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TIMED RESTRICTED FEEDING RESTORES THE RHYTHMS OF EXPRESSION OF THE CLOCK PROTEIN, PERIOD2, IN THE OVAL NUCLEUS OF THE BED NUCLEUS OF THE STRIA TERMINALIS AND CENTRAL NUCLEUS OF THE AMYGDALA IN ADRENALECTOMIZED RATS

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Abstract—Feeding schedules that limit food availability to a set time of day are powerful synchronizers of the rhythms of expression of the circadian clock protein Period 2 (PER2) in the limbic forebrain in rats. Little is known, however, about the mechanisms that mediate the effect of such timed restricted feeding (TRF) schedules on the expression of PER2. Adrenal glucocorticoids have been implicated in the circadian regulation of clock genes expression in peripheral tissues as well as in the control of the rhythms of expression of PER2 in certain limbic forebrain regions, such as the oval nucleus of the bed nucleus of the stria terminalis (BNSTov) and central nucleus of the amygdala (CEA) in rats. To study the possible involvement of glucocorticoids in the regulation of PER2 expression by TRF, we assessed the effect of adrenalectomy on TRF-entrained PER2 rhythms in the limbic forebrain in rats. Adrenalectomy selectively abolished the rhythms of PER2 in the BNSTov and CEA in normally fed rats, as previously shown, but had no effect on TRF-entrained PER2 rhythms in the same structures. These findings show that the effect of TRF on PER2 rhythms in the limbic forebrain is independent of adrenal glucocorticoids and demonstrate that the involvement of glucocorticoids in the regulation PER2 rhythms in the limbic forebrain is not only region specific, as previously shown, but also state dependent.

Key words: adrenalectomy, corticosterone, anticipatory activity, limbic forebrain, circadian rhythms, immunocytochemistry.

The circadian clock protein, Period 2 (PER2), is expressed rhythmically in regions of the brain important in stress, motivation and homeostatic regulation. These include the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), central nucleus of the amygdala (CEA), basolateral amygdala (BLA) and dentate gyrus (DG), areas of the limbic forebrain known to be sensitive to glucocorticoid hormones (Amir et al., 2004; Lamont et al., 2005). We showed previously that surgical removal of the adrenal glands and the daily rhythmic replacement of glucocorticoids abolishes and restores, respectively, the rhythmic expression of PER2 in the BNSTov and CEA in rats (Amir et al., 2004; Lamont et al., 2005; Segall et al., 2006). Adrenalectomy had no effect on PER2 rhythms in the BLA or DG, demonstrating that the effect of glucocorticoids in the limbic forebrain is region specific. More recently, we found in a separate series of experiments that the rhythmic expression of PER2 in all of these regions of the limbic forebrain, glucocorticoid sensitive or not, can be synchronized by timed restricted feeding (TRF) schedules suggesting that the mechanisms that mediate the effect of glucocorticoids and TRF on PER2 expression in these regions are dissociable (Verwey et al., 2007; Waddington Lamont et al., 2007). To explore this hypothesis directly, we examined the effect of TRF in PER2 rhythms in the limbic forebrain of intact and adrenalectomized (ADX) rats.

EXPERIMENTAL PROCEDURES

All experimental procedures were in accordance with the Animal Care Committee of Concordia University and followed the guidelines set by the Canadian Council on Animal Care. Every effort was made to reduce the number of animals used and to minimize potential suffering. Male Wistar rats weighing 225–250 g were purchased from Charles River Canada (St. Constant, Quebec, Canada). The rats were housed individually in clear plastic cages equipped with a running wheel, under a 12h:12h light/dark (LD) cycle. The cages were housed in sound-attenuated and lightproof isolation chambers equipped with a computer-controlled lighting system (VitalView, Mini-Mitter, Sunriver, OR, USA). Running-wheel activity was collected by VitalView software (Mini-Mitter) and analyzed with Circadia software.

Bilateral adrenalectomies were performed under isoflurane anesthesia via the dorsal approach, 1 week following arrival to the laboratory. ADX rats were given free access to 0.9% saline drinking solution throughout the experiment. Plasma corticosterone levels were measured on tail blood samples collected at the end of the study using ELISA to verify successful adrenalectomy.

During TRF schedules standard rat chow was presented at zeitgeber time (ZT) 4 and removed at ZT7 each day for 10 days. On the final day of the TRF schedule, rats were deeply anesthetized with an overdose of sodium pentobarbital (~100 mg/kg) at one of four ZTs (ZT5, 11, 17, 23). They were then 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

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Abbreviations: AdLib, *ad libitum*; ADX, adrenalectomized; ANOVA, analysis of variance; BLA, basolateral amygdala; BNSTov, oval nucleus of the bed nucleus of the stria terminalis; CEA, central nucleus of the amygdala; DG, dentate gyrus; LD, light/dark cycle; PER2, Period 2; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator-1alpha; SCN, suprachiasmatic nucleus; TRF, timed restricted feeding; ZT, zeitgeber time.

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(pH 7.3). Serial coronal brain sections (50 μm) were taken using a vibratome.

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, USA). 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 (v1.8, S. D. Barrett, http://www.ImageSXM.org.uk). Cells immunopositive for PER2 were counted using the captured images. For analysis, the mean number of PER2-immunoreactive cells per region was calculated for each animal from the counts of six unilateral images showing the highest number of labeled nuclei, as previously described (Amir et al., 2004). Differences between groups were revealed with analyses of variance (ANOVA). Alpha level was set at 0.05 for all analyses.

RESULTS

Examples of circadian wheel running activity rhythms in intact and ADX rats housed under a 12-h LD schedule with free access to food (*ad libitum*, AdLib) or under TRF are shown in Fig. 1. Under AdLib conditions all rats, whether intact or ADX, exhibited robust wheel running activity rhythms entrained to the 12-h LD cycle. Under TRF both intact and ADX rats exhibited changes in daily running patterns and developed anticipatory running wheel bouts which began 2–3 h before daily food presentation (Fig. 1).

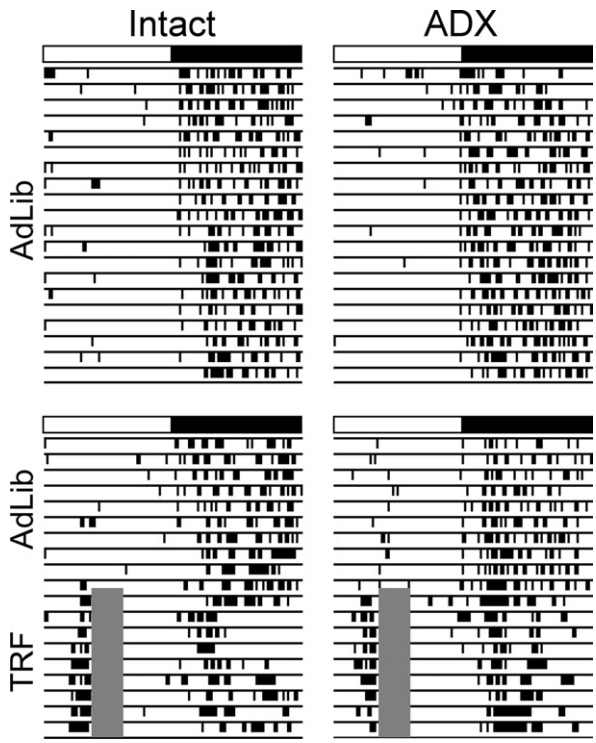

Fig. 1. Actograms of wheel-running activity from representative intact and ADX rats given free access to food (AdLib) or placed under TRF for 10 days. The daily presentation of food occurred from ZT4-7 (4–7 h after lights-on; illustrated by rectangles). All rats were housed under a 12-h LD cycle which is illustrated by the bars at the top of each actogram. The vertical marks indicate periods of activity of at least 10 wheel-revolutions/10 min. Successive days are plotted from top to bottom.

There were no noticeable differences in the pattern or magnitude of food anticipatory running between ADX and intact rats, consistent with previous evidence that circulating glucocorticoids are not critical for the development or expression of food anticipation under TRF (Stephan et al., 1979; Boulos et al., 1980).

The daily patterns of PER2 expression in the suprachiasmatic nucleus (SCN) and limbic forebrain of intact and ADX rats with free access to food (AdLib) or under TRF are shown in Fig. 2. In AdLib rats with intact adrenals, PER2 expression in the SCN, BNSTov and CEA peaked around the time of transition from day to night (ZT11) and that in BLA and DG peaked around the time of transition from night to day (ZT23, Fig. 2, left panel). Adrenalectomy selectively blunted the rhythm of PER2 expression in BNSTov and CEA (one-way ANOVA across time of day: BNSTov, $F[3,13]=2.08$, $P=0.1$; CEA, $F[3,13]=2.35$, $P=0.1$) without affecting rhythms in the SCN, BLA and DG (SCN, $F[3,13]=233.7$, $P<0.0001$; BLA, $F[3,13]=42.41$, $P<0.0001$; DG, $F[3,12]=11.67$, $P<0.0007$), as previously described (Fig, 2, left panel) (Amir et al., 2004; Lamont et al., 2005; Segall et al., 2006). Examples of PER2 in the SCN and BNSTov in freely fed, ADX rats are shown in Fig. 3. In intact rats, as expected, TRF shifted and synchronized the rhythms of PER2 in all regions with peak expression seen 12 h after food presentation (ZT17). In ADX rats TRF produced a pattern of PER2 expression in the BNSTov and CEA similar to that seen in intact rats (one-way ANOVA across time of day: BNSTov, $F[3,13]=24.98$, $P<0.0001$; CEA, $F[3,13]=54.57$, $P<0.0001$), and as in intact rats, these rhythms were synchronized with those in BLA and DG (Fig. 2, right panel). Examples of PER2 in the SCN and BNSTov in ADX rats under TRF are shown in Fig. 3. The results from two-way ANOVAs carried out for each brain region to assess differences between intact and ADX rats as a function of feeding condition (AdLib or TRF) and time of day are shown in Table 1.

DISCUSSION

Adrenal glucocorticoids can induce and entrain the expression of clock genes in tissues and cells *in vitro* and have been proposed as potential synchronizers of circadian clock gene rhythms in peripheral tissues *in vivo* (Balsalobre et al., 2000a,b; Reddy et al., 2007). Furthermore, they have been found to be essential circadian regulators of rhythmic PER2 expression in the BNSTov and CEA in rats (Segall et al., 2006). Based on these observations one might have predicted that the effect TRF on PER2 rhythms in the BNSTov and CEA would be attenuated or even completely blocked in the absence of adrenal glucocorticoids. Contrary to this, however, we found that the expression and synchronization of behavioral and limbic forebrain PER2 rhythms by TRF is not affected by ADX. This finding is consistent with our hypothesis outlined above that the mechanisms that mediate the effect of glucocorticoids and TRF on PER2 expression in these regions are dissociable.

Our finding that the pattern of light-entrained behavioral rhythms and the development and expression of food