Long Double-Stranded RNA-Mediated Suppression of PER2 in the SCN Disrupts Circadian Locomotor Activity and PER2 Rhythms in the Limbic Forebrain

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ABSTRACT

Long Double-Stranded RNA-Mediated Suppression of PER2 in the SCN Disrupts Circadian Locomotor Activity and PER2 Rhythms in the Limbic Forebrain

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Studies with targeted disruption in the Period2 (*Per2*) gene suggest that the PER2 protein participates in the regulation of circadian behavioral rhythms. Moreover, it has been shown that direct suppression of PER2 expression in the suprachiasmatic nucleus (SCN) with antisense oligonucleotides disrupts photic resetting of the SCN clock. The effect of such suppression on behavioral rhythms is unknown. Here double-stranded RNA (dsRNA) to *Per2* was used to transiently suppress PER2 expression in the SCN of adult rats. Bilateral infusions of dsRNA into the SCN (6 μg/side) disrupted circadian wheel running activity rhythms for up to 10 days in experimental rats housed in constant darkness; whereas, control infusions into the SCN or dorsal infusions of dsRNA to Per2 had no effect. Relative to controls PER2 suppression in the SCN was evident 12 days post-dsRNA infusion; however, maximal suppression was observed at day 3. In addition to the suppression of PER2 expression in the SCN, a blunted PER2 rhythm was observed in the oval nucleus of the bed nucleus of the stria terminalis, central nucleus of the amygdala and dentate gyrus. These results provide direct evidence that the expression of PER2 in the SCN is essential for the maintenance of circadian locomotor activity rhythms and for the expression of PER2 rhythms in the limbic forebrain in rats. The specificity of this effect was validated by demonstrating no difference between cFos expression in any of the above areas in control rats and rats treated with dsRNA to *Per2*.

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Introduction

Since the beginning of the Cambrian era, which took place over 500 million years ago, the length of the Earth's day has been roughly 24 hours (Tauber, Last, Olive, & Kyriacou, 2004). Over the eons, all life forms on Earth, from the smallest prokaryotic bacteria to the largest and most complex eukaryotic organisms, have adapted and learned to incorporate, to the best of their ability, these daily cycles, or circadian rhythms, which are in part a response to daylight or dark. However, environmentally-determined cyclical oscillations, such as changes in daylight, temperature, and availability of food, serve primarily to refine and adjust physiologically-determined circadian rhythms: In the absence of these external cues, the internal rhythms gradually drift out of phase with the environment (Yamazaki, et al., 2000). The most evident of these biological rhythms is the sleep-wake cycle but there are other equally-important rhythms as well: locomotor-activity rhythms, daily internal temperature fluctuations, hormone secretion, protein synthesis and apoptosis to name a few (Lowrey & Takahashi, 2000).

Extensive research on *rattus norvegicus* (Kramer & Poort, 1971), *mus musculus* (Abraham et al., 2006), *phodopus sungorus* (Antle, LeSauteur, & Silver, 2005; Hoffmann, Illnerová, & Vaneček, 1981), *arvicanthis ansorgei* (Caldelas, Poirel, Sicard, Pevet, & Challet, 2003), *pan troglodytes* (Anestis & Bribiescas, 2004) and *homo sapiens* (Roenneberg, Kumar, & Merrow, 2007) has shown that mammals have a light-entrainable central pacemaker, located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN synchronizes all other circadian oscillators in the brain and the periphery which ultimately give rise to behavioral and physiological rhythms (Albrecht, in press; Lowrey & Takahashi, 2000). Despite great strides made in the field of circadian

research in recent years, the mechanisms through which the SCN entrains subordinate oscillators are still poorly understood (Reppert & Weaver, 2002). While measuring the rhythmic expression of the clock protein PERIOD2 (PER2), our laboratory has uncovered circadian oscillators in the oval nucleus of the bed nucleus of the stria terminalis (BNST-ov), central nucleus of the amygdala (CEA), basolateral amygdala (BLA) and dentate gyrus of the hippocampus (DG; Amir, Lamont, Robinson, & Stewart, 2004; Lamont, Robinson, Stewart, & Amir, 2005).

Studies in mutant mice with targeted disruption in the *Per2* gene suggest that the PER2 protein participates in the regulation of circadian behavioral rhythms (Zheng, et al., 1999). In fact, a number of studies reported complete circadian arrhythmicity in *Per1/Per2* double mutant mice (Albrecht, Zhend, Larkin, Sun, & Lee, 2001; Zheng, et al., 1999). Mutant studies are limited when one is looking for specific gene silencing because the genes of interest are knocked out in virtually all cells of the organism and there are numerous developmental confounds. Other studies have used antisense oligonucleotides (ODNs) in order to induce temporary *Per2* gene silencing in cultured SCN neurons (Sugiyama, Yoshioka, & Ikeda, 2004). Wakamatsu and colleagues (2001) have transiently suppressed *Per2* gene expression in mice by repeatedly performing unilateral ODNs microinjections into the lateral ventricle. Although this showed that suppression of *Per2* can lead to changes in locomotor activity, evidence for a direct link between circadian rhythms in behavior and PER2 expression in the SCN is lacking. While ODNs are able to successfully suppress gene expression, their effect is short lasting and lacks specificity (Kurreck, 2003). The purpose of this thesis is to elucidate the role of PER2 in the SCN by investigating the transient suppression of *Per2* expression

in the SCN of adult rats using long double-stranded RNA (dsRNA) to *Per2*, which is longer-lasting and has greater specificity than antisense ODNs.

The suprachiasmatic nucleus and light entrainment

In mammals, the focal point of the circadian system is located in the SCN, a small bilateral structure of the anterior hypothalamus containing approximately 10,000 neurons per hemisphere, which is commonly referred to as the master clock (Reppert & Weaver, 2002; Stephan & Zucker, 1972; van den Pol, 1980). This is the case because it has been shown that lesioning the SCN results in complete loss of circadian rhythms (Abe, Kroning, Greer, Critchlow, 1979). Furthermore, it has also been shown that surgically transplanted fetal SCN tissue was able to fully restore behavioral rhythms in animals whose SCN had previously been lesioned (Ralph, Foster, Davis, & Menaker, 1990). Moreover, Guo and colleagues (2006) found that SCN transplants from wild-type hamsters were also able to restore circadian rhythmicity in *tau* mutants, which are normally arrhythmic. It is also interesting to note that each SCN neuron is able to independently generate its own circadian rhythm when removed from the SCN (Liu & Reppert, 2000; Welsh, Logothetis, Meister, & Reppert, 1995), yet within the intact SCN neurons couple to form strong synchronized circadian rhythms such as metabolic activity (Schwartz & Zimmerman, 1991) and gene expression (Hastings & Herzog, 2004; Reppert & Weaver, 2002).

Photic (light) information enters the retina and is transmitted via the retinohypothalamic tract (RHT; Panda & Hogenesch, 2004), which is distinct from the pathway involved in the interpretation of visual stimuli, directly to the SCN (Berson, Dunn, & Takao, 2002). While photic information affects the SCN the most (Wakamatsu

et al., 2001), it is also true that a number of non-photic cues such as one's health (Hastings, Reddy, & Maywood, 2003), social interactions (Roenneberg, Kumar, & Merrow, 2007), feeding schedules (Verwey, Khoja, Stewart, & Amir, 2007), and olfactory cues (Amir, Cain, Sullivan, Robinson, & Stewart, 1999) have been found to also have a significant impact on the circadian system. However, these cues affect mostly molecular oscillators located in brain regions other than the SCN (Abe et al., 2002).

Feedback loops and clock genes

The SCN regulates and coordinates overt behavioral and physiological rhythms in the brain and peripheral tissues (Peirson et al., 2006). All circadian rhythms appear to arise from the interaction of a small set of core clock genes which make up the molecular clockwork (Ko & Takahashi, 2006; Lowrey & Takahashi, 2004). Circadian clocks express an autonomous oscillator which features positive and negative feedback loops that can be entrained to environmental synchronizers via various input pathways in order to give rise to overt rhythms. The main clock genes involved in this oscillatory system include *Clock* and *Bmal1*, comprising the positive arm, and *Cryptocrome* (*Cry1*, *Cry2*) and *Period* (*Per1*, *Per2*, *Per3*), comprising the negative arm of the feedback loop (Ko & Takahashi, 2006; Reppert & Weaver, 2002). Clock genes and their respective feedback loops are not solely expressed in the SCN; in fact, they are found in many other tissues and organs throughout the body (Glossop & Hardin, 2002). However, SCN-lesioning experiments have shown that although various organs such as the lungs and the liver still exhibit circadian rhythms in gene expression, acute arrythmicity occurs once these peripheral oscillators are deprived of outputs from the SCN for several cycles (Yamazaki

et al., 2000). These findings support the idea that the SCN's main role is to maintain the rhythms of self-sustained cellular oscillators both in the brain and in the periphery.

Most experiments investigating the mechanisms generating circadian oscillation at the molecular level have been conducted with peripheral tissue because of the ease of access and the large amount of tissue available. Certain types of cells, such as liver cells (Reddy & Maywood, 2007; Reppert & Weaver, 2002), islets of Langerhans cells (Allaman-Pillet et al., 2004; Mühlbauer, Wolgast, Finckh, Peschke, & Peschke, 2004) or exocrine cells from the pancreas (Kramer & Poort, 1971) for instance, have rapid division rates making them excellent candidates for *in vitro* studies. While these studies are invaluable when trying to pinpoint the function of certain circadian oscillators, it is only by also being able to conduct these studies *in vivo*, that one can drive more complete conclusions of the exact function of these oscillators. After all, only when looking at the entire organism, is it possible to understand the multitude of interacting feedback loops from the various tissues and organs, which may interact with outputs from the SCN.

From a phylogenic point of view, the *Per* genes are thought to be the most rapidly evolving genes regulating circadian oscillators (Tauber et al., 2004). Given the fact that they are also among the genes that affect circadian oscillations the most, it is not surprising to see that such a vast part of the circadian literature has been focusing on understanding their function (Lowrey & Takahashi, 2000; Reppert & Weaver, 2002).

Mutant experiments

Mutant *Per2* experiments have found that mice without *Per2* function have transient rhythms with a shortened period of 22 hr (Zheng et al., 1999). While considering a different allele of the *Per2* gene, Bae and colleagues (2001) even reported

complete arrythmicity in *Per2* mutant mice placed in a normal light/dark (LD) cycle. Furthermore, most mutant mice lose rhythmicity when placed in constant darkness (Zheng et al., 2001). One should also note that *Per1* and *Per2* double mutants exhibit complete loss of their circadian rhythms (Albrecht, Zheng, Larkin, Sun, & Lee, 2001; Zheng et al., 1999). Despite the fact that mutant *Per1* and *Per2* mice are morphologically indistinguishable from their wild-type counter-parts, suggesting that these genes are not essential for proper cell differentiation during development, these animals display various symptoms of premature aging: more rapid decline in fertility, loss of soft tissues, development of kyphosis, hyperplasia of the salivary glands (Lee, 2006; Lowrey & Takahashi, 2000), and a slight increase in the number of cancerous tumors (Granda et al., 2005). Due to the fact that *Per2* mutants seem to experience such a wide range of symptoms, a more specific method of targeting *Per2* is needed if one is to study its impact on circadian rhythms exclusively. While one can observe severe disruption of circadian rhythms in *Per2* mutants, one cannot entirely exclude the potentially additive disruptive effects due in part to the development of kyphosis, an arching of the back which can slightly reduce mobility, on locomotor activity. Due to these and other confounds there is little concrete evidence for a direct link between *Per2* expression in the SCN and the disruption of circadian rhythms in behavior.

Gene silencing with antisense oligonucleotides

In order to link *Per2* expression in the SCN to circadian rhythms, one requires local silencing of the gene and this can be achieved through the use of antisense ODNs (Wakamatsu et al., 2001). These are short single-stranded (typically 19 to 25 base-pair-long) molecules which are complementary to specific messenger RNA (mRNA)

sequences. During transcription, mRNAs are formed from a DNA strand as various enzymes, such as RNA polymerase, copy the genetic code of specific genes. Subsequently, the mature mRNA leaves the cell nucleus and is expelled into the cytoplasm. It is then intercepted by ribosomes which attach to it and subsequently trigger translation of the genetic code contained within the single-stranded mRNA into the various proteins it codes for (Fire, 1999). At the same time, transfer RNA (tRNA) supplies the ribosomes with the required amino acids to constitute new proteins. However, if one were to inject antisense ODNs in the neighboring region of a cell, the antisense would be able to pass across the cellular membrane's pores and penetrate into the cytoplasm. There, the antisense has a high affinity to bind with its complementary base pairs located on mRNA strands. Once it detects a suitable piece of mRNA, the antisense attaches to it. Ribosomes are unable to translate double-stranded mRNA and thus the proteins for which the mRNA is coding for will not be formed. As a result, one is able to inhibit protein synthesis by injecting antisense ODNs into the surrounding tissue of a region of interest. The disadvantages of this technique are threefold: 1) It has been reported that in some cases only seven nucleotides binding to the proper complimentary mRNA strand suffice to halt translation (Fire et al., 1998). Hence, one can shut down the synthesis of multiple proteins which have partial complimentarity to the ODN used. Animal studies have shown that this can have potentially lethal side effects ranging from decreased heart rate to significant drops in white blood cell counts (Dias & Stein, 2002). 2) ODNs attach to the mRNA through the formation of hydrogen bonds, however, given the shortness of each antisense strand, not many bonds form and as a result the suppression effect is only short lasting as various enzymes, known as

RNases, degrade the hydrogen bonds (Dias & Stein, 2002). Once the hydrogen bonds are broken, ribosomes are again able to attach to the now single-stranded mRNA and recommence protein synthesis. 3) Each ODN is only able to bind to one mRNA strand and is generally degraded in a few hours (Choleris et al., 2007). Therefore, repeated injections with high concentrations are required in order to have a longer lasting and more potent effect. By increasing the concentrations however, one also increases the likelihood of cytotoxicity and off-target effects (Fire et al., 1998).

RNA interference

Another method of silencing genes is through the use of double-stranded RNA (dsRNA) to activate what is now termed the RNA interference (RNAi) pathway. There are two sub-types of RNAi: 1) Post-transcriptional gene silencing (PTGS) in which transcription of the target locus is unaffected but the half-life of the target RNA is significantly decreased (Montgomery, Xu, & Fire, 1998). 2) Transcriptional gene silencing (TGS) in which the structure of the chromatin template which forms the DNA strands is modified (Matzke et al., 1989). The latter is usually longer lasting than the former because RNA polymerase is unable to commence transcription. Therefore, if one is interested in silencing a given gene only for a few days, it is preferred to use PTGS by injecting long dsRNA.

Most long dsRNA are processed into short interference RNA (siRNA) rather effectively by an RNase-like enzyme named Dicer; however, dsRNA can induce a non-specific interferon response in mammalian cells when it is injected in large quantities (Minks, West, Denvin, & Baglioni, 1979; Pestka & Langer, 1987). Dicer recognizes dsRNA as it penetrates the cytoplasm and cleaves it into small siRNA fragments

(Dykxhoorn, Novina, & Sharp 2003). Other enzymes, such as Argonaute bind to the siRNAs and form the RNA-induced silencing complex (RISC), a catalytic complex responsible for target mRNA cleavage (Schwarz et al., 2003). Each RISC contains only one of the two strands of the siRNA; some suggest that both strands are able to successfully induce RNAi if they are able to find an appropriate target (Martinez, Patkaniowska, Urlaub, Lührmann, & Tuschl, 2002), while others maintain that only the antisense strand which is less tightly paired to its complement, is incorporated into RISC while the other is degraded (Schwarz et al., 2003). Either way, RNAi through the use of long dsRNA is an effective method of transiently suppressing gene expression in a given structure for several days (Bhargava, Dallman, Pearce, & Choi, 2004).

Rationale and objectives

Although it has been shown that direct suppression of *Per2* gene expression in the SCN with antisense ODNs disrupts regular function of the clock (Sugiyama et al., 2004), the effect of suppression of *Per2* in the SCN on behavioral rhythms is unknown. In order to attempt to clarify the role of *Per2* in the SCN on locomotor activity and PER2 expression in SCN-targeted structures such as the CEA, BNST-ov and DG, I performed bilateral injections of dsRNA to *Per2* into the SCN of male rats.

In experiment 1, rats were placed in constant darkness after bilateral SCN microinjections of long dsRNA to *Per2* in order to assess the effects of transient gene suppression on locomotor activity. Experiment 2 investigated the masking effect of light on PER2 expression in the SCN and limbic forebrain. This was achieved by placing rats, following surgery, back under the same LD cycle they were previously entrained to. Finally, experiment 3 looked at the specificity of the long dsRNA to *Per2* used, by

labeling alternate brain sections for PER2 and cFos immunoreactivity. Unlike PER2 (Beaulé, Houle, & Amir, 2003) or cFos expression in the shell of the SCN (SCN-sh; Beaulé, Arvanitogiannis, & Amir, 2001), cFos expression in the core of the SCN (SCN-co) is regulated by entraining light (Beaulé & Amir, 1999). Therefore, cFos immunoreactive nuclei were counted separately in the SCN-sh and SCN-co.

Method

Subjects

Adult male Wistar rats (Charles River Laboratories, St-Constant, QC) weighing between 315 gm and 425 gm on the day of surgery were used in these experiments. The rats were housed individually in clear plastic cages (50 cm X 27 cm X 37 cm) which were equipped with running wheels. The rats had *ad libitum* access to food and water during the entire experiment. The room in which the animals were housed was maintained at approximately 21 °C throughout the testing period. All procedures were in accordance with the Canadian Council on Animal Care guidelines and were approved by the Animal Care Committee at Concordia University.

Apparatus

All cages were individually housed within sound- and light-proof enclosures (66 cm X 66 cm X 44 cm). Each enclosure contained a fluorescent light source (outputting ≈ 300 lux), which was computer-controlled using VitalView (MiniMitter Co. Inc., Sunriver, OR), as well as a ventilation system to ensure proper airflow. Running-wheel activity was recorded using a magnetic switch connected to the VitalView system. Actograms were generated using Actiview (v1.2, MiniMitter Co. Inc., Sunriver, OR) and

the data were subsequently analyzed using Circadia Software (Behavioral Cybernetics, Cambridge, MA) in order to display and analyze running-wheel activity levels throughout the experiments.

Procedure

Each rat was entrained to a 12:12 hr LD cycle for at least 10 days before any manipulation took place. Throughout this period, locomotor activity was measured. This served a dual purpose: It provided a baseline activity level for all animals while at the same time serving as a measure of light entrainment for each individual. Rats were randomly divided into 2 groups: The experimental group received bilateral long double-stranded RNA (dsRNA) to *Per2* injections (6 μg in a volume of 1.5 μl per side) into the SCN, while the control group received bilateral injections of ß-globin dsRNA (1.5 μl per side) into the SCN. After surgery, the rats were placed back into their respective home cages. In experiment 1, rats (n = 16) were placed back in constant darkness for 46 days while their activity levels were recorded. A second group of rats (n = 6) were placed in constant dark for 21 days. Afterwards, they were placed under a 12:12 hr LD cycle which was the reverse of the one which they had initially been entrained to. In experiment 2, rats (n = 24) were placed back in the same LD cycle and were perfused three, six or 12 days after dsRNA microinfusions. A second group (n = 2) was bilaterally infused with dsRNA 1.5 mm dorsal to the SCN. Finally, in experiment 3, rats (n = 16) were entrained to a 12:12 hr LD cycle for 10 days. Afterwards, eight rats were bilaterally injected with dsRNA to *Per2* and perfused six days later while the others were given placebo injections. Half of the rats of either group were perfused at ZT1 while the others

were perfused at ZT13. Alternate slices were stained for cFos and PER2 in order to test the specificity of the long dsRNA used in the present experiments.

dsRNA synthesis

Long dsRNA specific for *Per2* was amplified by reverse transcription polymerase chain reaction and cloned in TopoII vector (Invitrogen, Burlington, ON) by Dr. Aditi Bhargava and her research team at the University of California, San Francisco. The resulting dsRNA was then placed in eppendorf tubes containing 6 μg of lyophilized dsRNA each.

dsRNA reconstitution

Each eppendorf tube containing 6 μg of lyophilized dsRNA was reconstituted by adding 1 μl of RNase-free water. The tubes were let to sit at room temperature for 15 min after vortexing for 15 s and briefly centrifuging the mixture. Subsequently, 0.5 μl of Lipofectamine (Invitrogen) was added to each tube, followed by a brief 5 s of vortexing and centrifuging. Finally, the mixture was let to incubate at room temperature for 30 min before being ready to be injected into the SCN.

Surgery and injections

The rats were deeply anesthetized with a solution of ketamine/xylazine with concentrations of 100 mg/ml and 20 mg/ml respectively, administered intraperitoneally (0.15 ml/100 gm). Once anesthetized, the rats were securely fitted into a stereotaxic apparatus (Stoelting, Wood Dale, IL). The surface of the scalp was disinfected with iodine and a 1.5 cm long incision was made at the center of the skull. Two small holes were drilled at the appropriate coordinates from bregma for the SCN: anterior/posterior, -

1.2 mm; and medial/lateral, ± 1.8 mm. Two Hamilton 2.0 µl syringes were loaded with 1.5 µl dsRNA each and using the stereotaxic, two specially-designed needle tips were slowly lowered to – 8.3 mm below dura at a 10 ° angle in order to avoid hitting the sinuses. Once the proper depth was reached, the dsRNA was bilaterally injected over a 10 min interval using an infusion pump (Harvard Apparatus, Holliston, MA). Afterwards, an additional 2 min interval was given in order to allow the surrounding tissue to completely absorb any remaining dsRNA. The needles were then retracted and the holes in the skull were filled with sterile bone wax and subsequently covered with a layer of sticky wax. Hibitane (Bioglan), an antifungal and antiseptic cream, was then applied locally in order to prevent infection and the lesion was sutured. The rats were then given a 0.15 ml (0.075 ml in each leg) intramuscular injection of Procilin (MTC Bimeda, Cambridge, ON), an antibiotic and analgesic. Finally, each rat received a 2.0 ml (1.0 ml per side) subcutaneous injection of saline 0.9 % in order to facilitate re-hydration. After awaking, each rat was taken back to its home cage where it remained for the rest of the experiment.

Perfusions

The rats were deeply anesthetized with an overdose of sodium pentobarbital (≈ 100 mg/kg, Somnotol, MTC Bimeda) administered intraperitoneally and perfused intra-cardially with 300 ml of cold saline solution (0.9 % NaCl), followed by 300 ml of cold 4 % paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3). Subsequently, the brains were post-fixed for 24 hr in 4 % paraformaldehyde at 4 °C. The following day, the brains were sliced using a vibratome (Vibratome, St-Louis, MO) into 50 µm thick coronal sections while being placed in cold trizma buffered saline (TBS). After a series of three

10 min TBS rinses to remove any remaining traces of paraformaldehyde, the sections were transferred into Watson's cryoprotectant and maintained at -20 °C until immunocytochemistry was performed.

Immunocytochemistry

Brain sections were taken out of Watson's cryoprotectant and rinsed in six 10 min cold TBS washes to remove any excess cryoprotectant. Brain sections were then transferred into quenching solution, consisting of 90 % TBS and 10 % hydrogen peroxide (H_2O_2) for 30 min at room temperature. Three 10 min rinses in cold TBS followed. Sections were then transferred into pre-block solution (0.3 % Triton X in TBS and 5.0 % normal goat serum) for 1 hr at 4 °C. Sections were transferred directly into a primary antibody solution, consisting of rabbit polyclonal antibody raised against PER2 diluted to 1:2000 in Triton X-100 in TBS and normal goat serum (NGS), where they were incubated for 48 hr at 4 °C. Sections were rinsed for three times 10 min in cold TBS. Brain sections were incubated in a secondary antibody solution, constituted of biotinylated anti-rabbit IgG made in goat (Vector Laboratories, Burlington, ON) diluted 1:200 in Triton TBS and 3 % NGS, for 1 hr at 4 °C. Another three 10 min rinses in cold TBS followed. The sections were then transferred into ABC solution (avidin-biotin-peroxidase complex) in TBS for 2 hr at 4 °C (Vectastain Elite ABC Kit, Vector Laboratories). Sections were then rinsed for three 10 min periods in cold TBS followed by an additional 10 min rinse with cold 50 mM Tris-HCL. Afterwards, sections were incubated for 10 min in 0.05 % diaminobenzidine (DAB) in Tris-HCL. Following this step, sections were incubated for 10 min in DAB diluted in 50 mM Tris-HCL with 0.01 % H_2O_2 and 8 % Nickel Chloride ($NiCl_2$). Finally, the sections were rinsed with cold

TBS for three times 10 min and then mounted on gel-coated slides the following day. The next day, slides were dehydrated in alcohols of various concentrations for 10 min each, soaked in Citrosolve (Fischer Scientific, Houston, TX) for at least 30 min and coverslipped with Permount (Fischer Scientific).

Data analysis

Behavior

Actograms generated by the VitalView monitoring system were visually analyzed in order to ascertain entrainment and locomotor activity.

Brain

The coverslipped slides were inspected under a light microscope (Leitz Laborlux S) and images of the SCN, CEA, the BNSTov and DG were captured using a Sony XC-77 video camera connected to a Scion LG-3 frame grabber using NIH Image software (v1.63). Bilateral images were captured at 20X magnification using a 400 X 400 μm template. Cells which were immunoreactive for PER2 were counted using Image SXM software (v1.79) in all experiments. The average of the 6 sections containing the highest numbers of labeled nuclei was used to calculate the final immunoreactivity of each area in each subject. The same procedure was used to count cFos immunoreactivity in experiment 3.

Experiment 1

Rats were first placed on a 12:12 hr LD cycle for two weeks. In order to observe the effect of long dsRNA to *Per2* on overall locomotor activity and any potential shifts in free running rhythms, rats were then placed in constant darkness for 46 days following bilateral dsRNA infusions, while their activity levels were recorded. Rats were perfused

at circadian time (CT) 1, which corresponds to 1 hr after the subjective activity offset. A separate group of rats were placed in constant dark for 21 days. Afterwards, they were placed under a 12:12 hr reversed LD cycle for 14 days in order to ascertain that the SCN was still responsive to photic cues. Animals were then perfused at ZT1, 1 hr after activity offset (lights on).

Experiment 2

To examine the effect of long dsRNA to *Per2* on locomotor activity and in order to measure the degree of silencing as well as the length of PER2 suppression, rats were perfused at ZT13 (1 hr after activity onset) three, six and 12 days after bilateral dsRNA infusions in the SCN while activity levels were monitored throughout. The masking effect of light on the SCN was tested by placing the rats back under the same LD cycle following surgery. The resulting differences in counts of immunoreactive cell nuclei for PER2 were analyzed using a 2-way ANOVA with days (three, six or 12) and treatment (experimental or control) as the independent variables. The alpha level was set at .05 for all analyses. A second group was bilaterally infused with dsRNA 1.5 mm dorsal to the SCN in order to test whether locomotor disruption and PER2 suppression was not due to spreading of the dsRNA up the injection tract.

Experiment 3

In order to determine whether the long dsRNA to *Per2* was in fact as specific as believed and that it was not also suppressing the expression of other genes, PER2 and cFos immunoreactivity was assessed in alternate sections in rats perfused at ZT1 or ZT13, six days after microinfusions. The resulting differences in counts were analyzed using a 2-way ANOVA with perfusion time (ZT1 and ZT13) and treatment (experimental

or control) as the independent variables for PER2 and cFos. All analyses were evaluated for significance at $\alpha = .05$.

Results

Experiment 1

Behavior in constant darkness

After being initially placed on a 12:12 hr LD cycle for 14 days, 20 rats were placed in constant darkness while their running wheel activity was measured every 10 min. Initially, locomotor activity was relatively random; however, all rats became entrained in a matter of days. The criterion for entrainment was defined as a pattern of running wheel activity which was almost exclusively limited to the dark phase. Two weeks post-entrainment rats were anesthetized and bilaterally infused with dsRNA to *Per2* ($n = 16$) or β-globin ($n = 4$; shown by the arrow in each one of the double-plotted actograms in Figure 1). Rats were then placed back in their home cages under constant darkness in order to assess the effect of dsRNA on free running activity. Four of the 16 experimental rats were excluded due to unilateral or bilateral dsRNA infusion misses. As can be seen in the sample actograms (Figure 1A), experimental rats showed an arrhythmic running pattern up to 8 days post-surgery. Conversely, control rats (Figure 1B) exhibited disruption in their running patterns for the first two days after surgery only; a period of time corresponding with the time needed for recovery. Thus, on the third day post-surgery, control rats were active during their usual endogenous dark period whereas the experimental rats were entirely arrhythmic. Activity levels were monitored up to 46 days

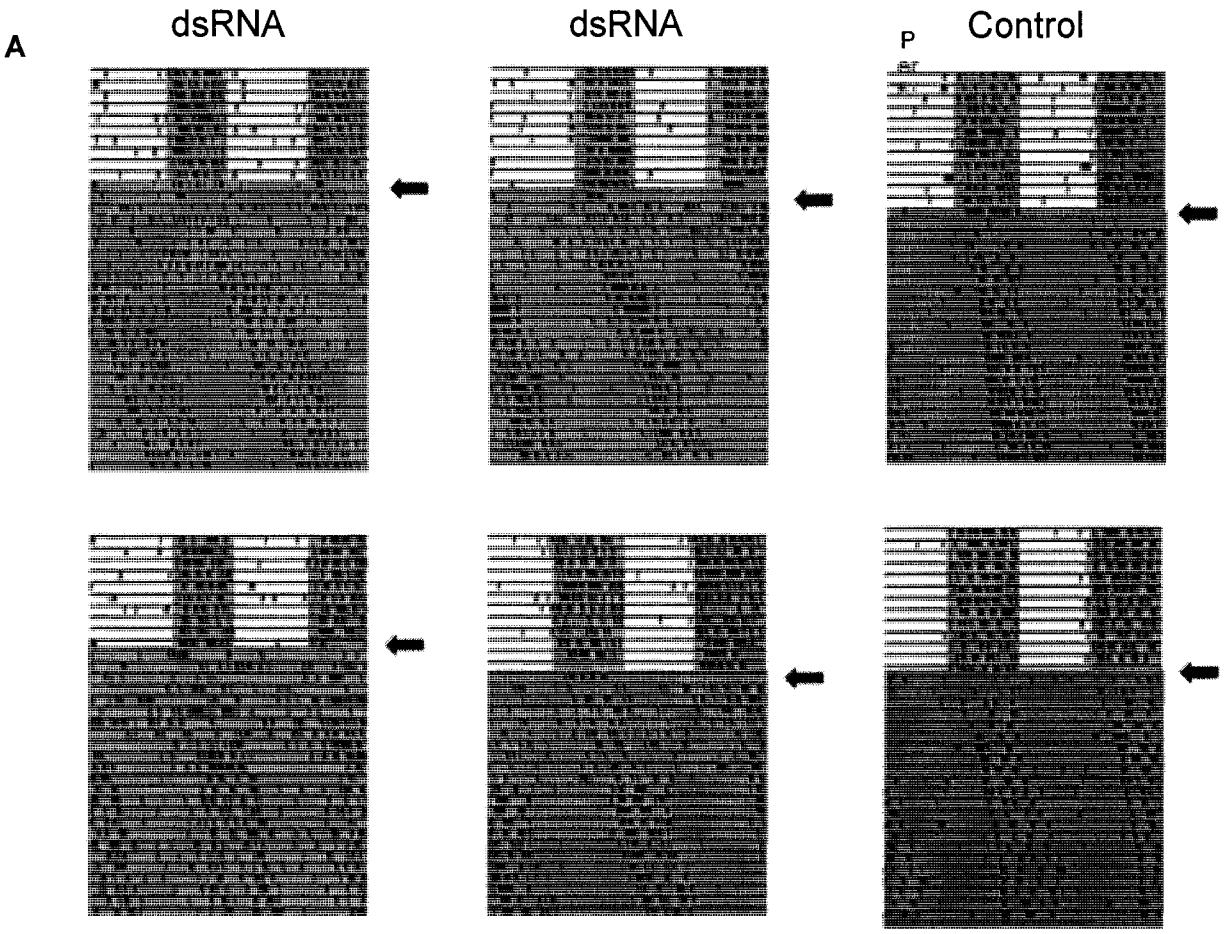

Figure 1. Representative double-plotted (48 hr) actograms showing wheel running activity rhythms before and after bilateral intra-SCN infusions of (A) long dsRNA to *Per2* or (B) control solution (β-globin). The day of infusion is marked with an arrow. Rats were initially entrained to a 12:12 hr LD cycle and then returned to constant darkness following SCN infusions. White actogram portions indicate lights on while grey actogram portions indicate lights off. Black marks show periods of activity of at least 10 wheel rotations/10 min. Successive days are plotted from top to bottom.

following surgery before all rats were sacrificed.

Constant darkness and light/dark cycle reversal

Six additional rats (experimental: $n = 3$; control: $n = 3$) were subject to the same experimental conditions as described above except that following surgery, rats were placed in constant darkness for 21 days and then exposed to a reverse 12:12 hr LD cycle for 14 days before being sacrificed. As shown in Figure 2, all rats were fully entrained within the first 2 days following exposure to the new LD cycle. This indicates that dsRNA did not have any long-lasting secondary side effects on the experimental rats' ability to entrain to a novel LD cycle.

Experiment 2

Behavior

As in experiment 1, rats were placed on a 12:12 hr LD cycle for 14 days. After bilateral infusions of dsRNA to *Per2* or β-globin, rats were placed back in home cages and kept on the same lighting schedule for the remainder of the experiment. Rats were then perfused at ZT13, three days (experimental: $n = 4$; control: $n = 4$), six days (experimental: $n = 4$; control: $n = 4$) and 12 days (experimental: $n = 4$; control: $n = 4$) after surgery. Figure 3 shows sample double-plotted actograms of each one of the three perfusion days, both for the experimental (A) and control rats (B). Upon inspection of Figure 3 it is apparent that experimental rats remained arrhythmic, to some degree, for up to eight days post-surgery. Interestingly the rats in this experiment showed less arrhythmicity than rats in experiment 1. This may be due to the masking effect of light which was able to entrain cells which still expressed *Per2* mRNA and synchronize their PER2 expression.

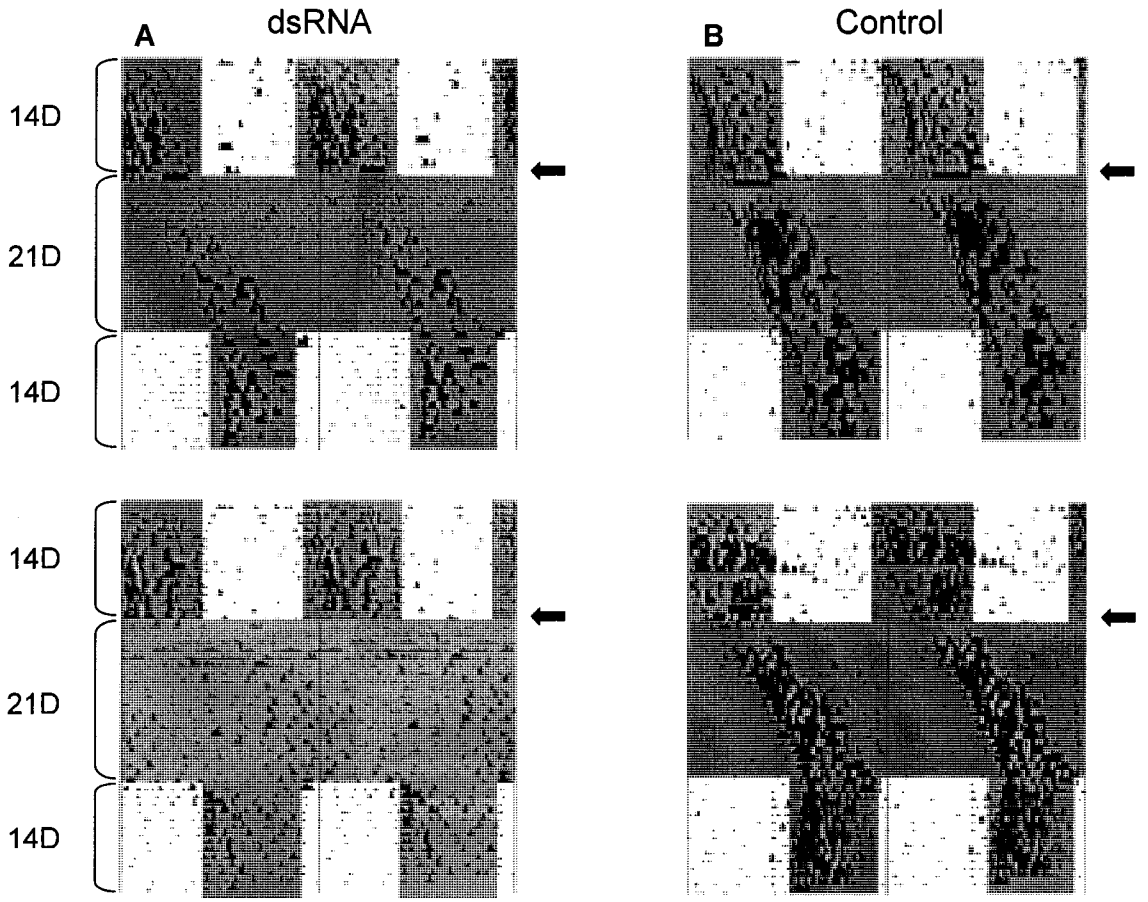

Figure 2. Representative double-plotted actograms showing wheel running activity rhythms before and after bilateral intra-SCN infusions as well as post-infusion light entrainment. (A) long dsRNA to *Per2* or (B) control solution (β-globin) were infused on the day marked with the arrow. Rats were entrained to a 12:12 hr LD cycle for 14 days and then released into constant darkness for 21 days following SCN infusions. Rats were then placed on a reverse 12:12 hr LD cycle in order to assess whether the SCN neurons could still entrain to light. White portions of the actogram indicate lights on while the grey portions indicates lights off. Black marks show periods of activity of at least 10 wheel rotations/10 min. Successive days are plotted from top to bottom.

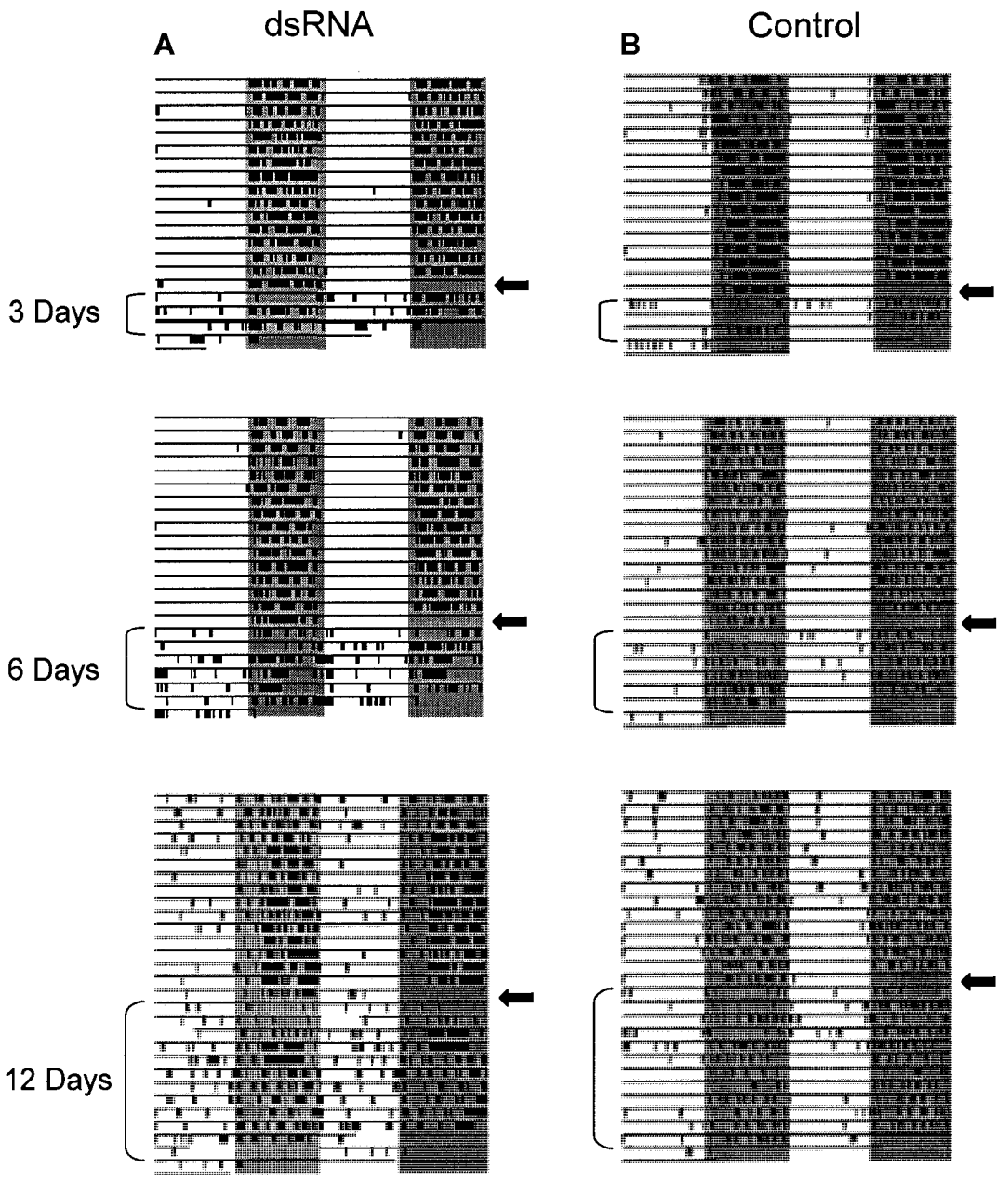

Figure 3. Double-plotted actograms showing wheel running activity in rats perfused at ZT13, three, six or 12 days after bilateral intra-SCN infusions of (A) dsRNA to *Per2* or (B) control solution (β-globin). The arrow indicates the day of infusion.

PER2 Immunocytochemistry

SCN

All rats were perfused at ZT13 in order to measure the rate of suppression of PER2 expression in experimental animals. Figure 4 provides sample photomicrographs which correspond to the respective actograms found in Figure 3. Mirroring the recovery of rhythmicity previously demonstrated in Figure 3 a recovery in the number of PER2 immunoreactive (PER2-ir) nuclei was apparent as time post-surgery increased for rats in the experimental condition (Figure 4A). Further analyses of these data show significantly less PER2-ir in experimental rats when compared to controls, $F(1, 18) = 43.04$, $p < .01$, $\eta^2 = .71$. This suppression of PER2-ir was significant at all time points (Day 3: $F(1, 6) = 42.60$, $p < .01$, $\eta^2 = .88$; Day 6: $F(1, 6) = 6.79$, $p < .05$, $\eta^2 = .53$; Day 12: $F(1, 6) = 10.90$, $p < .05$, $\eta^2 = .65$) suggesting that dsRNA to *Per2* did successfully suppress PER2 expression in the SCN. This suppression appeared transient in nature as rats perfused three, six and 12 days after surgery had a mean PER2-ir suppression, relative to control rats, of 59 %, 39 % and 26 % respectively (Figure 5A).

BNST-ov

Figure 5B shows the mean expression of PER2-ir nuclei in the BNST-ov of rats perfused at ZT13, three, six and 12 days after bilateral microinfusions of dsRNA to *Per2* into the SCN. Here a significant effect of perfusion day, $F(2, 18) = 5.26$, $p < .05$, $\eta^2 = .37$, and experimental treatment, $F(1, 18) = 131.05$, $p < .01$, $\eta^2 = .88$, was evident. Upon further investigation dsRNA was shown to significantly suppress PER2-ir on all perfusion days (Day 3: $F(1, 6) = 43.05$, $p < .01$, $\eta^2 = .88$; Day 6: $F(1, 6) = 39.40$, $p < .01$, $\eta^2 = .87$; Day 12: $F(1, 6) = 86.74$, $p < .01$, $\eta^2 = .94$. However, PER2-ir was significantly

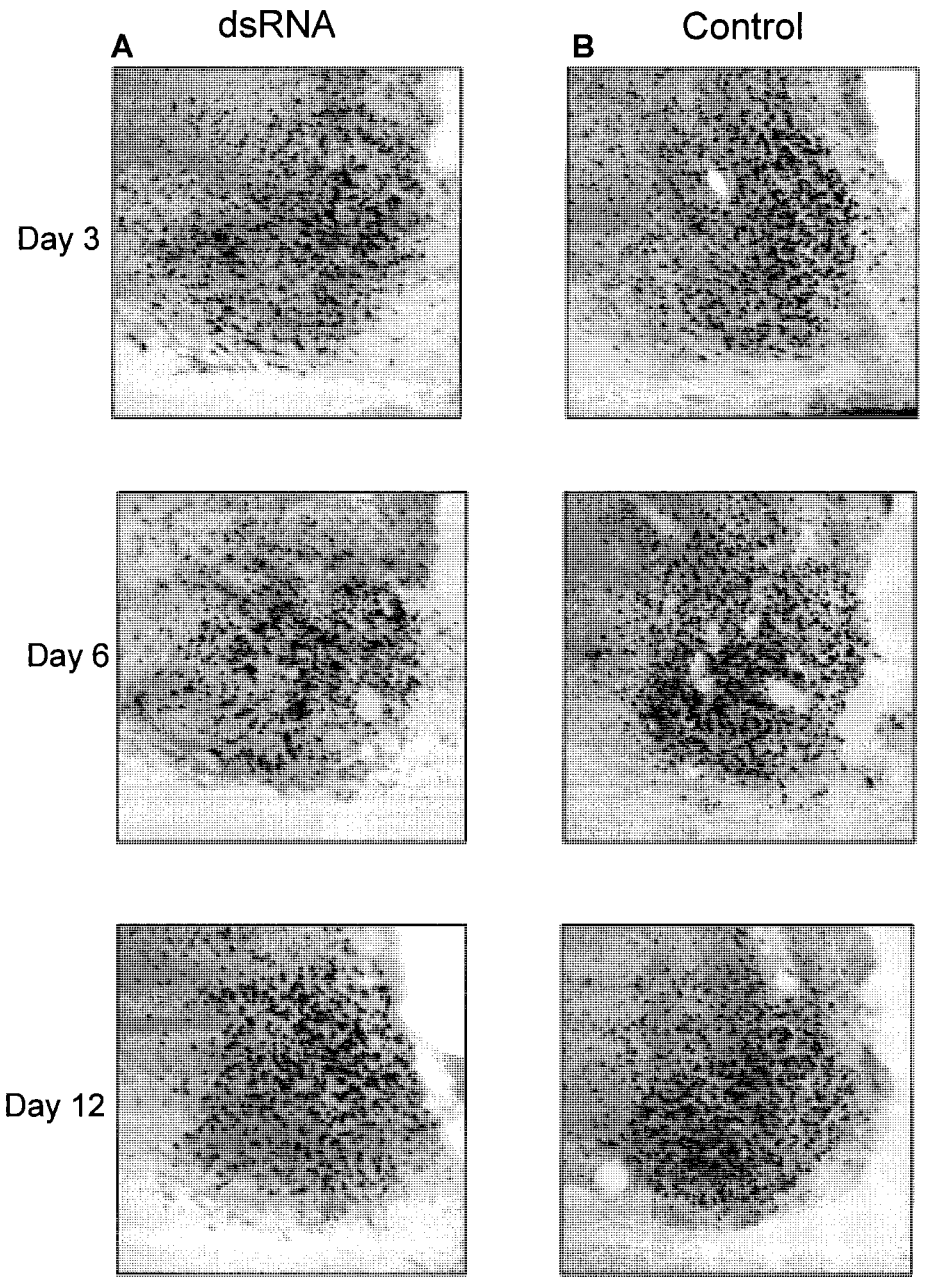

Figure 4. Photomicrographs showing examples of PER2 immunoreactivity in the SCN (unilaterally) in rats killed at ZT13, three, six or 12 days after intra-SCN infusions of (A) dsRNA to *Per2* or (B) control solution (β-globin). These photomicrographs correspond to the actograms displayed in Figure 3.

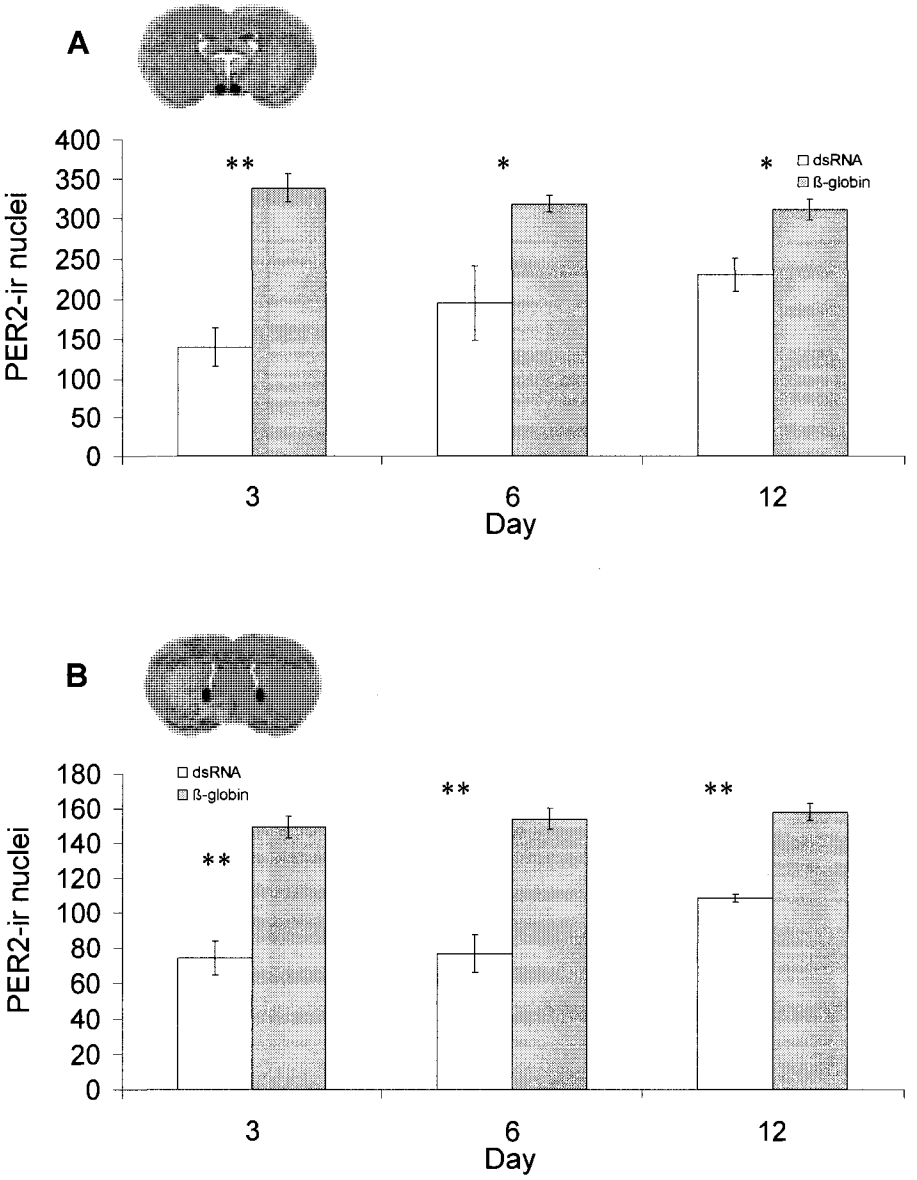

Figure 5. PER2 immunoreactive nuclei (Means ± *SE*) in the (A) SCN and (B) BNST-ov in rats killed at ZT13 three ($n = 8$), six ($n = 8$) or 12 days ($n = 8$), after bilateral intra-SCN infusions of dsRNA to *Per2* (white bars) or control solution (β-globin; gray bars). Black regions on the brain maps indicate relative locations of the areas under investigation. * $p < .05$. ** $p < .01$.

greater in the experimental rats on day 12, as compared to day 3, $t(6) = -3.52, p < .05$.

CEA

Figure 6A shows the mean expression of PER2-ir nuclei in the CEA of experimental and control rats perfused at ZT13, three, six and 12 days after bilateral microinfusions of dsRNA to *Per2* into the SCN. A between subjects ANOVA revealed a significant main effect of perfusion day, $F(2, 18) = 5.11, p < .05, \eta^2 = .36$, as well as a significant main effect of treatment, $F(1, 18) = 55.17, p < .01, \eta^2 = .75$. However, there was no significant interaction between treatment and time of perfusion. Further analysis revealed a suppression of PER2-ir in the experimental group on all perfusion days (Day 3: $F(1, 6) = 25.83, p < .01, \eta^2 = .81$; Day 6: $F(1, 6) = 21.30, p < .01, \eta^2 = .78$; Day 12: $F(1, 6) = 8.84, p < .05, \eta^2 = .60$). However, a significant recovery of PER2 expression in the experimental rats was observed between day 3 and day 12, $t(6) = -4.62, p < .01$.

DG

Figure 6B shows the mean expression of PER2-ir nuclei in the DG of experimental and control rats perfused at ZT13, three, six and 12 days after bilateral microinfusions of dsRNA to *Per2* into the SCN. A between subjects ANOVA revealed a main effect of treatment, $F(1, 18) = 37.87, p < .01, \eta^2 = .68$, but no significant effect of perfusion day or treatment by perfusion day interaction. Contrary to findings in the BNST-ov and the CEA experimental rats showed increased PER2-ir in the DG relative to control rats. Further analyses were performed in order to assess the effect of SCN dsRNA infusion over time. These analyses revealed a significant difference between experimental and control rats on all perfusion days (Day 3: $F(1, 6) = 23.32, p < .01, \eta^2 = .80$; Day 6: $F(1, 6) = 6.91, p < .05, \eta^2 = .54$; Day 12: $F(1, 6) = 14.58, p < .01, \eta^2 = .71$).

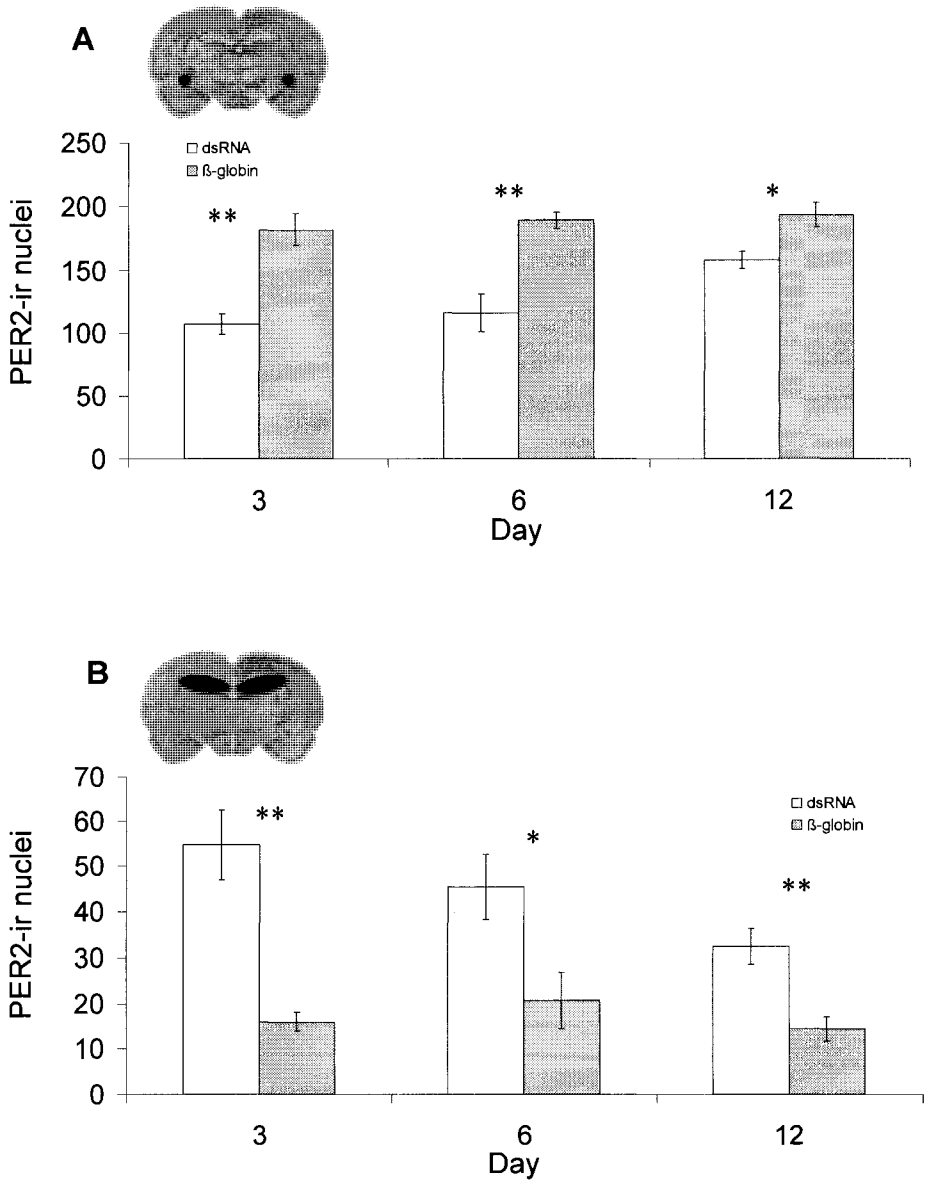

Figure 6. Mean number of PER2 immunoreactive nuclei (± *SE*) in the (A) CEA and (B) DG in rats killed at ZT13 three (n = 8), six (n = 8) or 12 days (n = 8), after bilateral intra-SCN infusions of dsRNA to *Per2* (white bars) or control solution (β-globin; gray bars). Black regions on the brain maps indicate relative locations of the areas under investigation.

* $p < .05$. ** $p < .01$.

The DG did show some recovery of PER2 rhythmicity, $t(6) = 2.56$, $p < .05$, contrary to the other brain regions which were investigated.

Dorsal controls

In order to verify the specificity of dsRNA two rats were bilaterally infused with dsRNA to *Per2* 1.5 mm dorsally to the SCN. Figure 7 displays the actograms of these two rats when entrained to a 12:12 hr LD cycle for 14 days prior to surgery and 6 days post-surgery. These actograms are virtually identical to those of rats having received β-globin control injections, suggesting that dsRNA to *Per2* disrupts circadian function through a direct effect on the SCN.

Experiment 3

Behavior

Running wheel activity was monitored throughout the course of the experiment. The resulting actograms were exact replications of the findings in experiment 2 (data not shown). Rats were perfused either at ZT1 or ZT13, six days after surgery.

PER2 immunocytochemistry

SCN

Figure 8 shows representative photomicrographs from the SCN of rats which were bilaterally infused with (A) dsRNA to *Per2* in the SCN at ZT1 and ZT13, and of rats bilaterally infused with (B) β-globin in the SCN at ZT1 and ZT13. A between subjects ANOVA revealed a significant main effect of perfusion time, $F(1, 12) = 71.30$, $p < .01$, $\eta^2 = .86$, treatment group, $F(1, 12) = 4.79$, $p < .05$, $\eta^2 = .29$, and a perfusion time by treatment interaction, $F(1, 12) = 24.43$, $p < .01$, $\eta^2 = .67$. Overall PER2-ir was greater at ZT13. However, at ZT1 rats in the experimental group displayed significantly more

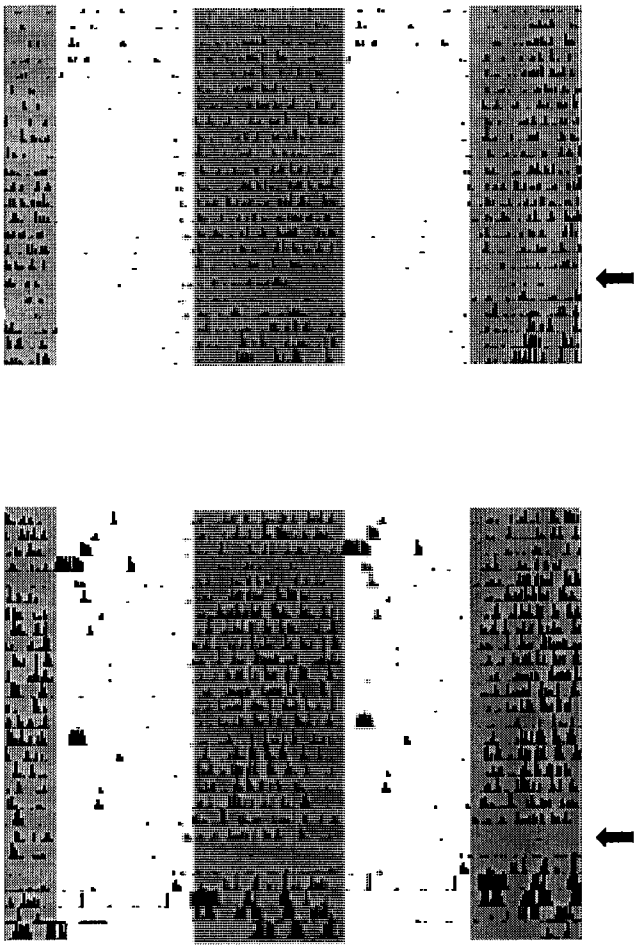

Figure 7. Double-plotted actograms showing wheel running activity in dorsal control rats ($n = 2$) which were bilaterally injected 1.5 µm above the SCN with dsRNA to *Per2*.

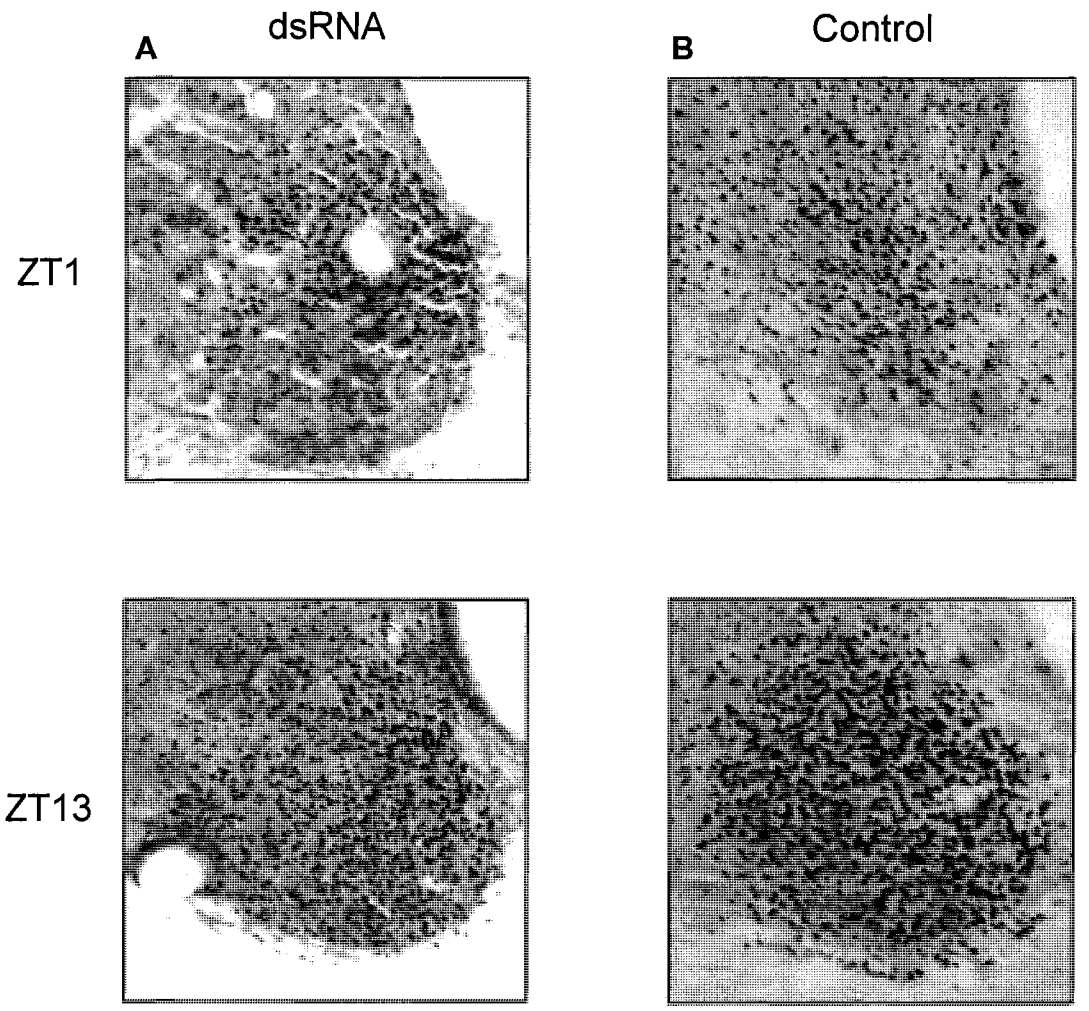

Figure 8. Photomicrographs showing examples of PER2 immunoreactivity in the SCN (unilaterally) in rats killed at ZT1 and ZT13, six days after intra-SCN infusions of (A) dsRNA to *Per2* or (B) control solution (β-globin).

PER2-ir than rats in the control group, $t(6) = 5.04$, $p < .01$ (Figure 9A).

Limbic Forebrain Regions

The mean PER2-ir nuclei at ZT1 and ZT13, for rats perfused six days after surgery is displayed in Figure 9. For both the BNST-ov (B) and the CEA (C) rats displayed significantly more PER2-ir at ZT13 than ZT1, $F_{BNST\text{-}ov}(1, 12) = 38.46, p < .01$, $\eta^2 = .76$; $F_{CEA}(1, 12) = 59.14, p < .01$, $\eta^2 = .83$. Moreover, a significant effect of treatment was also observed. BNST-ov and CEA PER2-ir was significantly reduced in the dsRNA group, $F_{BNST\text{-}ov}(1, 12) = 5.47, p < .01$, $\eta^2 = .76$; $F_{CEA}(1, 12) = 26.97, p < .01$, $\eta^2 = .69$. Furthermore for the CEA a significant interaction between perfusion time and treatment was observed, $F(1, 12) = 9.76, p < .01$, $\eta^2 = .62$. Investigation of this interaction revealed a significant suppression of PER2-ir in experimental rats at ZT13, $t(6) = 6.23, p < .01$. Similar to the BNST-ov and the CEA there was a significant effect of perfusion time, $F(1, 12) = 93.75, p < .01$, $\eta^2 = .89$, and treatment, $F(1, 12) = 15.89$, $p < .01$, $\eta^2 = .57$, when investigating DG PER2-ir. Moreover a significant interaction between time of perfusion and treatment was also observed, $F(1, 12) = 30.25, p < .01$, $\eta^2 = .72$. However, contrary to findings in the BNST-ov and CEA a significant blunting of PER2-ir was observed in experimental relative to control rats at ZT1, $t(6) = 5.01$, $p < .01$.

cFos immunocytochemistry

SCN

Figure 10 shows representative photomicrographs of cFos-ir in the SCN of rats which were bilaterally infused with (A) dsRNA to *Per2* in the SCN at ZT1 and ZT13, and of rats bilaterally infused with (B) β-globin in the SCN at ZT1 and ZT13.

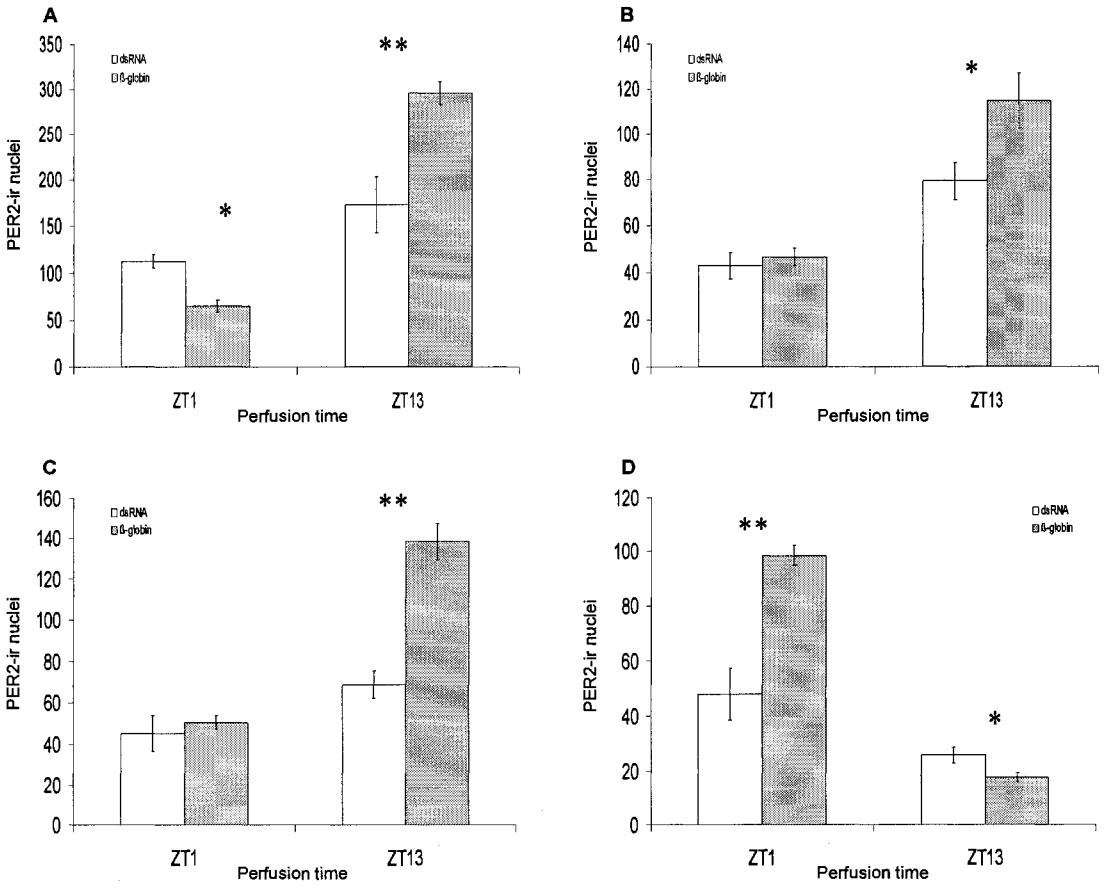

Figure 9. PER2 immunoreactive nuclei (Means ± *SE*) in the (A) SCN, (B) BNST-ov, (C) CEA and (D) DG in rats sacrificed at ZT1 ($n = 8$) or ZT13 ($n = 8$), 6 days after bilateral intra-SCN infusions of dsRNA to *Per2* (white bars) or control solution (β-globin; gray bars).

* $p < .05$. ** $p < .01$.

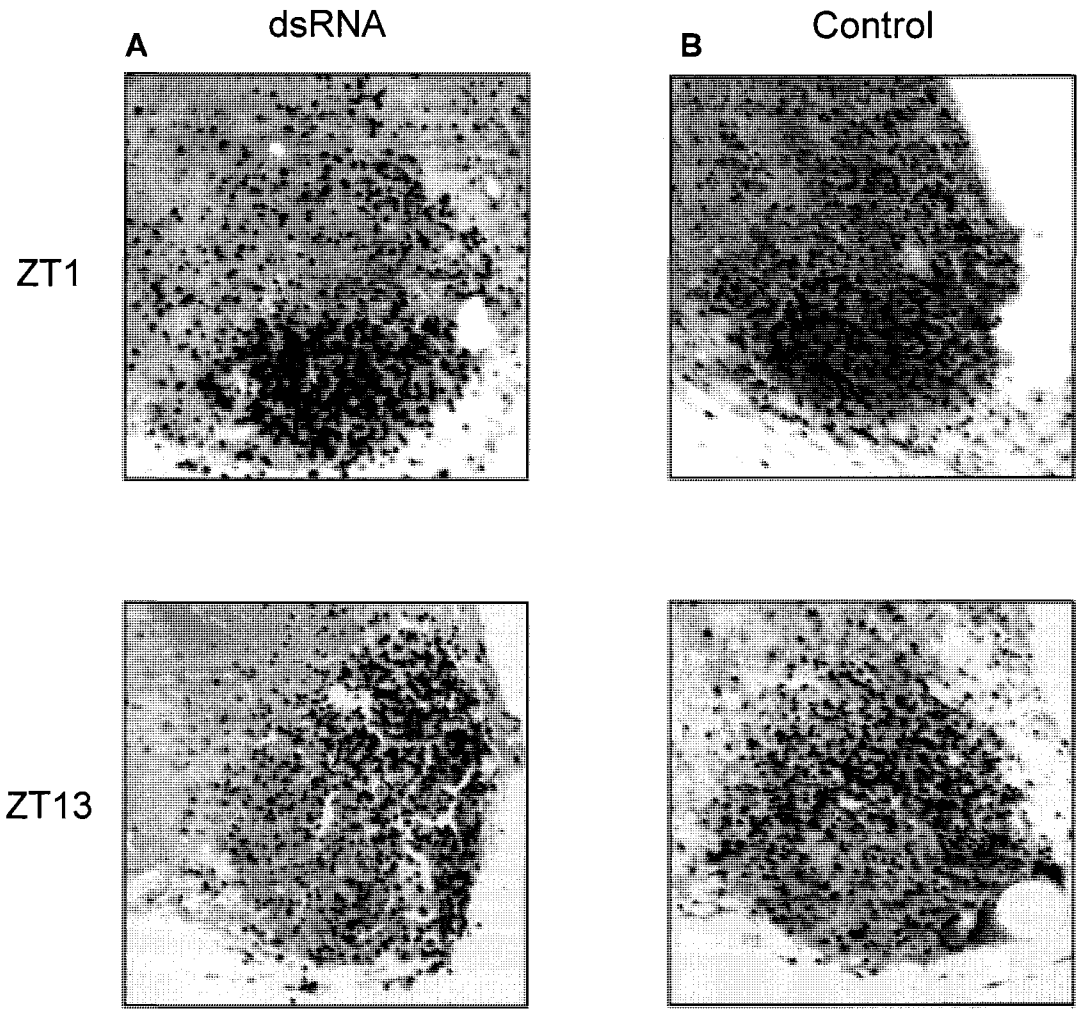

Figure 10. Photomicrographs showing examples of cFos immunoreactivity in the SCN (unilaterally) in rats killed at ZT1 and ZT13, six days after intra-SCN infusions of (A) dsRNA to *Per2* or (B) control solution (β-globin).

Figure 11A shows a schematic, drawn on top of a representative photomicrograph of cFos-ir in the SCN, depicting the separation that exists between the shell and the core. As seen in Figure 11B the mean expression of cFos-ir in the SCN-sh or SCN-co (Figure 11C) was not significantly different for experimental and control rats. However, as demonstrated previously for PER2-ir, perfusion time did have a significant effect on cFos-ir in both the SCN-co, $F(1, 12) = 157.90$, $p < .01$, $\eta^2 = .93$ and SCN-sh, $F(1, 12) = 13.46$, $p < .05$, $\eta^2 = .53$. In both regions cFos-ir was reduced at ZT13.

Limbic Forebrain Regions

Figure 12 shows the mean expression of cFos-ir in the BNST-ov (A), CEA (B) and DG (C) of experimental and control rats perfused at ZT1 or ZT13, six days following bilateral microinfusions of dsRNA to *Per2* into the SCN. In all regions a significant main effect of perfusion time was observed ($F_{\text{BNST-ov}}(1, 12) = 157.90$, $p < .01$, $\eta^2 = .93$; $F_{CEA}(1, 12) = 25.13$, $p < .01$, $\eta^2 = .68$; $F_{DG}(1, 12) = 19.67$, $p < .01$, $\eta^2 = .62$); however, there were no significant main effects of treatment and no significant interactions. Furthermore, in all regions cFos-ir was greatest at ZT1.

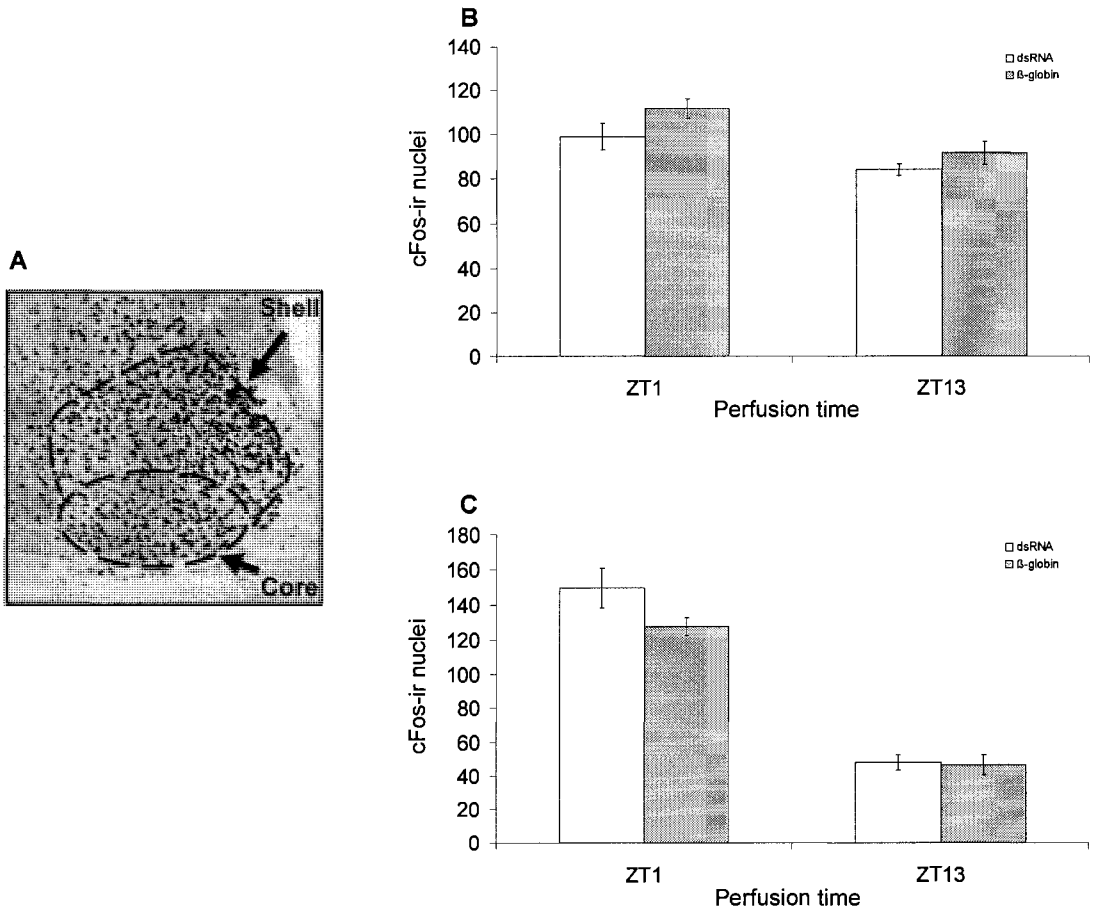

Figure 11. (A) Sample photomicrograph showing cFos expression as well as a schematic of the two sub-sections of the SCN: the shell and the core, indicated by the arrows. cFos immunoreactive nuclei (Means ± *SE*) in the (B) SCN shell and (C) SCN core in rats sacrificed at ZT1 ($n = 8$) or ZT13 ($n = 8$), six days after bilateral intra-SCN infusions of dsRNA to *Per2* (white bars) or control solution (β-globin; gray bars).

* $p < .05$. ** $p < .01$.

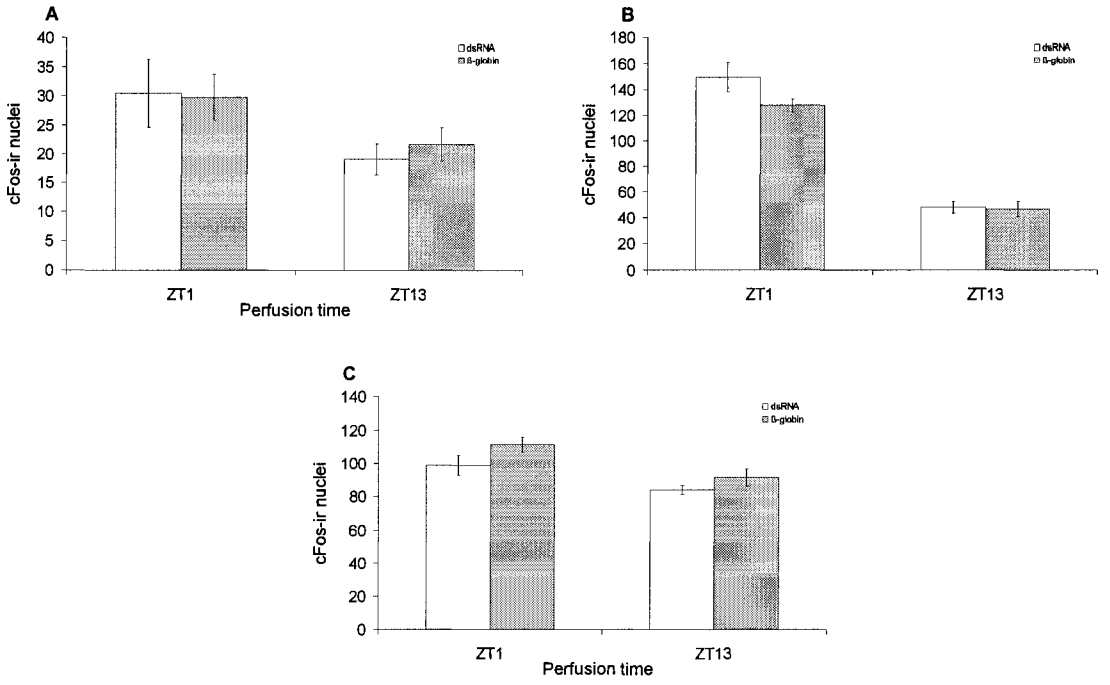

Figure 12. Mean number of cFos immunoreactive nuclei (± *SE*) in the (A) BNST-ov, (B) CEA and (C) DG in rats sacrificed at ZT1 ($n = 8$) or ZT13 ($n = 8$), six days after bilateral intra-SCN infusions of dsRNA to *Per2* (white bars) or control solution (β-globin; gray bars).

Discussion

Mutant studies have suggested that PER2 plays a key role in the regulation of circadian rhythms in behavior (Spoelstra, Albrecht, van der Horst, Brauer, & Daan, 2004; Zheng et al., 1999). However, evidence for a direct link between circadian rhythms in behavior and PER2 expression in the SCN is lacking due to developmental confounds is mutants (Sumova et al., 2006; Zueger et al., 2006) and limitations associated with ODNs (Wakamatsu et al., 2001). In order to circumvent these confounds, the present thesis examined whether dsRNA to *Per2,* infused locally through bilateral infusions into the area of the SCN, could suppress both circadian wheel running and PER2 protein expression. These infusions produced a transient disruption in locomotor activity rhythms in rats housed on a 12:12 hr LD cycle and in constant darkness. Moreover, this disruption in rhythmicity was correlated with a reduction in PER2-ir in the SCN and various limbic forebrain regions.

In experiment 1, it was found that bilateral SCN microinjections of long dsRNA to *Per2* were effective at disrupting PER2 rhythmicity in the SCN for up to eight days. The specificity of this effect was demonstrated by showing no effect of dsRNA when injected a few millimeters off-target (dorsal controls) or when a unilateral or bilateral injection was missed.

The behavioral data show that rats can become entrained to a reverse LD cycle very rapidly after having been exposed to constant darkness for an extended period of time. Moreover, rats given bilateral dsRNA infusions into the SCN were able to entrain, as quickly as control rats given only a vehicle, to a reverse LD cycle after the eight days during which the dsRNA is effective. This demonstrates a transient effect of dsRNA

suppression.

Experiment 2 investigated the masking effect of light on PER2 expression in the SCN and limbic forebrain. As expected, once experimental rats were placed post-surgery on a LD cycle, light masked part of their behavior and therefore, they seemed to be better entrained than the rats in experiment 1, running more during the dark phase. By perfusing the rats at ZT13, three, six and 12 days post-surgery and quantifying PER2-ir in the SCN, BNST-ov, CEA and DG, I was able to determine the course of the effectiveness of the dsRNA to *Per2*. It was found that PER2 was suppressed 59 %, 39 % and 26 % after, three, six and 12 days respectively. These findings further support the behavioral results showing that dsRNA is transient. These results also help us to better interpret some of the findings in experiment 1: Although experimental rats were arrhythmic in constant darkness for up to eight days, once they started free-running, their period corresponded almost perfectly to that of control rats, which had been free-running ever since the animals underwent surgery. This re-synchronization with the free-running periods of the controls can be explained by the fact that maximal PER2 suppression was only 59 %. Therefore, there were many SCN neurons which were still expressing PER2; since each individual SCN neuron is able to maintain its own rhythm (Reppert & Weaver, 2002), these neurons were able to remain rhythmic while neighboring neurons had their activity suppressed. Once the neighboring neurons were able to express PER2 again, they were able to re-synchronize with the neurons that had not lost rhythmicity. Given that in order to increase the degree of PER2 suppression, one would require injecting larger quantities of dsRNA, which would ultimately become cytotoxic (Fire, 1999), one cannot completely suppress PER2 expression in the SCN by using this method.

The PER2-ir counts from experiment 2 combined with the behavioral data from experiments 1 and 2 illustrate that there is a threshold effect of PER2 suppression; at which point behavioral rhythmicity is lost. This threshold seems to be in the vicinity of 39 % because at six days post-surgery, some rats are still arrhythmic while others start regaining rhythmicity. These findings support previous research concluding that various posttranscriptional and posttranslational mechanisms are in place, which can ultimately delay clock gene expression to various degrees throughout the day (Harms, Kivimae, Young, & Saez, 2004), thus resulting in a blunted rhythm.

Finally, experiment 3 looked at the specificity of the long dsRNA to *Per2* sequence used, by labeling alternate brain sections for PER2 and cFos in rats perfused at ZT1 or ZT13. It was found that PER2 was significantly suppressed but cFos expression was unaltered. An interesting finding was the fact that while PER2 expression was suppressed at ZT13; it was actually significantly higher than in controls at ZT1. A possible interpretation would be that since there is a six-hour delay between peak *Per2* mRNA levels and PER2 expression (Harms et al., 2004; Takahashi, 2004), by disrupting the circadian clock through PER2 suppression, PER2 levels are reduced at ZT13. Due to this reduction, the feedback mechanisms no longer function normally and thus produce a smaller troth; hence PER2 expression at ZT1 results in being higher than in controls.

A possible limitation of these experiments is the fact that the noted effect could be due to a shift in the usual phase of PER2 expression, which may be the result of posttranscriptional and/or posttranslational processes such as phosphorylation, ubiquitylation and protein degradation (Tamanini, Yagita, Okamura, & van der Horst, 2005). However, if this were the case, then the dsRNA would have merely caused a shift

in locomotor activity because animals whose phases are shifted by being placed on a different LD cycle for instance, will exhibit a gradual shift in locomotor activity, as can be seen during the entrainment phase of these experiments. Yet no such shift occurred; instead, locomotor arrhythmicity has been noted. Nevertheless, perfusing more animals at two additional time points could provide a better understanding of this matter and might help clear the issue at hand. Another possible criticism regarding the present thesis can be made when considering recent findings by Fujimoto, Yagita, and Okamura (2006), who reported that PER2 expression was possible without previous expression of its mRNA. This suggests that the circadian machinery's feedback loops are more complex than initially thought, however, there is not enough data to verify this speculation.

The current thesis has shown that long dsRNA to *Per2* can be used to transiently suppress PER2 expression in the SCN in vivo. Furthermore, it was shown that this suppression resulted in loss of locomotor circadian rhythmicity. The specificity of the dsRNA used was tested by measuring the amount of cFos-ir in alternate brain slices, which led to the conclusion that the dsRNA was in fact specific to *Per2* because it did not alter cFos expression. Future studies could investigate the effects of dsRNA to *Per2* infused into the SCN at additional time points such as ZT7 and ZT19 in order to draw a clearer picture of circadian PER2 fluctuations. Additionally, it would be of interest to investigate the effects of dsRNA infusions directly into other structures such as the BNST-ov, CEA, DG and even the dorsal medial hypothalamus, which plays a key role in restricted feeding. Finally, dsRNA can be used to look at the role played by PER2 in specific structures and its effects on metabolism since a number of studies (Liu et al., 2007; Wijnen et al., 2006) have already outlined the importance of clock genes therein.

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APPENDICES

APPENDIX A: Experiment 2 PER2 ANOVA Tables

Table A1

Descriptive Statistics

Perfusion	Treatment		SCN	BNST-ov	CEA	DG
Day 3	dsRNA	*M*	139.50	74.00	107.00	54.68
		SE	24.67	9.55	8.40	7.75
	β-globin	*M*	337.75	149.00	182.25	15.95
		SE	17.73	6.28	12.20	2.05
Day 6	dsRNA	*M*	194.50	76.25	116.00	45.35
		SE	46.19	10.70	14.71	7.12
	β-globin	*M*	317.75	153.75	189.25	20.60
		SE	10.27	6.16	5.95	6.17
Day 12	dsRNA	*M*	229.50	108.50	158.00	32.40
		SE	20.74	2.25	7.18	3.92
	β-globin	*M*	310.25	158.00	193.50	14.45
		SE	12.95	4.81	9.54	2.59

Table A2

Analysis of Variance by Treatment for PER2 Immunoreactivity in the SCN

Source	*df*	*F*	η^2	*p*
Days (D)	2	0.78	.08	.47
Treatment (T)	1	43.04**	.71	.00
D X T	2	2.82	.24	.09
Error	18	(2506.18)		

Note. Value enclosed in parentheses represents mean square errors.
** $p < .01$.

Table A3

Analysis of Variance by Perfusion Day for PER2 Immunoreactivity in the SCN

Days	Source	*df*	*F*	η^2	*p*
Day 3	Treatment	1	42.60**	.88	.00
	Error	6	(1845.29)		
Day 6	Treatment	1	6.79*	.53	.04
	Error	6	(4477.92)		
Day 12	Treatment	1	10.90*	.65	.02
	Error	6	(1195.96)		

Note. Values enclosed in parentheses represent mean square errors.
* $p < .05$. ** $p < .01$.

Table A4

Analysis of Variance by Treatment for PER2 Immunoreactivity in the BNST-ov

Source	*df*	*F*	η^2	*p*
Days (D)	2	5.26*	.37	.02
Treatment (T)	1	131.04**	.88	.00
D X T	2	2.31	.20	.13
Error	18	(207.58)		

Note. Value enclosed in parentheses represents mean square errors.
* $p < .05$. ** $p < .01$.

Table A5

Analysis of Variance by Perfusion Day for PER2 Immunoreactivity in the BNST-ov

Days	Source	*df*	*F*	η^2	*p*
Day 3	Treatment	1	43.05**	.88	.00
	Error	6	(261.33)		
Day 6	Treatment	1	39.40**	.87	.00
	Error	6	(304.92)		
Day 12	Treatment	1	86.73**	.94	.00
	Error	6	(56.50)		

Note. Values enclosed in parentheses represent mean square errors.
** $p < .01$.

Table A6

Analysis of Variance by Treatment for PER2 Immunoreactivity in the CEA

Source	*df*	*F*	η^2	*p*
Days (D)	2	5.11*	.36	.02
Treatment (T)	1	55.17**	.75	.00
D X T	2	2.45	.21	.11
Error	18	(409.14)		

Note. Value enclosed in parentheses represents mean square errors.
* $p < .05$. ** $p < .01$.

Table A7

Analysis of Variance by Perfusion Day for PER2 Immunoreactivity in the CEA

Days	Source	*df*	*F*	η^2	*p*
Day 3	Treatment	1	25.83**	.81	.00
	Error	6	(438.46)		
Day 6	Treatment	1	21.30**	.78	.00
	Error	6	(503.79)		
Day 12	Treatment	1	8.84*	.60	.02
	Error	6	(285.17)		

Note. Values enclosed in parentheses represent mean square errors.
* $p < .05$. ** $p < .01$.

Table A8

Analysis of Variance by Treatment for PER2 Immunoreactivity in the DG

Source	*df*	*F*	η^2	*p*
Days (D)	2	2.72	.23	.09
Treatment (T)	1	37.87**	.68	.00
D X T	2	1.92	.18	.18
Error	18	(116.73)		

Note. Value enclosed in parentheses represents mean square errors.
** $p < .01$.

Table A9

Analysis of Variance by Perfusion Day for PER2 Immunoreactivity in the DG

Days	Source	*df*	*F*	η^2	*p*
Day 3	Treatment	1	23.32**	.80	.00
	Error	6	(128.60)		
Day 6	Treatment	1	6.91*	.54	.04
	Error	6	(177.41)		
Day 12	Treatment	1	14.58*	.71	.01
	Error	6	(44.19)		

Note. Values enclosed in parentheses represent mean square errors.
* $p < .05$. ** $p < .01$.

APPENDIX B: Experiment 3 PER2 ANOVA Tables

Table B1

Descriptive Statistics

Perfusion	Treatment		SCN	BNST-ov	CEA	DG
ZT1	dsRNA	*M*	112.71	43.04	45.21	48.13
		SE	7.12	5.59	8.68	9.36
	ß-globin	*M*	65.25	46.75	50.63	98.5
		SE	6.17	3.73	3.16	3.69
ZT13	dsRNA	*M*	173.05	79.21	68.71	25.92
		SE	30.5	8.08	6.49	2.97
	ß-globin	*M*	295.96	114.79	138.5	17.88
		SE	13.01	12.15	9.07	1.67

Table B2

Analysis of Variance by Treatment for PER2 Immunoreactivity in the SCN

Source	*df*	*F*	η^2	*p*
ZT	1	71.30**	.86	.00
Treatment (T)	1	4.79*	.29	.05
ZT X T	1	24.43**	.67	.00
Error	12	(1188.04)		

Note. Value enclosed in parentheses represents mean square errors.
* $p < .05$. ** $p < .01$.

Table B3

Analysis of Variance by Treatment for PER2 Immunoreactivity in the BNST-ov

Source	*df*	*F*	η^2	*p*
ZT	1	38.46**	.76	.00
Treatment (T)	1	5.47*	.31	.04
ZT X T	1	3.60	.23	.08
Error	12	(282.39)		

Note. Value enclosed in parentheses represents mean square errors.
* $p < .05$. ** $p < .01$.

Table B4

Analysis of Variance by Treatment for PER2 Immunoreactivity in the CEA

Source	*df*	*F*	η^2	*p*
ZT	1	59.14**	.83	.00
Treatment (T)	1	26.97**	.69	.00
ZT X T	1	19.76**	.62	.00
Error	12	(209.72)		

Note. Value enclosed in parentheses represents mean square errors.
** $p < .01$.

Table B5

Analysis of Variance by Treatment for PER2 Immunoreactivity in the DG

Source	*df*	*F*	η	*p*
ZT	1	93.75**	0.89	.00
Treatment (T)	1	15.89**	0.57	.00
ZT X T	1	30.25**	0.72	.00
Error	12	(112.79)		

Note. Value enclosed in parentheses represents mean square errors.
** $p < .01$.

Table B6

Simple Effects of Treatment on PER2 Immunoreactivity at ZT1

Area	Source	*df*	*F*	*p*
SCN	Between Groups	1	25.39**	.00
	Within Groups	6	(177.41)	
CEA	Between Groups	1	0.34	.58
	Within Groups	6	(170.65)	
DG	Between Groups	1	25.08**	.00
	Within Groups	6	(202.39)	

Note. Values enclosed in parentheses represent mean square errors.
** $p < .01$.

Table B7

Simple Effects of Treatment on PER2 Immunoreactivity at ZT13

Area	Source	*df*	*F*	*p*
SCN	Between Groups	1	13.74*	.01
	Within Groups	6	(2198.68)	
CEA	Between Groups	1	39.16**	.00
	Within Groups	6	(248.79)	
DG	Between Groups	1	5.57	.06
	Within Groups	6	(23.20)	

Note. Values enclosed in parentheses represent mean square errors.
* $p < .05$. ** $p < .01$.

Table B8

Simple Effects of Perfusion Time on PER2 Immunoreactivity for dsRNA

Area	Source	*df*	*F*	*p*
SCN	Between Groups	1	3.71	.10
	Within Groups	6	(1961.38)	
CEA	Between Groups	1	4.70	.07
	Within Groups	6	(234.99)	
DG	Between Groups	1	5.12	.06
	Within Groups	6	(192.75)	

Note. Values enclosed in parentheses represent mean square errors.

Table B9

Simple Effects of Perfusion Time on PER2 Immunoreactivity for B-globin

Area	Source	*df*	*F*	*p*
SCN	Between Groups	1	256.69**	.00
	Within Groups	6	(414.71)	
CEA	Between Groups	1	83.73**	.00
	Within Groups	6	(184.46)	
DG	Between Groups	1	395.87**	.00
	Within Groups	6	(32.84)	

Note. Values enclosed in parentheses represent mean square errors.
** $p < .01$.

APPENDIX C: Experiment 3 cFos ANOVA Tables

Table C1

Descriptive Statistics

Perfusion	Treatment		SCN-sh	SCN-co	BNST-ov	CEA	DG
ZT1	dsRNA	*M*	98.92	149.54	30.46	40.92	4.34
		SE	5.98	11.29	5.87	5.59	0.88
	ß-globin	*M*	111.5	127.46	29.75	44.42	6.00
		SE	4.33	5.12	3.99	4.66	0.93
ZT13	dsRNA	*M*	84.21	48.09	19.04	20.88	2.00
		SE	2.59	4.67	2.69	3.34	0.19
	ß-globin	*M*	91.63	46.79	21.63	21.75	2.42
		SE	5.26	5.88	2.89	2.92	0.33

Table C2

Analysis of Variance by Perfusion Day for cFos Immunoreactivity in the SCN-sh

Source	*df*	*F*	η^2	*p*
ZT	1	13.46**	.53	.00
Treatment (T)	1	4.50	.27	.06
ZT X T	1	0.30	.02	.59
Error	12	(88.84)		

Note. Value enclosed in parentheses represents mean square errors.
** $p < .01$.

Table C3

Analysis of Variance by Perfusion Day for cFos Immunoreactivity in the SCN-co

Source	*df*	*F*	η^2	*p*
ZT	1	157.90**	.93	.00
Treatment (T)	1	2.60	.18	.13
ZT X T	1	2.06	.15	.18
Error	12	(210.06)		

Note. Value enclosed in parentheses represents mean square errors.
** $p < .01$.

Table C4

Analysis of Variance by Perfusion Day for cFos Immunoreactivity in the BNST-ov

Source	*df*	*F*	η^2	*p*
ZT	1	5.79*	.33	.03
Treatment (T)	1	0.05	.00	.82
ZT X T	1	0.16	.01	.69
Error	12	(65.91)		

Note. Value enclosed in parentheses represents mean square errors.
* $p < .05$.

Table C5

Analysis of Variance by Perfusion Day for cFos Immunoreactivity in the CEA

Source	*df*	*F*	η^2	*p*
ZT	1	25.13**	.68	.00
Treatment (T)	1	0.26	.02	.62
ZT X T	1	0.10	.01	.76
Error	12	(72.59)		

Note. Value enclosed in parentheses represents mean square errors.
** $p < .01$.

Table C6

Analysis of Variance by Perfusion Day for cFos Immunoreactivity in the DG

Source	*df*	*F*	η^2	*p*
ZT	1	19.67**	.62	.00
Treatment (T)	1	2.44	.17	.14
ZT X T	1	0.87	.07	.37
Error	12	(1.78)		

Note. Value enclosed in parentheses represents mean square errors.
** $p < .01$.