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Double-stranded RNA-mediated suppression of PER2 expression in the suprachiasmatic nucleus disrupts circadian locomotor activity in rats

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List of Abbreviations

ANOVA: Analysis of variance

BNSTov: Oval nucleus of the bed nucleus of the stria terminalis

bp: Base pairs

cDNA: Complementary deoxyribonucleic acid

CEA: Central nucleus of the amygdala

DD: Constant darkness

DG: Dentate gyrus

dsRNA: Double-stranded ribonucleic acid

LD: Light-dark cycle

MMLV-RT: Moloney murine leukemia virus reverse transcriptase

PCR: Polymerase chain reaction

Per2: Period 2

RT: Reverse transcriptase

RNAi: RNA interference

SCN: Suprachiasmatic nucleus

ZT: Zeitgeber time

ABSTRACT

Circadian behavioral rhythms in mammals are controlled by a central clock located in the suprachiasmatic nucleus (SCN). PER2, the protein product of the clock gene, Per2, is expressed rhythmically in the SCN (Beaule et al., 2003) and has been implicated in the control of circadian behavioral rhythms based on the evidence that genetic mutations in the Per2 abolish free running locomotor activity rhythms in mice (Zheng et al., 1999, Bae et al., 2001). Such mutations eradicate PER2 expression in the SCN and disrupt the SCN molecular clockwork, however, they also affect PER2 in the rest of the brain and body leaving open the possibility that the changes in behavioral rhythms might be influenced, at least in part, by disruptions in PER2 functioning outside the SCN. We used RNAi-mediated transient knockdown of Per2 to study the effect of selective suppression of PER2 expression in the SCN, per se, on behavioral circadian rhythms. We found that transient suppression of PER2 in the SCN disrupted free running locomotor activity rhythms for up to 10 days in rats. Infusions of control dsRNA into the SCN or infusions of dsRNA to Per2 immediately dorsal to the SCN had no effect. These results constitute evidence for a direct link between PER2 expression in the SCN and the expression of behavioral circadian rhythms in mammals.

Keywords: Clock genes, Circadian rhythm, Oval nucleus of the bed nucleus of the stria terminalis, Central nucleus of the amygdala, Dentate gyrus, RNAi

Studies in mutant mice with targeted disruptions in the Per2 gene have led to the conclusion that PER2 is essential for the expression of behavioral circadian rhythms (Zheng et al., 1999, Bae et al., 2001). These rhythms are controlled by the SCN, however, there is no direct anatomical evidence linking PER2 expression in the SCN to behavioral circadian rhythms. Furthermore, mutations in Per2 can influence behavioral processes that are independent of the SCN. Thus changes in behavioral rhythms might be confounded by behavioral consequences of genetic disruptions of PER2 functioning outside the SCN (Abarca et al., 2002, Spanagel et al., 2005, Feillet et al., 2006). RNA interference (RNAi) is a powerful tool to selectively and transiently suppress the expression of proteins in specific regions of adult, developmentally intact brain, in vivo (Backman et al., 2003, Bai et al., 2003, Bhargava et al., 2004, Thakker et al., 2004, Musatov et al., 2006, Lasek et al., 2007). We used long dsRNA to Per2 to examine the effect of local and highly specific suppression of PER2 in the SCN, per se, on free running locomotor activity rhythms in rats. We also assessed the effect of suppression of PER2 in the SCN on the daily fluctuations in PER2 expression in areas of limbic forebrain known to be under the control of the SCN (Amir et al., 2004, Lamont et al., 2005b).

EXPERIMENTAL PROCEDURES

Animals and housing

Experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee, Concordia

University. Male Wistar rats (300-350g; Charles River Laboratories, St. Constant, QC, Canada) were individually housed in cages with running wheels and had free access to food and water. All cages were housed within sound- and light-proof enclosures equipped with a fluorescent light source and ventilation system. Running-wheel activity was monitored using VitalView software (Mini Mitter Co. Inc., Sunriver, OR) and analyzed with Circadia software (Behavioral Cybernetics, Cambridge, MA) as described (Amir et al., 2004).

dsRNA synthesis for RNAi

dsRNA to Per2 was synthesized using previously described methods (Bhargava et al., 2004). Briefly, rat brain total RNA (2 µg) was reverse-transcribed using random hexamers and MMLV-RT in a 20 µl volume (Applied Biosystems, Branchburg, NJ) as per manufacturer's specifications. Subsequent PCR was performed using 5 µl RT and Per2-specific primers (derived from GenBank # NM_031678.1). The primer set used was as follows: Per2 sense primer 5’cactgtaagaaggacgcctt3’ and antisense primer 5'aaggcgtccttcttacagtg3'. The 503 bp PCR products was analyzed by agarose gel electrophoresis, cloned in pTopo4 (Invitrogen, Carlsbad, CA) and sequenced to confirm identity. The Per2 construct was linearized with SpeI or NotI and sense and antisense RNA were transcribed from these linearized templates using T7 and T3 RNA polymerases respectively as described earlier (Bhargava et al., 2004). Rat ß-globin cDNA sequences were used as nonspecific control dsRNA in order to validate the sequence-specific effects of dsRNA to Per2 as discussed previously (Bhargava

et al., 2004). For infusions, 20 µg of control or 10 µg of Per2 long dsRNAs (1 µl) were mixed with 0.5 µl of lipofectAMINE (Gibco, BRL; (Dalby et al., 2004)) and the mix was incubated at room temperature for 30 minutes before use as previously described (Bhargava et al., 2004).

Microinfusions

Rats were anaesthetized with a ketamine (100 mg/ml) / xylazine (20 mg/ml) mixture (1.5 ml/kg, i.p.). A 30 gauge needle was lower into the SCN using the following stereotaxic coordinates: 1.2 mm posterior to Bregma; 1.8 mm lateral to the midline; 9.3 mm below the surface of the skull. All rats received bilateral infusions. Each infusion of dsRNA to Per2 consisted of 6 µg/1.5 µl/side (the highest concentration possible) or ß-globin (1.5 µl/side) into the SCN was made over a 10-min interval using an infusion pump. Needle placements were determined histologically at the end of the study.

Tissue preparation and immunocytochemistry

Rats were anaesthetized with sodium pentobarbital (Somnotol, ~ 100 mg/kg) and 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). Brains were postfixed in 4% paraformaldehyde and stored at 4°C overnight. Serial coronal sections (50 µm) were collected using a vibratome and stored in Watson's cryoprotectant solution. Immunocytochemistry for PER2 was performed as previously described (Amir et al., 2004) using an affinity purified rabbit polyclonal

antibody raised against PER2 (1:800, Alpha Diagnostics International, San Antonio, TX). Immunocytochemistry for Fos was performed as described (Beaule et al., 2001) using a polyclonal cFos antibody raised in rabbit (1:100,000, Oncogene Sciences, Boston, MA).

Data analysis

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 or Fos were counted. The mean number of immunoreactive cells per region was calculated for each animal from the counts of 6 unilateral images showing the highest number of labeled nuclei. Data were analyzed using analysis of variance (ANOVA). Alpha level was 0.05.

RESULTS and DISCUSSION

We first assessed the effect of local infusions of dsRNA on PER2 in the SCN. Groups of rats (n=4/group) housed under 12:12h light-dark cycle (LD) were given bilateral infusions of dsRNA to Per2 or ß-globin during the middle of the day and perfused 3, 6 or 12 days later, at zeitgeber time (ZT) 13, time of peak expression of PER2 in the SCN (ZT0 indicates time of lights on under LD). Examples of PER2 expression in the SCN are shown in Fig. 1. Infusions of dsRNA to Per2 significantly suppressed PER2 expression in the SCN at all time intervals tested ($F(1, 18) = 43.04$, $p < .01$). The decrease in PER2 was transient, with maximal

suppression (59% relative to control rats infused with dsRNA to ß-globin) seen three days after the infusion. After six days the levels of PER2 were 36% below, whereas 12 days after the infusions they were 26% below those in the SCN of controls (Fig. 1).

In separate groups of rats, we assessed the effect of bilateral infusions of dsRNA into the SCN on free-running activity rhythms. Rats housed under LD for two weeks were given infusions of dsRNA to Per2 or ß-globin during the daytime and placed in constant darkness (DD) for 45 days. Representative actograms are shown in Fig. 2. Bilateral infusions of dsRNA to Per2 aimed at the SCN disrupted circadian wheel-running activity rhythms in 11 of 16 rats. Failure to affect circadian rhythms in the remaining five rats was associated with one or both inaccurate injector placements. Infusions of dsRNA to ß-globin into the SCN (n=8) or infusions of dsRNA to Per2 immediately dorsal to the SCN (n=2) had no effect. As evident in the actograms, affected rats exhibited fragmented activity patterns throughout the circadian cycle that lasted up to 9 days. Infusions of dsRNA into the SCN had no effect on body weight or general health. Periodgram analysis on the portions of the actograms showing fragmented wheel-running patterns revealed no rhythms in the circadian range (data not shown). After this period all affected rats displayed normal free running rhythms that were indistinguishable from those of control rats, indicating that the behavioral effects seen were not due to permanent SCN damage. The time course of loss and recovery of behavioral circadian rhythms following infusions of

dsRNA to Per2 in the SCN closely mirrors that of the loss and recovery of PER2 in the SCN, consistent with the hypothesis that the expression of behavioral rhythms are linked to the expression of PER2 in the SCN.

In a final experiment we studied the effect of dsRNA infusions into the SCN on the daily variation in PER2 expression in three limbic forebrain areas, the oval nucleus of the bed nucleus of the stria terminalis (BNSTov) central nucleus of the amygdala (CEA) and the dentate gyrus (DG). We have shown previously that the PER2 rhythms in these areas are controlled by the SCN (Amir et al., 2004, Lamont et al., 2005b). Accordingly we hypothesized that suppression of PER2 expression in the SCN would disrupt the daily variation in PER2 in the limbic forebrain. Groups of rats (n=4/group) housed under LD were treated with dsRNA to Per2 or ß-globin and perfused 6 days later at ZT1 or ZT13, times of minimal and maximal expression of PER2 in the SCN, BNSTov and CEA, and times of maximal and minimal expression in the DG. To study the specificity of the dsRNA, alternate brain sections were stained for Fos. Fos is induced in the core region of the SCN by light exposure in the morning, whereas in the evening expression of Fos in the core is low. In the shell region of the SCN Fos expression is generally high during the day and low at night (Beaule and Amir, 1999, Guido et al., 1999, Beaule et al., 2001).

As shown in Figs. 3 and 4, infusions of dsRNA to Per2 blunted the daily variation in PER2 in the SCN by suppressing expression at ZT13, consistent with the

findings shown in Fig. 1. The total levels of PER2 in the BNSTov, CEA and DG were not affected, however, the daily variation was significantly blunted in all three areas (Fig. 4), further demonstrating that these limbic forebrain PER2 rhythms depend on the integrity of the SCN clock. The results from ANOVAs carried out on the data from each area are shown in Table 1. It can be seen that dsRNA Treatment had no effect on the overall levels of PER2 in any region outside the SCN. The significant effects of Time reflect the daily variation, whereas the Treatment x Time interactions reflect the blunting of the daily variation in dsRNA treated groups. Contrary to these effects on PER2 expression, infusions of dsRNA to Per2 into the SCN had no effect on the daily pattern of expression of Fos in the SCN or limbic forebrain (Figs. 3, 4). In both the experimental and control groups Fos expression in the core region of the SCN was high at ZT1 and low at ZT13, whereas in the shell region Fos tended to be lower at ZT1 than ZT13. Similarly, Fos expression in the BNSTov, CEA and DG did not vary between treatment groups (Figs. 4). These results show that the suppression of PER2 in the SCN results from specific action of the dsRNA on Per2 and is not due to non-specific suppression of gene expression.

The present results demonstrate that local bilateral infusions of dsRNA to Per2, which was found to partially and transiently suppress PER2 in the SCN, are sufficient to disrupt free-running locomotor activity rhythms in rats housed in constant darkness. Importantly, dsRNA reduced PER2 expression in the SCN in rats housed under a LD cycle showing that it was effective under conditions in

which light might enhance PER2 expression in the SCN. Thus, the effects of dsRNA in DD may have been even greater, consistent with the complete, but reversible behavioral disruption in experiment 2. These results extend previous findings in Per2 mutant mice by providing anatomical evidence for a first direct link between PER2 expression in the SCN, per se, and circadian behavioral rhythms (Zheng et al., 1999, Bae et al., 2001). Furthermore, the present results show that suppression of PER2 in the SCN blunts the expected daily variation in expression of PER2 in the BNSTov, CEA and DG. This finding is consistent with our earlier results showing that bilateral lesions of the SCN, which disrupt wheel-running rhythms, blunt PER2 rhythms in the limbic forebrain in rats (Amir et al., 2004, Lamont et al., 2005b). Moreover, it is consistent with the evidence that constant light housing, which blunts the rhythm of PER2 in the SCN, disrupts locomotor activity rhythms and rhythms of PER2 in the limbic forebrain (Amir et al., 2004, Lamont et al., 2005a). It is important to point out that the loss of circadian rhythms of PER2 in the limbic forebrain, per se, could not account for the disruption of behavior rhythms seen in this study. For example, both adrenalectomy and thyroidectomy completely blunt the circadian rhythms of PER2 expression in the BNSTov and CEA without affecting circadian locomotor activity rhythms (Amir and Robinson, 2006, Segall et al., 2006). Finally, we found that infusions of dsRNA to Per2 into the SCN had no effect on Fos induction by light in the core region of the SCN or on the constitutive expression of Fos in the shell region. Furthermore, it had no effect on expression of Fos in the BNSTov, CEA and DG. These results not only confirm that the effect of the

dsRNA to Per2 is specific, but also show that the expression of Fos and PER2 in the SCN and limbic forebrain can be dissociated as we have shown previously (Beaule and Amir, 2003, Verwey et al., 2007).

PER2 is expressed rhythmically in many brain regions outside the SCN (Shieh, 2003, Sellix et al., 2006) and evidence from Per2 mutant mice indicates that PER2 participates in behavioral processes that are independent of the SCN, such as food anticipatory behavior (Feillet et al., 2006), sensitization to the behavioral activating effects of cocaine (Abarca et al., 2002), and alcohol preference (Spanagel et al., 2005). The importance of these extra-SCN rhythms is underscored by the evidence that they can be influenced directly by changes in circulating hormones (Amir and Robinson, 2006, Perrin et al., 2006, Segall et al., 2006) and other perturbations that affect behavioral state, such as restricted feeding (Lamont et al., 2005a) and exposure to drugs (Yamamoto et al., 2005). There is currently, however, no evidence to link directly the expression of PER2 within specific brain areas to specific behavioral or physiological outputs. The present experiments show that long dsRNA-mediated RNAi works effectively to achieve transient, tissue specific knockdown of PER2 expression in the brain of adult, developmentally intact rats. As such, our dsRNA provides a powerful new tool to elucidate the behavioral and neural correlates of PER2 within select brain regions.

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Figure captions

Fig. 1

Examples of PER2 expression on one side of the SCN of experimental (dsRNA/Per2) and control (dsRNA/b-globin) rats killed at ZT13, three, six and 12 days after infusion (Scale bar = 200μm). Right panel, graphs showing mean (±SEM) number of PER2-immunoreactive (PER2-IR) cells in the SCN in experimental and control rats (n=4/group). Asterisks indicate significant difference from control ($p<0.05$).

Fig. 2

Double-plotted actograms of wheel-running activity of representative experimental (dsRNA/Per2) and control (dsRNA/Per2) rats before and after treatment. Each horizontal line represents a 48 hr period; successive days are plotted from top to bottom. Vertical lines indicate periods of activity of at least 10 wheel-revolutions/10 min. Animals were initially housed in a 12L:12D light-dark cycle (LD). They were transferred to constant darkness (DD) following the infusions (indicated with a star). The schedule of the light-dark cycle is indicated by the bar above each record. The period of loss of wheel running circadian rhythm is indicated with a black rectangle on the actogram.

Fig. 3

Examples of PER2 and Fos expression on one side of the SCN of experimental (dsRNA/Per2) and control (dsRNA/b-globin) rats killed at ZT1 or ZT13, six days after infusion (n=4/group). Broken white lines on the Fos images point to the core-shell boundaries. Scale bar = 200μm.

Fig. 4

PER2 and Fos expression (means±SEM) in the SCN, BNSTov, CEA and DG of experimental (dsRNA/Per2) and control (dsRNA/b-globin) rats as a function of time of day, six days after intra-SCN infusions. Brain maps indicating the location of regions under study are shown on the left. Asterisks indicate significant difference from corresponding control group ($p<0.05$).

Table 1

Table 1: Results from ANOVAs carried out to assess the effect of Treatment (dsRNA/Per2 vs. dsRNA/□-globin), Time of Day (ZT1 vs. ZT13) and Treatment x Time of Day interactions on PER2 expression in each brain area under study

Brain area	Treatment	Time of Day	Treatment x Time
SCN	$F_{1,12}$=4.84, P<0.05.	$F_{1,12}$=71.55, P<0.0001	$F_{1,12}$=24.61, P<0.0003
BNSTov	$F_{1,12}$=3.42, n.s.	$F_{1,12}$=28.69, P<0.0002	$F_{1,12}$=38.21, P<0.0001
CEA	$F_{1,12}$=1.71, n.s.	$F_{1,12}$=22.58, P<0.0005	$F_{1,12}$=10.37, P<0.007
DG	$F_{1,12}$=2.91, n.s.	$F_{1,12}$=71.19 P<0.0001	$F_{1,12}$=7.45, P=0.02

Figure-1

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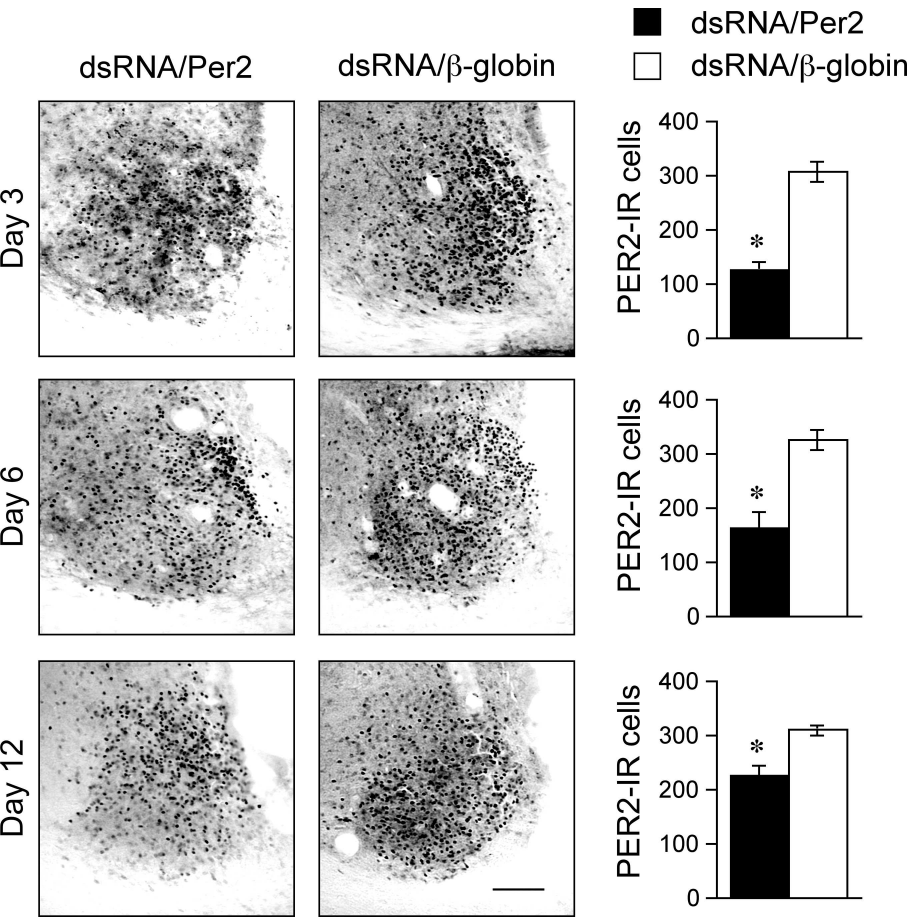

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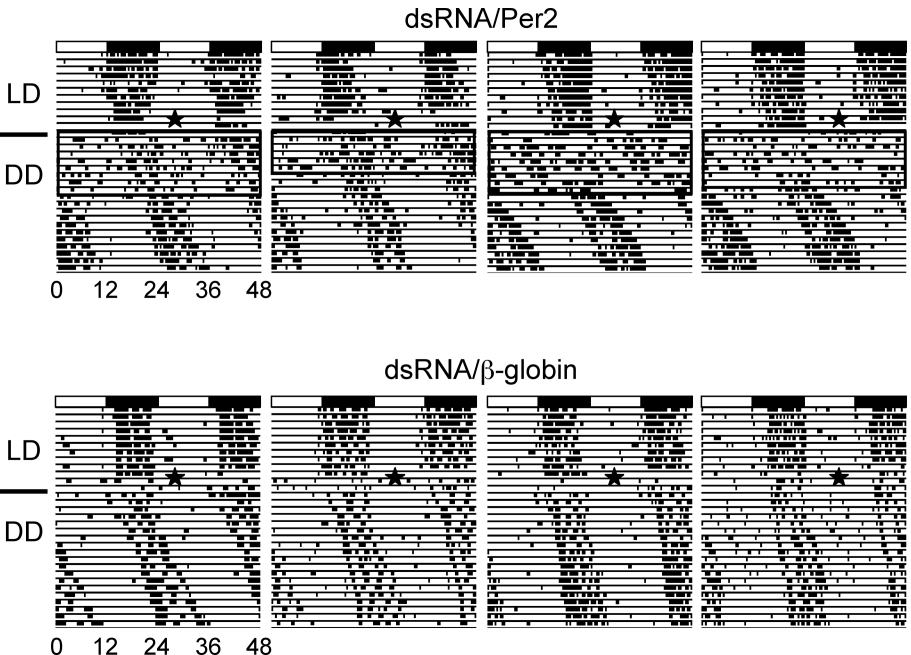

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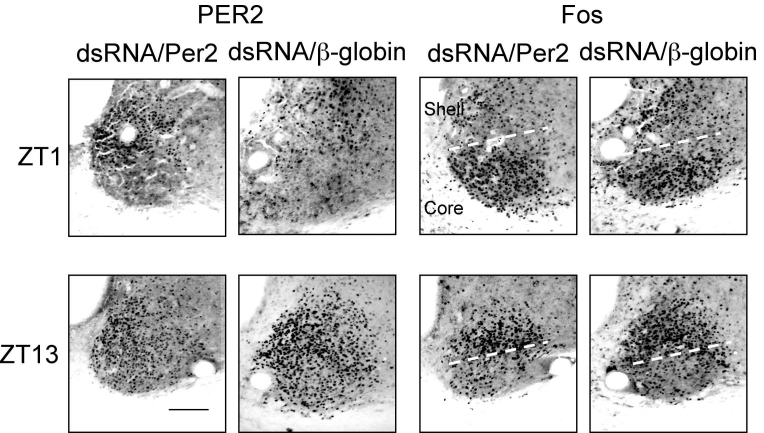

Figure-4

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