DAILY RHYTHMS OF GLUCOCORTICOIDS CONTROL THE CIRCADIAN EXPRESSION OF THE CLOCK PROTEIN, PERIOD2, IN OVAL NUCLEUS OF THE BED NUCLEUS OF THE STRIA TERMINALIS AND CENTRAL NUCLEUS OF THE AMYGDALA IN THE RAT

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Abbreviations

SCN, suprachiasmatic nucleus; BNSTov, oval nucleus of the bed nucleus of the stria terminalis; CEA, central nucleus of the amygdala; BLA, basolateral amygdala; DG, dentate gyrus; PER2, Period2; ZT, zeitgeber time; ANOVA, analysis of variance; RIA, radioimmunoassay; CORT, corticosterone; ENK, enkephalin; CRH, corticotropin-releasing hormone

Abstract - Glucocorticoid hormones are thought to play a role in the regulation of clock genes involved in the generation of circadian rhythms in peripheral tissues (Balsalobre et al., 2000a, Balsalobre et al., 2000b). Little is known about the role of glucocorticoids in the control of clock genes in the brain although there is evidence that clock genes in the suprachiasmatic nucleus (SCN), the master circadian pacemaker in mammalian brain, are invulnerable to glucocorticoids (Balsalobre et al., 2000a). We investigated the involvement of the adrenal glucocorticoid, corticosterone, in the control of the clock protein, PER2, in forebrain nuclei known to be sensitive to glucocorticoids, stressors and drugs of abuse, the oval nucleus of the bed nucleus of the stria terminalis (BNSTov) and the central nucleus of the amygdala (CEA) (Schulkin et al., 1998, Day et al., 1999, Day et al., 2001, Erb et al., 2001, Engstrom et al., 2003). We found earlier that the daily rhythm of PER2 in these nuclei is uniquely dependent on the integrity of the adrenal glands (Amir et al., 2004, Lamont et al., 2005). We now show that in the absence of the adrenals, a treatment that establishes a daily rhythm of corticosterone, but not one that provides constant corticosterone levels, restores the rhythm of PER2 in the BNSTov and CEA. These results underscore the importance of circadian glucocorticoid signaling in PER2 rhythms in the BNSTov and CEA and suggest a novel mechanism whereby stressors, drugs of abuse, and other abnormal states that affect the patterns of circulating glucocorticoids can alter the functional output of these nuclei.

Key Words: Adrenalectomy, Corticosterone, Circadian clock, Suprachiasmatic nucleus, circadian rhythms

We have shown that the PER2 protein is expressed rhythmically in several key structures of the rat limbic forebrain. These include two anatomically and functionally related nuclei of the central extended amygdala, the BNSTov and CEA, and two functionally related cortical-like structures, the basolateral amygdala (BLA) and dentate gyrus (DG) (Amir et al., 2004, Lamont et al., 2005). Furthermore, we found that although the rhythms of expression of PER2 in these structures are all under the control of the SCN, the rhythms in the BNSTov and CEA and those in the BLA and DG are diametrically opposite in phase, with those in the BNSTov and CEA peaking in the evening, in phase with the SCN rhythm, and those in the BLA and DG peaking in the morning. Most importantly, the PER2 rhythms in the BNSTov and CEA and those in the BLA and DG were found to be differentially affected by adrenalectomy (Amir et al., 2004, Lamont et al., 2005). Adrenalectomy abolished the PER2 rhythms in the BNSTov and CEA, but had no effect on the those in BLA and DG, suggesting that glucocorticoids play a selective role in the regulation of the rhythms in BNSTov and CEA. To study further this issue, we assessed the effect of corticosterone replacement via the drinking water (a manipulation that reinstates the daily rhythm of corticosterone) or via time-release pellets (to induce constant levels of corticosterone) on the daily pattern of expression of PER2 immunoreactivity in the BNSTov and CEA, as well as in the SCN, BLA and DG in sham operated and adrenalectomized rats.

EXPERIMENTAL PROCEDURES

Animals and Housing

All experimental procedures were approved by the Animal Care Committee of Concordia University in accordance with the guidelines set by the Canadian Council on Animal Care. Bilaterally adrenalectomized (n=64) and intact (n=32) male Wistar rats weighing 225-250g were purchased from Charles River Canada (St. Constant, Quebec). They were housed individually in clear plastic shoebox cages, under a 12h:12h light/dark (LD) schedule, and were provided with *ad libitum* access to food and either 0.9% saline or saline containing corticosterone (Sigma, 25 mg/L).

Sham adrenalectomy

Thirty-two rats underwent sham adrenalectomy under Isoflurane anesthesia in which bilateral incisions were made in the skin and immediately closed with stainless steel wound clips.

Pellet implantation

Thirty-two bilaterally adrenalectomized rats were anesthetized with isofluorane and a small incision was made in the skin of the scruff region. A pocket was made by separating the skin from the muscle tissue and a 100 mg 30-day slow release pellet was implanted. Sixteen rats received corticosterone pellets (Innovative Research of America (Sarasota, Florida), while the remaining 16 rats

received cholesterol control pellets. The skin wounds were closed with stainless steel wound clips.

Plasma collection and corticosterone determination

Rats were placed in plastic restraining devices and tail-clipped for rapid blood collection (1.5 ml). Sample were centrifuged at 4 °C, 13,000 rpm for ten min, and the plasma was extracted and stored at –80 °C. Plasma corticosterone levels were assessed in duplicates using radioimmunoassay (RIA).

Tissue preparation and immunocytochemistry

Rats were injected with an overdose of sodium pentobarbital (~100 mg/kg) at one of four zeitgeber times (ZT1, ZT7, ZT13, ZT19; ZT0 denotes time of light on) and were perfused intracardially with 300 ml of cold saline (0.9% NaCl) followed by 300 ml of cold, 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3). Following perfusion, brains were postfixed in 4% paraformaldehyde and stored at 4° C overnight. Serial coronal brain sections (50 μm) containing regions of interest were collected from each animal using a vibratome and stored in Watson's Cryoprotectant solution at -20 °C. Immunocytochemistry for PER2 was performed as previously described (Amir et al., 2004) using an affinity purified rabbit polyclonal antibody raised against PER2 (1:800, ADI, San Antonio, TX). Stained sections were mounted onto gel-coated slides dehydrated in a series of alcohols and Citrisolv (Fisher), and cover-slipped.

Data Analysis

PER2-stained brain sections were examined under a light microscope and images were captured using a Sony XC-77 video camera, a Scion LG-3 frame grabber, and Image SXM software (v1.6, S. D. Barrett, http://www.ImageSXM.org.uk). Cells immunopositive for PER2 were counted manually using a 400 X 400 μm template (SCN, BNSTov, CEA, BLA) or 200 X 400 μm template (DG). For analysis, the mean number of PER2 immunoreactive cells per region was calculated for each animal from the counts of 6 unilateral images showing the highest number of labeled nuclei. Differences between groups were revealed with analysis of variance (ANOVA). Alpha level was set at 0.05 for all analyses

RESULTS AND DISCUSSION

As expected, in sham operated rats that were given saline as the drinking fluid (four weeks), PER2 expression in SCN, BNSTov and CEA peaked at ZT13, whereas in BLA and DG, PER2 peaked at ZT1 as shown by the photomicrographs in Fig. 1a and the data in Fig. 1b. Furthermore, corticosterone administration in the drinking water (four weeks) had no effect on PER2 rhythms in sham operated rats in any of the regions studied.

Fig. 2a shows the daily pattern of PER2 expression within all areas in bilaterally adrenalectomized rats that received saline or corticosterone in the

drinking fluid (four weeks). As expected, in adrenalectomized rats given saline, the rhythm of PER2 in BNSTov and CEA was abolished (BNSTov, $F[3,9]=.83$, $p=.5$); CEA, $F[3,9]=.4$, $p=.75$), whereas in SCN, BLA and DG adrenalectomy had no effect (SCN,$F[3,9]=25.24$, $p<.0001$; BLA, $F[3,9]=10.37$, $p=.001$; DG, $F[3,9]=35.22$, $p<0001$). In contrast, in adrenalectomized rats given corticosterone, the rhythm of PER2 expression in BNSTov and CEA was completely restored (BNSTov, $F[3,9]=29.63$, $p<.0001$; CEA, $F[3,9]=8.73$, $p=.005$). Results of corticosterone RIA performed on plasma taken from adrenalectomized rats receiving corticosterone in the drinking fluid showed that rats self-administered the hormone mainly during the night (in μg/dL: ZT1=6.1±2.0; ZT7=2.8±1.3; ZT13=11.05±2.6; ZT19=6.7±2.2; $F[3,10]=2.97$, $p=.08$). Adrenalectomized rats receiving saline as the drinking fluid exhibited negligible levels of circulating corticosterone (in μg/dL: ZT1=0.14±.04; ZT7=.60±.30;ZT13=2.1±.74; ZT19=.95±.43; $F[3,10]=.83$, $p=.50$).

Although these findings demonstrate that corticosterone can restore the rhythmic expression of PER2 in BNSTov and CEA of adrenalectomized rats, they do not tell us whether it is the rhythm of corticosterone that is critical or whether the mere presence of corticosterone is sufficient. To address this issue, we assessed the expression of PER2 in two additional bilaterally adrenalectomized groups that were implanted subcutaneously with time-release pellets containing corticosterone or cholesterol.

It is apparent from the data shown in Fig. 2b that corticosterone replacement by time-release pellets (30-day, 100 mg) did not restore rhythmic PER2 expression in either BNSTov or CEA. The ANOVAs revealed no effect of either Treatment (BNSTov, $F[1,12]=.06$, $p=.80$; CEA,$F[1,12]=.18$, $p=.67$) or Treatment x Time interactions (BNSTov, $F[3,12]=1.68$, $p=.22$; CEA,$F[3,12]=1.13$, $p=.37$). Note that PER2 expression in all other regions was rhythmic and unaffected by pellet replacement. Results of the RIA showed that adrenalectomized rats receiving corticosterone via time-release pellets had constantly high levels of plasma corticosterone (in μg/dL: ZT1=13.34±.95; ZT7=14.7±1.07; ZT13=11.62±3.29; ZT19=11.92±2.13; $F[3,12]=.46$, $p=.71$). Adrenalectomized rats treated with cholesterol pellets exhibited negligible levels of circulating corticosterone (in μg/dL: ZT1=1.7±.93; ZT7=2.12±.98; ZT13=.80±.27; ZT19=1.25±.38; $F[3,7]=.32$, $p=.80$).

Taken together the results of these experiment show that following adrenalectomy the rhythms of expression of the clock protein, PER2, in the BNSTov and CEA can be restored by a daily rhythm in corticosterone level, but not by a constant level of corticosterone achieved by time-release pellets. These findings suggest that the rhythm of expression of PER2 in these nuclei is controlled by the daily fluctuation of corticosterone secretion from the adrenal gland. It is important to note that the rhythm of corticosterone induced by drinking in adrenalectomized rats and that seen under basal conditions in intact rats follow similar circadian patterns, both of which are under the control of the

SCN (Moore and Eichler, 1972, Szafarczyk et al., 1983, Satinoff and Prosser, 1988, Engeland and Arnhold, 2005). Thus, corticosterone could serve as mediator between the SCN and the rhythmic expression of clock genes not only in peripheral tissues, as previously proposed (Balsalobre et al., 2000a Balsalobre, 2000 #12, Bittman et al., 2003, Yamamoto et al., 2005), but also in select brain nuclei such as the BNSTov and CEA.

The mechanism by which corticosterone controls PER2 rhythms in the BNSTov and CEA is unknown. Both the BNSTov and CEA are sensitive to glucocorticoids (Honkaniemi et al., 1992, Makino et al., 1994b, Makino et al., 1994a, Pompei et al., 1995, Watts and Sanchez-Watts, 1995, Schulkin et al., 1998, Lechner and Valentino, 1999), but they are not unique in that respect. Indeed, as is the case with BNSTov and CEA, the hippocampus and BLA contain glucocorticoid receptors, activation of which affect diverse physiological and behavioral processes (Jacobson and Sapolsky, 1991, Ahima et al., 1992, Morimoto et al., 1996, Roozendaal and McGaugh, 1997, Sousa and Almeida, 2002, Conrad et al., 2004). Unlike the BNSTov and CEA, however, the rhythmic expression of PER2 in these regions is unaffected by adrenalectomy or by rhythmic or constant levels of replacement of corticosterone. Cells within the BNSTov and CEA express a variety of neuropeptides, including corticotropin-releasing hormone (CRH) and enkephalin (ENK) (Honkaniemi et al., 1992, Schulkin et al., 1998, Dong et al., 2001, Kozicz and Arimura, 2001, Kozicz, 2002). We have reported previously that in both nuclei, PER2 is expressed in

ENK neurons (Amir et al., 2004, Lamont et al., 2005). Thus, it is possible that the changes in the rhythm of PER2 expression observed after adrenalectomy and corticosterone replacement are due to a direct effect of glucocorticoids on these neurons. Although PER2 appears not to be expressed in CRH containing neurons, we cannot rule out an indirect effect of glucocorticoids on the rhythm of PER2 expression via their well known effects on these CRH neurons.

The finding that the BNSTov and the CEA exhibit daily rhythms in PER2 that depend on rhythmic glucocorticoid signaling suggest that abnormalities in the daily rhythms of circulating glucocorticoids induced by events such as chronic exposure to stressors or to drugs of abuse, as well abnormalities induced by disease states, such as Cushing's disease, could directly affect the local expression of circadian clock genes. Because the BNSTov and CEA have important roles in the control of emotional and motivational states, such persistent disruptions of local circadian functioning could lead to the development of abnormal autonomic, endocrine and behavioral responses to motivationally and emotionally significant stimuli.

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Figure captions

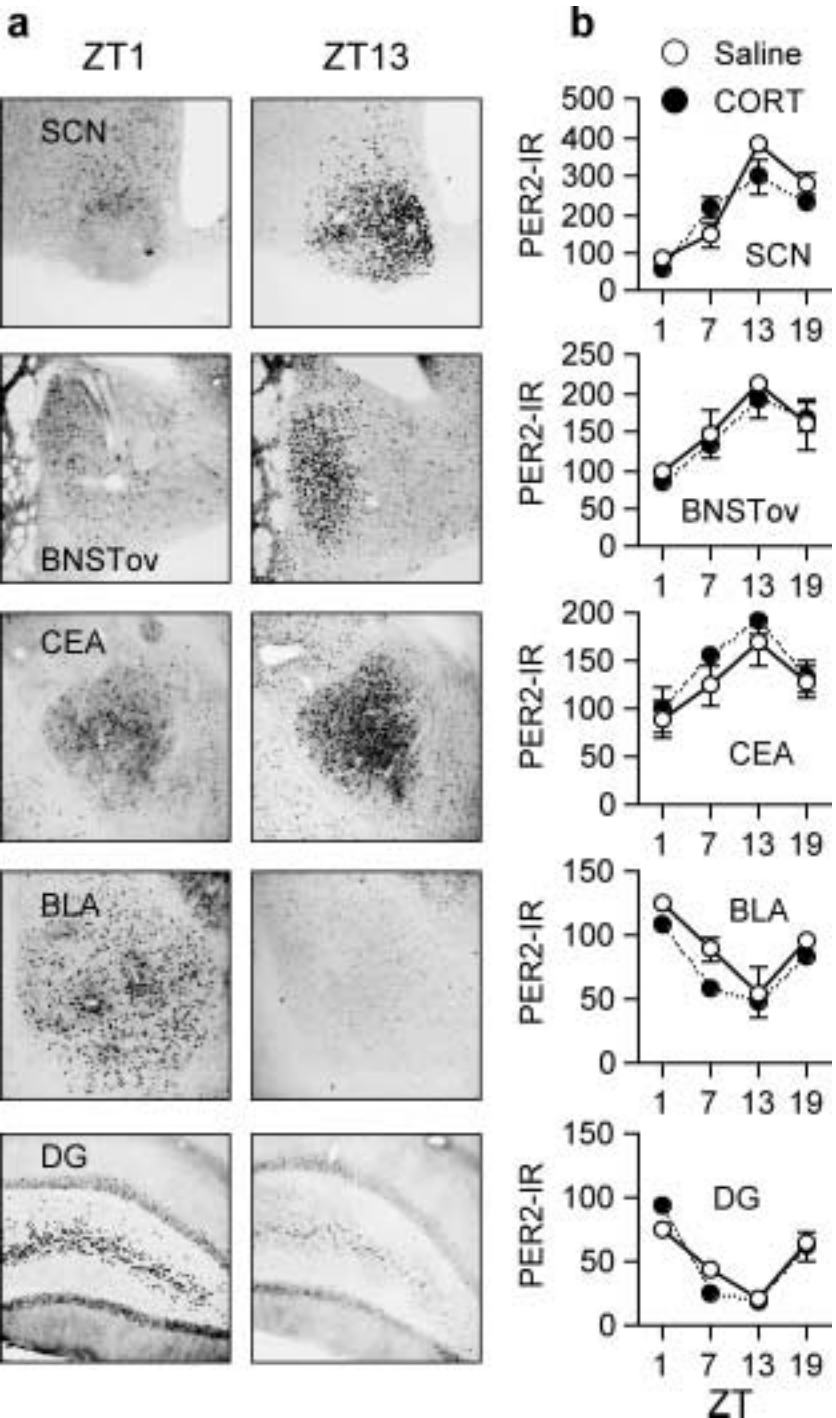
Fig. 1

a, Examples of PER2 expression in the SCN, BNSTov, CEA, BLA and DG at ZT1 and ZT13 in sham-operated rats given 0.9% saline (four weeks) as the drinking fluid (magnification: x20); b, Number of PER2 – immunoreactive cells in the SCN, BNSTov, CEA, BLA and DG as a function of zeitgeber time (ZT) in sham-operated rats given 0.9% saline or saline containing corticosterone (CORT, 25 mg/L) as the drinking fluid (four weeks) (Means + S.E. are shown, n=3-5 per point).

Fig. 2

a, Number of PER2 – immunoreactive cells in the SCN, BNSTov, CEA, BLA and DG as a function of zeitgeber time (ZT) in groups of adrenalectomized rats given 0.9% saline or saline containing corticosterone (CORT, 25 mg/L) as the drinking fluid (four weeks) (Means + S.E. are shown, n=3-4 per point); b, Number of PER2 – immunoreactive cells in the SCN, BNSTov, CEA, BLA and DG as a function of zeitgeber time (ZT) in groups of adrenalectomized rats given 0.9% saline as the drinking fluid and a subcutaneous time-release pellet (100 mg, 30-day) containing cholesterol (Chol) or corticosterone (CORT) (Means + S.E. are shown, n=3-4 per point).

Figure


a
ZT1
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BNSTov
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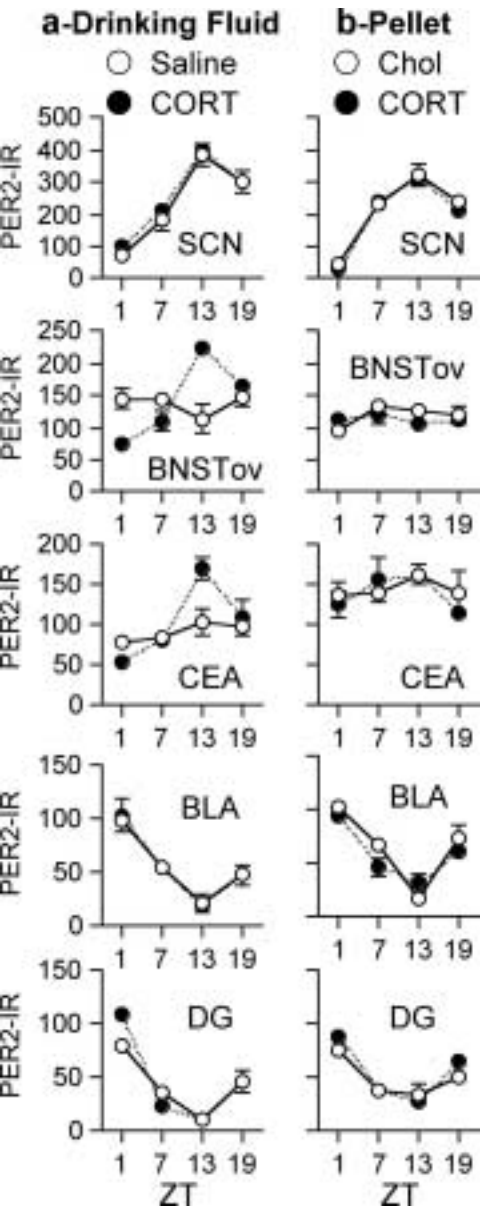
Figure


a-Drinking Fluid
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Saline
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