bearing neurons (Delfs et al., 1994), as well as by cholinergic interneurons (Jabourian et al., 2005), implies that the actions of morphine in modulating PER2 expression, at least within this region, would presumably be indirect.

Glutamate

Glutamate release from fibers in the retinohypothalamic tract regulates expression of clock genes in the SCN. Given the abundance of evidence indicating a role for DA in modulating the effects of glutamate on cellular activity in several different brain regions, including those examined in the present studies, glutamatergic signaling is likely implicated in the changes in PER2 observed following both selective manipulations of DA levels and manipulations of catecholamine levels in general. For example, depletion of DA content in the striatum using 6-OHDA lesions or AMPT is associated with a rapid loss of dendritic spines on D2 receptor-expressing medium spiny neurons, on which glutamatergic corticostriatal fibers form synapses (Day et al., 2006). Similarly, repeated exposure to drugs of abuse also induces changes in glutamatergic signaling in terminal regions of the mesolimbic DA system (e.g., Sepulveda et al., 2004; Fu et al., 2007; Kourrich et al., 2007; Dumont et al., 2008). In the SCN, calcium-dependent modulation of the cAMP-CREB pathway following stimulation of NMDA receptors has been shown to regulate *fos* expression in response to

light (Schurov et al., 1999), and perhaps a similar mechanism might operate in regions such as the dorsal striatum in the regulation of PER2 expression. Interestingly, blockade of voltage-gated calcium channels containing the cav1.3 subunit has been shown to prevent the loss of dendritic spines on striatopallidal medium spiny neurons following a 6-OHDA lesion or AMPT treatment, suggesting that the effect of DA depletion on glutamatergic regulation of these cells involves changes in calcium signaling (Day et al., 2006).

Melatonin

Although the involvement of melatonin signaling in the effects of DA on PER2 expression was not discussed previously, evidence suggests that these two systems are closely interrelated. Melatonin synthesis and release from the pineal gland are prominent circadian signals that are downstream of SCN control, and which in turn influence the organization of the locomotor activity rhythm, the activity of the SCN (for a review, see Cassone, 1990), and the expression of clock genes. For example, removal of circulating melatonin in mice through pinealectomy abolishes *Per1* mRNA and PER1 protein rhythms in the dorsal and ventral striatum (Uz et al., 2003). However, this regulation of the clock by melatonin appears to be both tissue- and gene-specific, as pinealectomy was not found to affect *Per1* or *Per2* mRNA in the SCN (Oishi et al., 2000), or the rhythms of PER2 expression in SCN, dorsal striatum, BNSTov, CEA, or DG in rats (Amir et al., 2006), even though each of these regions expresses melatonin receptors (Laudon et al., 1988; Musshoff et al., 2002; Uz et al., 2003).

Importantly, there is evidence for a mutually inhibitory relationship between melatonin and DA in tissues such as the retina. The rhythms of DA and melatonin release in the retina are 180 degrees out of phase with each other, and stimulation of melatonin receptors during the dark phase prevents DA release, whereas stimulation of D2 receptors during the light phase inhibits melatonin release (for a review, see Tosini et al., 2008). Behavioral evidence also suggests that this mutual antagonism operates in the striatum. For example, the reduction of melatonin by pinealectomy in mice enhances the expression of locomotor sensitization to cocaine during the dark phase (Uz et al., 2003), whereas injections of high doses of melatonin inhibit locomotor activity (Chuang and Lin, 1994). It has also been proposed that an imbalance between melatonin and DA levels contributes to the circadian disruptions and motor deficits observed in Parkinson's disease (PD) (Willis, 2008). Thus, it is possible that the reduction in PER2 expression in the dorsal striatum following depletion of DA or catecholamines in general could be attributable in part to a dysregulation of melatonin signaling. To examine this possibility, it would be of interest to investigate whether blockade of melatonin receptors in the dorsal striatum, for example, would influence the loss of PER2 expression in this region following a unilateral 6-OHDA lesion.

Functional significance of DA modulation of PER2 expression

The findings of the present thesis suggest a number of implications for the role of circadian clockwork in brain regions outside of the SCN, specifically the dorsal striatum, and provide further insight into the mechanisms involved in the

regulation of circadian rhythms in general. These implications are discussed in the following sections. In particular, the finding that a shift in the normal rhythm of PER2 expression in the dorsal striatum was associated with a loss of the normal increase in wheel running at night suggests that one role of a striatal clock is to regulate the circadian locomotor activity rhythm. More generally, the responsiveness of PER2 to DA signaling suggests that events that activate midbrain DA neurons, such as rewards, can influence the rhythmic activity of the dorsal striatum. Importantly, however, this responsiveness also renders the circadian clock in this region vulnerable to potentially maladaptive signals that distort normal dopaminergic activity, such as drugs of abuse.

PER2 in the dorsal striatum regulates daily wheel-running activity

Several findings from the present experiments indicate that the daily fluctuation in PER2 expression in the dorsal striatum is associated with the daily wheel-running activity rhythm. First, both daily injections of quinpirole in 6-OHDA lesioned rats and morphine injections in intact rats dramatically suppressed nighttime wheel running in addition to shifting the daily rise in PER2 in the dorsal striatum away from its normal peak in the early morning. Notably, quinpirole injections in naïve rats did not affect the normal PER2 rhythm in the dorsal striatum, nor did they markedly suppress nighttime activity. Second, the observation from the double immunolabeling study that PER2 expression in the dorsal striatum is primarily found in D2 receptor- and enkephalin-positive cells suggests that rhythmic PER2 expression in this region is confined to striatopallidal neurons. Importantly, this projection pathway from the dorsal

striatum is implicated in the regulation of locomotor activity (Albin et al., 1989; Durieux et al., 2009).

Interestingly, other studies have illustrated the importance of D2 receptor stimulation and rhythmic clock gene expression in the dorsal striatum for normal activity rhythms. For example, D2 receptor knock-out mice show less of a day-night difference in their locomotor activity and exhibit reduced masking of activity by light (Doi et al., 2006). Although the disruption of rhythmic activity in these mice has been attributed to the loss of D2 receptors in the retina, the rhythms of *Per2* and *dbp* transcription, as well as BMAL1 phosphorylation in the striatum of these mice are also significantly blunted (Sahar et al., 2010). A number of other findings in the literature have demonstrated an association between changes in clock gene rhythms in the striatum and changes in the rhythm of locomotor activity (e.g., Masubuchi et al., 2000). An interesting illustration of this association is found in studies of the diurnal grass rat (*Arvicanthis niloticus*). Normally, this rodent is active during the light phase; however, if given access to a running wheel, some grass rats develop a nocturnal activity pattern. Notably, it has been reported that the phase of PER1 expression in the striatum varies according to the activity rhythm of the individual animal, such that expression of PER1 peaks during the inactive phase irrespective of whether this inactivity occurs during the light or dark (Ramanathan et al., 2008).

Related to the involvement of a striatal clock in the regulation of activity rhythms is the expression of a so-called 'anticipatory' activity rhythm in advance of a predictable, salient event, such as food delivery to food-restricted rats.

Bouts of anticipatory activity have been reported to develop in advance of daily, timed injections of drugs of abuse, and we report a similar, albeit modest effect in our morphine experiments. Some have emphasized the importance of anticipatory activity as a marker of entrainment by non-photic cues, such as restricted feeding schedules. However, the notion that the development of anticipatory running to a daily morphine injection contributed to the shift in PER2 expression observed in the dorsal striatum of these rats is undermined by other results, such as those from the quinpirole injection study in 6-OHDA-lesioned rats, in which daily quinpirole injections re-entrained the expression of PER2 in the lesioned striatum but did not elicit any anticipatory wheel running. Furthermore, 6-OHDA-lesioned rats that were injected with the D1 receptor agonist, SKF 81297, exhibited an increase in wheel-running activity at the time of the daily injection, but showed no changes in PER2 expression in the dorsal striatum of either hemisphere. Several studies of anticipatory running in food-restricted rodents suggest that this activity is regulated by the dorsomedial hypothalamus (Gooley et al., 2006; Fuller et al., 2008), although others have disputed this conclusion (Verwey et al., 2009). Taken together, these findings suggest that although the striatal clock is associated with the wheel-running activity rhythm, it is not clearly involved in the development of anticipatory activity.

Extracellular DA levels in the dorsal striatum: a clock-controlled output and entrainment signal

Finally, the finding that DA can affect the expression of clock genes in extra-SCN tissues is particularly intriguing in light of previous evidence showing that clock genes regulate the activity of midbrain DA systems themselves. Specifically, clock gene expression in the ventral tegmental area has been shown to regulate the firing rate of DA neurons (McClung et al., 2005; Roybal et al., 2007; Mukherjee et al., 2010), and the *Per2* gene is implicated in the expression of monoamine oxidase A in the striatum (Hampp et al., 2008). Behavioral studies indicate that responding for particular rewards follows a circadian rhythm (for a review, see Webb et al., 2009b); for example, rats given 24-h access to cocaine self-administer more drug during the dark phase than the light (Roberts et al., 2002; Bass et al., 2010), and male rats show a greater conditioned place preference during the mid-dark phase than during the light for an environment previously paired with access to a sexually receptive female (Webb et al., 2009c). The daily variation in responding to these rewards might be mediated in part by rhythmic clock gene expression in the midbrain (Roybal et al., 2007). Taken together with the present results that DA signaling can itself entrain the expression of PER2 in the striatum, these findings suggest that daily fluctuation in extracellular DA levels is both a clock-controlled output signal and an entrainment signal for rhythmic clock gene expression in the dorsal striatum.

That PER2 expression in the dorsal striatum can be modified independently of the LD schedule by stimulation of DA receptors suggests a

mechanism by which events that activate DA release, such as rewards, can influence the circadian functioning of an organism. This mechanism could allow an organism to adjust its daily pattern of activities to take advantage of predictable events that are rewarding or have adaptive value. Such a mechanism could also prove disadvantageous, however, in that it renders circadian systems vulnerable to potentially harmful stimuli that distort the normal pattern of DA signaling, such as late-night activity schedules associated with shift work, drugs of abuse, or diseases of catecholamine systems, as in the case of PD. Given the interaction between clock gene expression and activity of midbrain DA systems, a dysregulation of either DA tone or clock gene expression in regions such as the ventral tegmental area or the dorsal striatum could perhaps contribute to the circadian symptoms observed in mood disorders, substance abuse, and PD.

It also follows from the above suggestion, however, that treatments targeting the circadian clock might prove effective in alleviating certain pathologies. There is already some support for this proposal. For example, lithium treatment for bipolar disorder has been demonstrated to lengthen the period of the circadian clock as a result of the effect of lithium on the phosphorylation activity of GSK3ß (Martinek et al., 2001; Doble and Woodgett, 2003; Beaulieu et al., 2007). Also supporting circadian-oriented treatments are new clinical findings of an effect of melatonin agonists in the treatment of depression (Mendlewicz, 2009), as well as previous evidence showing roles for

light therapy and sleep deprivation in alleviating depression (Wirz-Justice and Van den Hoofdakker, 1999).

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