Pergamon

PII: S0306-4522(02)00100-8

Neuroscience Vol. 112, No. 3, pp. 677–685, 2002
© 2002 IBRO. Published by Elsevier Science Ltd
All rights reserved. Printed in Great Britain
0306-4522/02 $22.00+0.00

www.neuroscience-ibro.com

IS NOVEL WHEEL INHIBITION OF *PER1* AND *PER2* EXPRESSION LINKED TO PHASE SHIFT OCCURRENCE?

P. C. YANNIELLI, J. MCKINLEY BREWER and M. E. HARRINGTON*

Department of Psychology and Neuroscience Program, Smith College, Northampton, MA 01063, USA

Abstract—We studied whether access to a novel running wheel *in vivo* could reset the suprachiasmatic nuclei (SCN) *in vitro*. Golden hamsters were transferred to dim red light at Zeitgeber time (ZT) 4, given their first exposure to a running wheel for 3 h, and killed at either ZT7 or ZT9. Using a brain slice preparation, the SCN firing rate rhythm *in vitro* was advanced relative to controls only in the slices prepared at ZT9 (phase shift: 2.36 ± 0.06 h, $n = 4$) but not ZT7 (-0.26 ± 0.16 h, $n = 4$). Transitions to dim red light or brain slice preparation at ZT7 or ZT9 alone do not shift the rhythm. Hamsters with wheels had significantly lower levels of SCN *per1* mRNA compared with controls at ZT7, and lower *per2* mRNA when examined at ZT9.

We conclude that 3 h of novel wheel access appears to require some extended time *in vivo* in order for the SCN to be reset, even beyond the time when *per1* mRNA levels have been altered. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: circadian rhythms, hamster, non-photic entrainment, *per1*, *per2*.

Circadian rhythms can be synchronized to the 24-h cycles of the external environment through a complex process called entrainment. The most important external cue is light. Light has access to the hypothalamic suprachiasmatic nuclei (SCN), which functions as an endogenous circadian pacemaker in mammals, through a direct projection from retinal ganglion cells (Ebling, 1996), and resets circadian rhythm phase during the subjective night mainly through glutamate release.

Dark pulses, arousal, increased locomotor activity, sleep deprivation and certain social cues have been shown to be capable of phase shifting the overt rhythm in a non-photic manner; is not clear whether they share the same neurochemical mediators or even act through the same mechanism in affecting the phase of the clock. Phase resetting of the hamster circadian clock during the subjective day appears to be mediated by neuropeptide Y (NPY) afferents from the intergeniculate leaflet (IGL) (Biello et al., 1994; Janik and Mrosovsky, 1994; Harrington and Mistlberger, 2000), and/or by serotonergic afferents from the raphe nuclei (Morin, 1999). Either NPY or serotonin can reset internal rhythms in a manner similar to behavioral stimulation (Hastings et al., 1998). To focus on novel wheel-induced resetting in the golden hamster, IGL lesions, but not serotonergic lesions or serotonergic antagonists, are able to block resetting induced by novel wheel access (Antle et al., 1998; Meyer-Bernstein and Morin, 1998; Janik and Mrosovsky, 1994; Wickland and Turek, 1994).

The core mechanism of the mammalian biological clock that allows the generation of a self-sustained 24-h oscillation in the SCN has been recently described (Reppert and Weaver, 2001). The effect of light input to the clock is thought to occur through the activation of certain clock genes, Period 1 (*per1*) and Period 2 (*per2*), at times when those gene products are low. Both *mper1* and *mper2* are induced by light during the subjective night (Zylka et al., 1998). In turn, it was proposed that increases in activity, the associated arousal or their neurochemical consequences may ultimately work by decreasing levels of these circadian clock-related genes, at a time when these gene products are high. During the subjective day, both NPY and novel wheel running activity can decrease *mper1* and *mper2* mRNA levels in the SCN *in vivo* (Maywood et al., 1999; Maywood et al., 2002) and *in vitro* (Fukuhara et al., 2001). Systemic injections of 8-hydroxy-2-(di-*n*-propylamino)-tetralin (8-OH-DPAT), a 5-hydroxytryptamine-$1_{A/7}$ agonist, and short-acting benzodiazepines suppress *mper1* and *mper2* levels in the SCN of hamsters (Horikawa et al., 2000; Yokota et al., 2000). These drugs can induce phase advances of the wheel running rhythm during the subjective day and require the integrity of the raphe serotonergic projection to the SCN (Challet et al., 1998; Meyer-Bernstein and Morin, 1998; Mintz et al., 1997). However, it is not clear whether the ability to decrease the expression of *per*

*Corresponding author. Tel.: +1-413-585-3925; fax: +1-413-585-3786.

E-mail address: mharring@smith.edu (M. E. Harrington).

Abbreviations: 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)-tetralin; ACSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; DR, dim red light; CT, circadian time; IGL, intergeniculate leaflet of the thalamus; LD, light:dark schedule; NPY, neuropeptide Y; SCN, suprachiasmatic nuclei of the hypothalamus; SSC, saline sodium citrate; ZT, Zeitgeber time.

NSC 5549 4-6-02

genes in the middle of the day is causally related to phase shifts or if some other critical component of the clock is changing as well.

The daily oscillation in the firing rate of SCN neurons that can be observed for three to four circa-24-h cycles *in vitro* provides a unique system in which to examine the effect of resetting stimuli (Gillette, 1991). We demonstrated that it is feasible to measure the phase shifting effect of light *in vitro* using a brain slice preparation even when the stimulus has been applied *in vivo*, before killing the animal (Yannielli and Harrington, 2000). We also observed a peak in *per1* mRNA levels 1 h after, and a more delayed peak in *per2* mRNA levels 2 h after *in vivo* light stimulation (McKinley Brewer et al., 2002). Light-induced expression of *Per* genes *in vitro* was similar to what was described for hamsters *in vivo* (Horikawa et al., 2000; Yokota et al., 2001)

Transferring a hamster housed without a running wheel to a new cage with a wheel has been shown to induce significant phase advances of the circadian rhythm of general locomotor activity and body temperature when this takes place during the subjective day (Wickland and Turek, 1991; Gannon and Rea, 1995). The aim of the present study was to investigate the effect of the first exposure to a running wheel given *in vivo* on the phase of the neural firing rhythm measured *in vitro* in the SCN of the golden hamster. As well, we investigated the correlation between the occurrence of a phase advance and the suppression of the rhythmic peak in *per* gene expression.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

Male golden hamsters (LVG, Charles River, USA; 40–60 days old) were housed under a light:dark (LD) schedule of 14:10 h, with food and water available *ad libitum*. Zeitgeber time (ZT) was defined as ZT12 being the projected time of lights off in the animal room. Hamsters were administered an overdose of halothane anesthesia and decapitated during the subjective day at the stated ZTs. Hypothalamic slices (500 μm) containing the SCN were placed in a gas–fluid interface slice chamber (Medical Systems BSC with Haas top), continuously bathed (1 ml/min) in artificial cerebrospinal fluid (ACSF) containing 125.2 mM NaCl, 3.8 mM KCl, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 24.8 mM $NaHCO_3$, 10 mM glucose. ACSF (pH 7.4) supplemented with an antibiotic (gentamicin, 50 mg/l) and a fungicide (amphotericin, 2 mg/l) and maintained at 34.5°C. Warm, humidified 95% oxygen:5% carbon dioxide was continuously provided.

For *in situ* experiments the animals were treated in the same way as for electrophysiological studies except that the hypothalamus was immediately frozen on dry ice and stored at −70°C until sectioning.

All animal manipulations were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals.

In vivo treatment

Animals maintained under LD (150 lux during the light phase) for at least 3 weeks were individually transferred to dim red light (DR, 2×10^{-3} lux) conditions, with or without access to a novel running wheel (diameter: 17.5 cm) at ZT4. Since animals were naive to running wheels, this paradigm involves novelty as well as the wheel-induced locomotor activation (Wickland and Turek, 1991; Gannon and Rea, 1995). Lights remained off for the rest of the experiment. Animals were left undisturbed from ZT4 to ZT7, a total of 3 h (wheel ZT4–7), or from ZT4 to ZT9, a total of 5 h (wheel ZT4–9), and then dissected for electrophysiology or *in situ* hybridization studies. Half of the animals in the wheel ZT4–9 group had the wheel removed from the cage the last two h of DR. In our hands, most running activity occurs during the first 2 h after transfer to the novel wheel in DR (average time of running after being transferred: 126 ± 11 min, $n=16$), so that the amount of wheel running activity was not expected to differ between these groups. Controls were as follows: animals transferred to DR (no wheel) from ZT4 to ZT7 (control ZT4–7) or from ZT4 to ZT9 (control ZT4–9). Two additional treatments were added. Firstly, in order to evaluate the occurrence of phase shift when the treatment was applied in the colony room under standard lighting conditions, some animals were given running wheel access in the colony room from ZT4 to ZT9 (wheel LD ZT4–9). Secondly, as a control for any effect of the time of dissection on the phase shift occurrence, some animals were transferred to DR with running wheel access from ZT6 to ZT9 (wheel ZT6–9). Wheel running activity was recorded with ClockLab hardware and software (Actimetrics, Evanston, IL, USA). A summary of the experimental design is shown in Fig. 1.

Electrophysiological recordings

Extracellular single unit activity of SCN cells was detected with glass micropipette electrodes filled with ACSF, advanced through the slice using a hydraulic microdrive. The signal was fed into an amplifier and was monitored by an oscilloscope and audio monitor. Firing rate was analyzed using data acquisition software and a customized program for calculation of descriptive statistics. A number of experiments in each condition were recorded ‘blind’ where the person recording data had no knowledge of the treatment. In all cases only one SCN slice was recorded from each animal.

In situ hybridization

[^{35}S]UTP-labeled cRNA probes were transcribed from an ∼800-bp (GenBank No. AF249882, approx. nt 320–1120) Syrian hamster *per1* cDNA fragment cloned into pBluescript II KS(−) (Stratagene, USA) created by Drs. Lowrey and Takahashi (Northwestern University) and a 480-bp mouse *per2* fragment (GenBank No. AF035830 nt 9–489) inserted in the pCRII vector (Invitrogen, USA), a gift from Drs. Shearman and Reppert (Harvard Medical School). The *haper1* plasmid was linearized with *Apa*I or *Bst*XI and transcribed with T7 or T3 RNA polymerase for antisense and sense probes respectively. Similarly, *Not*I with SP6 and *Bam*HI with T7 were used to create *mper2* antisense and sense probes.

All *in situ* procedures were adapted, single-label, versions of a dual-label protocol (Petersen and McCrone, 1994). Slices were removed from the chamber at the appropriate time, quick-frozen on glass coverslips on dry ice and stored at −70°C. Cryostat-cut sections (20 μm, cut at 14 to −20°C) were thaw-mounted on Fisher Superfrost-Plus slides, warmed 1 min at 42°C, and returned to −70°C until fixed. Fixation included a 10-min room temperature thaw, 4% formalin in phosphate-buffered saline, and 0.25% acetic anhydride in 1× triethanolamine hydrochloride (pH 8.0), interspersed with 2× saline sodium citrate (SSC) rinses, dehydration with an ethanol series, delipidation with chloroform, and partial rehydration to 95% ethanol.

Slices were incubated with 1.0×10^6 d.p.m. probe per 25 μl hybridization buffer (0.01% sodium pyrophosphate, 10% dextran sulfate, 50% formamide, 4× SSC, 1× Denhardt’s solution (0.02% polyvinylpirolidone, 0.02% Ficoll, 0.02% bovine serum albumin), 0.5 mg/ml tRNA, 0.5 mg/ml heparin, and 0.4 mg/ml single-stranded sheared salmon sperm DNA) for 16–20 h at

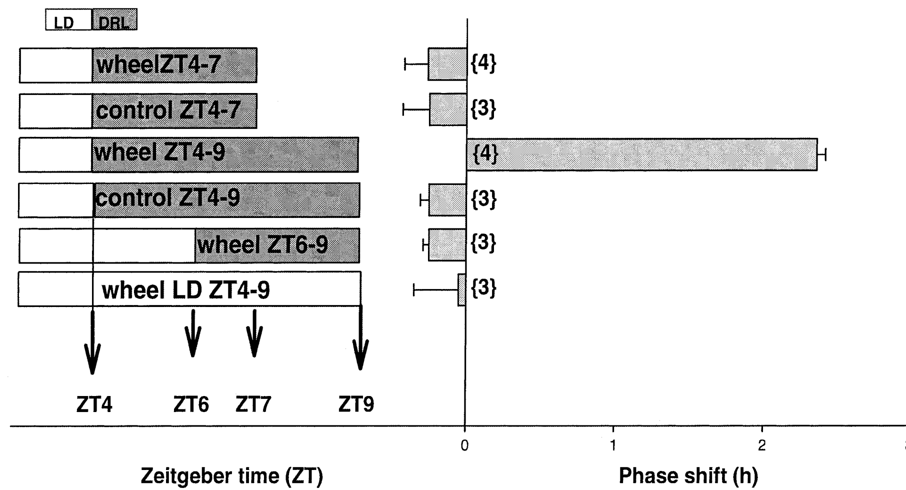

Fig. 1. Wheel access *in vivo* induced a phase advance of the firing activity rhythm on the second day *in vitro*. The rhythm of spontaneous firing rate was measured from SCN slices on the second day *in vitro* and phase measured relative to untreated control slices. The panel on the left shows the experimental design. The panel on the right shows the means ± S.E.M. of phase shifts (h) induced by the access to wheels for the first time for 3 or 5 h. The wheel pulse was applied under DR or in the colony room under standard lighting conditions, and the controls were animals that did not receive wheels but were transferred to the same room and the same lighting as the matching treated group. Average time of peak of control slices from untreated animals (ZT6.3 ± 0.2 h, $n=4$) was used to define the phase shift. Number of slices are shown in brackets. The phase shifting effect of the wheel pulse *in vivo* was significant only for slices prepared 5 h after the beginning of the pulse (group wheel ZT4–9, $P<0.05$ vs. all other groups, ANOVA followed by Student–Newman–Keuls test).

55°C. Alternate slices from each animal were hybridized with *per1* or *per2*.

Post-hybridization washes included multiple 1× SSC washes, 2× SSC/50% formamide at 52°C, then treatments with RNaseA (50 μg/ml) at 37°C and 2× SSC/50% formamide at 50°C, each preceded by a pair of 2× SSC rinses. Finally, slices were washed in an ascending series (70–95%) of ethanol/0.1× SSC baths with a 70% ethanol last rinse. Air-dried slides were opposed to Kodak BioMax MR film for 2–6 days.

Data analysis

For electrophysiological results, data were initially grouped into 1-h bins and an analysis of variance (ANOVA) test was used to determine if any bins differed from the others. If the ANOVA test indicated significant differences, data were smoothed using 1-h running means with a 15-min lag. The ZT of the middle of the 1-h bin with the highest mean firing rate after processing by this smoother was taken as the time of peak firing rate for that slice. Phase shifts of individual slices were measured relative to the average time of peak firing of control slices (animals housed under the LD cycle, killed between ZT4–9, mean time of peak: 6.4 ± 0.2, $n=4$). Means are reported ± S.E.M.

For *in situ* hybridization studies the incorporated radioactivity of each slice was quantified using the Scion Image for Windows (Scion Corp., Beta version 4.0.2) modification of NIH Image on a Dell OptiPlex GX110 with an Epson Expression 1600 scanner. A macro calculated an individual threshold two standard deviations above the mean optical density of a selected area of background hypothalamus for each slice, used that threshold to create a silhouetted duplicate image of areas in the slice above that threshold, independently determined the perimeter of the labeled SCN area, then applied that outline to the original grayscale image (minus threshold) to calculate a mean optical density and area for the labeled SCN. Slices ascertained as labeled above threshold in less than a 10-pixel area (< 1 μm) were labeled as 0. The mean optical density for each subject was the average over all SCN-containing sections from one animal (average per animal = 7 sections), and that number was incorporated in the group mean.

RESULTS

Fig. 1 summarizes the experimental design and the phase shifting effect of a wheel running pulse given *in vivo* and recorded *in vitro* on the firing activity rhythm in brain slices containing the SCN (bars). Brain slices from animals killed at ZT7, 3 h after transfer to DR, with or without running wheel access did not show statistically significant phase shifts of the firing activity rhythm (wheel ZT4–7; control ZT4–7). In contrast, brain slices from animals killed at ZT9, 5 h after transfer to DR with running wheel access, expressed a ~2-h phase advance, while the respective control group did not exhibit any shift in the firing activity rhythm (mean phase shift: wheel ZT4–9, 2.4 ± 0.3 vs. control ZT4–9, −0.25 ± 0.2). Removing the wheel for the last 2 h of DR in this group did not affect the magnitude of the phase shift (the time of peak on the second day *in vitro* for each individual slice were: wheel left until ZT9: time of peak ZT4.1 and ZT4.0; wheel removed from ZT7 to ZT9: time of peak ZT3.1 and ZT4; total $n=4$). Also, 5 h of running wheel access under a LD cycle had no effect on the phase of the rhythm measured *in vitro* (wheel LD ZT4–9). Dissection at ZT9 itself did not induce phase shifts, since brain slices from animals transferred to DR with running wheel access from ZT6 to ZT9 did not show phase shifts of the firing rhythm in the SCN (wheel ZT6–9).

Total wheel revolutions recorded for the novel wheel access groups showed that the wheel ZT4–7 group did not significantly differ from the wheel ZT4–9 group (2771 ± 482 vs. 1342 ± 463 revolutions; $t=2.13$, $P=0.09$, Student's *t*-test). In both cases the activity was concentrated in the first 2 h of wheel exposure. Total wheel revolutions recorded for the group wheel LD ZT4–9 and

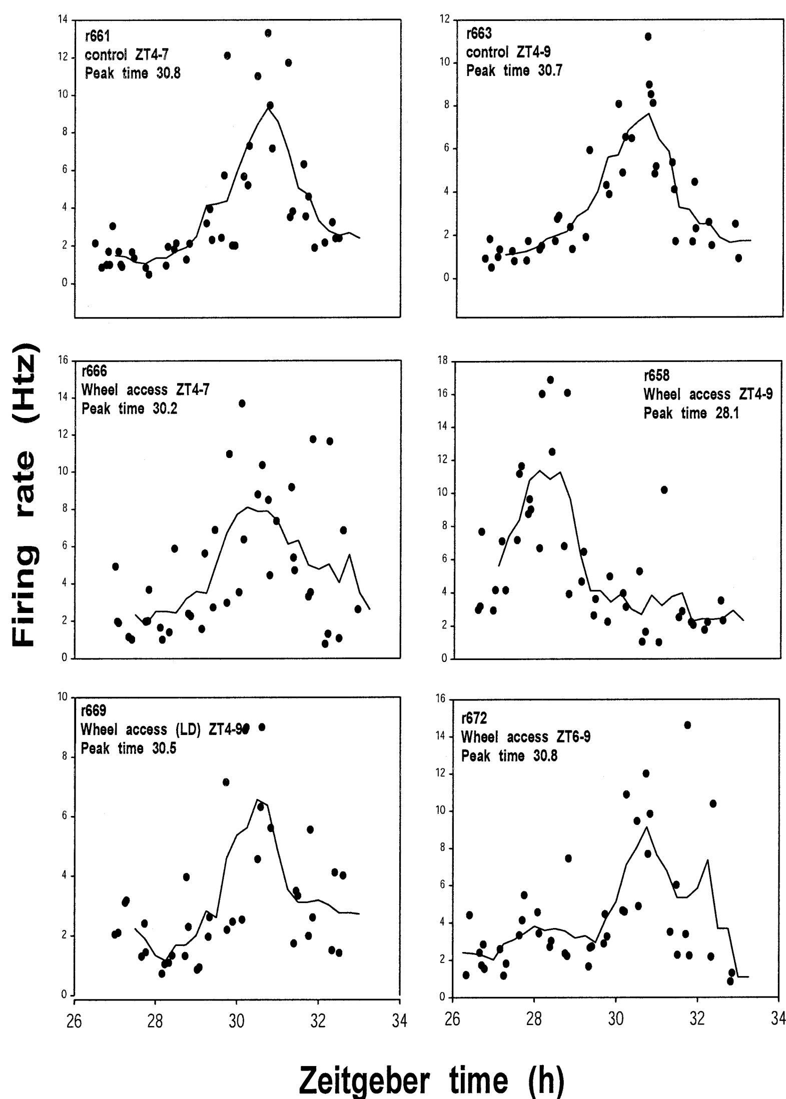

Fig. 2. Representative electrophysiological recordings. Representative individual recordings. The firing rate of individual neurons (dots) was plotted against the ZT of recording of the second day *in vitro*. Treatments were applied as stated in Experimental procedures. Peak times as indicated by the running mean smoother (line) and treatments are denoted in each graph showing a representative recording from one brain slice in that treatment group.

wheel ZT6–9 were 1681 ± 839 and 1307 ± 251 respectively. No significant differences were found for the total number of revolutions among the groups with access to a wheel ($P = 0.66$, ANOVA).

Representative electrophysiological recordings for each of these treatments are shown in Fig. 2.

Per1 mRNA was significantly decreased after 3 h of wheel access, even at the time when the SCN does not express a phase shift *in vitro* (wheel ZT4–7; see Fig. 3A). *Per1* expression in control slices of animals transferred to DR without running wheel access showed high levels of gene expression (control ZT4–7). Expression of *per2* in the same groups may have slightly decreased after 3 h of running wheel access, but this effect was not statistically significant. Activity for the novel wheel access group averaged 3178 ± 445 total counts, and was concentrated in the first 2 h of wheel access. Figure 3B shows representative *in situ* hybridized adjacent/alternate SCN slices for *per1* and *per2* detection.

Figure 4A summarizes the results of *per1* and *per2* mRNA levels in the SCN of animals transferred to DR with or without access to a running wheel, and dissected at ZT9, 5 h after the beginning of the stimulation. While expression of *per1* was almost identical at this time of dissection for both groups, i.e., with or without access to a running wheel (control ZT4–9 vs. wheel ZT4–9), *per2* levels were significantly lower after the wheel stimulation. Activity for the novel wheel access group averaged 2189 ± 468 counts, and was restricted to the first 3 h of DR exposure by manually blocking the wheels. Total activity counts during the 3 h of novel wheel running access did not differ significantly between groups killed at ZT7 or ZT9 (3178 ± 445 vs. 2189 ± 468 revolutions respectively; $t = 1.5$, $P = 0.17$, Student's t-test). Represen-

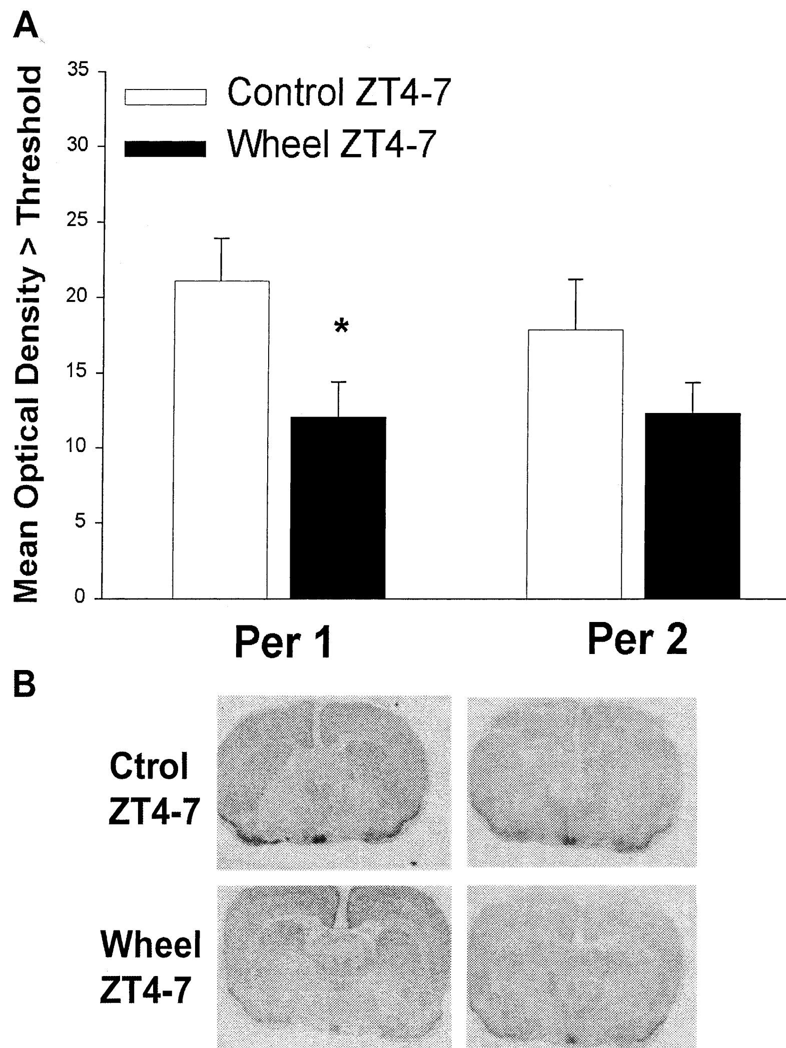

Fig. 3. *per1* and *per2* expression in the SCN of animals killed at ZT7, with or without running wheel access for 3 h. The effect of a novel wheel pulse for the first time on diurnal basal expression of *per1* and *per2* mRNA in hamster SCN was evaluated through *in situ* hybridization. After the treatment (i.e., novel wheel access from ZT4 to ZT7 and/or transference to DR) animals were killed by halothane overdose, the brains quickly dissected and frozen. Expression of *per1* and *per2* was measured as described in Experimental procedures. A: Optical density over threshold of ^{35}S-labeled *per1* and *per2* autoradiographs is plotted as mean ± S.E.M. Expression of *per1* was significantly decreased after 3 h of novel wheel access comparing with the effect of wheel access on *per2* levels ($*P<0.05$, ANOVA followed by Dunnet's test). The numbers were 4–5/group. B: Representative film images showing expression of *per1* (left panels) and *per2* (right panels) mRNA levels in the SCN at the time of the end of each treatment (ZT4–7, wheel access or control group). Coronal sections (20 μm) were examined by *in situ* hybridization using antisense cRNA probes at 1 h (*haper1*) and 2 h (*mper2)* after the treatment.

tative *in situ*-hybridized adjacent/alternate SCN slices for *haper1* and *mper2* detection are showed in Fig. 4B.

DISCUSSION

Surprisingly, novel wheel treatment did not phase shift the SCN *in vitro* unless dissection was delayed until 5 h after introduction of the wheel. This lag in expression of the shift allowed us to correlate the time at which the clock can maintain a phase shift while isolated under *in vitro* conditions with the timing of changes in levels of the two *period* genes examined. The present studies demonstrate that novel wheel inhibition of *per1* mRNA may not be linked to phase shift occurrence. Levels of *per1* mRNA were suppressed at ZT7, yet the shift was not expressed by the isolated SCN when dissection was at ZT7. Inhibition of *per2* mRNA, in contrast, appears more closely linked to these phase shifts. Levels of *per2* mRNA were suppressed at ZT9, when the dissection and isolation of the SCN did not interfere with the expression of the phase shift.

In similar '*in vivo*/*in vitro*' experiments we have exposed hamsters to a 5-min light pulse and dissected immediately after the end of the light. In this situation, the cultured SCN is phase shifted in the expected pattern (Yannielli and Harrington, 2000). While experiments using hamsters and mice demonstrate that light-induced

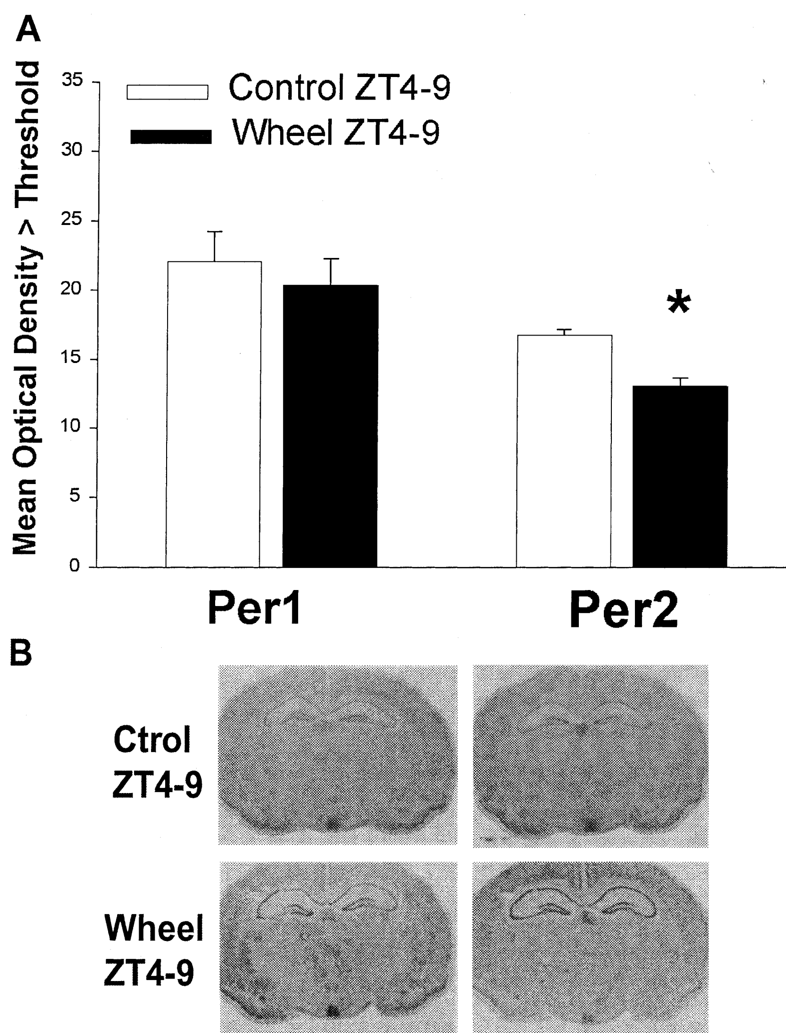

Fig. 4. *haper1* and *mper2* expression in the SCN of animals killed at ZT9, with or without running wheel access for 3 h. The effect of a novel wheel pulse for the first time on diurnal basal expression of *per1* and *per2* mRNA in hamster SCN was evaluated through *in situ* hybridization. After the treatment (i.e., novel wheel access from ZT4 to ZT9 and/or transference to DR) animals were killed after an halothane overdose, the brains quickly dissected and frozen. 20-μ slices were obtained, and expression of *per1* and *per2* was measured as described in Experimental procedures. Optical density over threshold of ^{35}S-labeled *per1* and *per2* autoradiographs is plotted as mean ± S.E.M. Expression of *per2* was significantly decreased after 5 h of novel wheel access, while expression of *per1* was unaffected at this time ($P < 0.05$, ANOVA followed by Dunnet's test). The numbers were 4–5/group. B: Representative film images showing expression of *per1* (left panels) and *per2* (right panels) mRNA levels in the SCN at the time of the end of each treatment (ZT4–9, wheel access or control group). Coronal sections (20 μm) were examined by *in situ* hybridization using antisense cRNA probes at 1 h (*haper1*) and 2 h (*mper2*) after the treatment.

resetting of the circadian oscillator occurs during ∼2 h (Best et al., 1999), evidently the molecular photo-entrainment process may be completed *in vitro*.

Why was the SCN *in vitro* not shifted when the dissection was performed at ZT7 after 3 h of initial wheel access? It is unlikely that running activity in the remaining 2 h (ZT7–9) is the crucial factor. Although it has been shown that the number of wheel revolutions is related to the magnitude of the phase shift in a related paradigm (Mrosovsky, 1991; Bobrzynska and Mrosovsky, 1998), in our animals, running in their first wheel was mainly concentrated in the first 2 h of wheel access, and only rarely did an animal run more than 3 h, even when the wheel was left inside the cage. As shown by Wickland and Turek (1991) extension of the time of access to a first novel wheel beyond the initial hour has little effect on phase shift magnitude. If running in the first hour is a sufficient stimulus for the phase shift, might the process of inducing the permanent change in phase of the clock take up to 4 h more?

Our results suggest that non-photic resetting could take between 3 and 5 h from the start of the wheel access to be able to withstand *in vitro* isolation. Similarly, 3 h of sleep deprivation advances the clock within 4 h of the start of the gentle handling used to accomplish the sleep deprivation, as shown by advanced timing of light-induced *c-fos* (Antle and Mistlberger, 2000). As well, triazolam injections have been shown to take several hours to induce a phase advance in hamsters (Joy and Turek, 1992). To our knowledge, the timing of light

induction of *c-fos* has not been examined following novel wheel treatment. The fact that light can still modulate the final expression of the non-photic-induced phase advance even when applied up to 4 h after the end of the wheel pulse (Mrosovsky, 1991) supports our suggestion that several hours are needed for these behaviorally induced phase shifts to be completed. Nevertheless, although novel wheel-induced phase shifts may take between 3 and 5 h to be fully expressed, other kinds of non-photic manipulations such as saline or melatonin injections may shift the clock faster (Mead et al., 1992; Sumova and Illnerova, 1996).

The regulation of *per1* and *per2* mRNA levels could be a common mechanism for resetting of the mammalian clock by photic and non-photic cues (Fukuhara et al., 2001; Horikawa et al., 2000; Maywood et al., 1999; Maywood et al., 2002; Yokota et al., 2000). In our paradigm, the time when the phase shift can be measured *in vitro* is correlated with statistically significant *per2*, but not *per1*, suppression. If suppression of one or both of the two *period* genes is causally related to the occurrence of a phase shift, then we assume the mRNA must be suppressed to a particular level and/or for a particular duration of time.

The levels of *per1* and to a lesser extent *per2* after wheel running access during the day (ZT4–7) are comparable to the levels of expression found at ZT20 during the dark phase (Maywood et al., 1999). A very similar relative level of suppression was reported for the serotonergic agonist 8-OH-DPAT injected at circadian time (CT)6 with mRNA levels assessed 2 h later (Horikawa et al., 2000). There may not be a perfect correlation between the level of mRNA suppression and phase shift magnitude: triazolam and 8-OH-DPAT induced similar magnitude suppressions of *per1* and *per2* mRNA, yet phase advances measured following triazolam injections were larger than those following 8-OH-DPAT injections (Horikawa et al., 2000). This finding suggests that duration of suppression could be another important factor.

The time course of *Per* gene suppression varies with the stimulus. Horikawa et al. (2000) reported that *mper1* and *mper2* were strongly inhibited when examined 2 h (but not 1 h) after 8-OH-DPAT and triazolam injections, and both genes returned to normal mRNA levels when examined 4 h after the injections. Injections of brotizolam suppressed *per1* both 1 and 2 h post-injection and *per2* only at 2 h post-injection (Yokota et al., 2000). Novel wheel access was reported in previous studies to reduce both *per1* and *per2* levels by 3 h after introduction of the wheel (Maywood et al., 1999; Maywood and Mrosovsky, 2001). NPY can decrease daytime *per1* and *per2* levels in the SCN *in vitro* 0.5 and 2 h after NPY application respectively (Fukuhara et al., 2001). Compared to the present study, this may suggest the inference that NPY is released only circa 3 h after presentation of the novel wheel. This is surprising since antiserum to NPY can block novel wheel shifts when presented prior to the start of the novel wheel treatment (Biello, 1995).

It should be noted that since our data reveal a correlation between two events more than a causal linkage, we can not rule out the possibility that any effect on Per1 and Per2 genes is indirectly tied to the non-photic phase shift. However, evidence *in vitro* and *in vivo* indicate that these genes participate in non-photic phase resetting in the SCN.

Since the phase resetting action of NPY in the subjective day does not require de novo synthesis of clock proteins (Hall et al., 1999), and novel wheel access may not alter PER1 protein levels (Maywood et al., 1999), it is possible that the action of NPY on levels of *per* gene mRNA in the subjective day does not play a causal role in the subjective daytime resetting action. This is supported by the lack of mid-subjective day resetting when *mper1* antisense was administered to the suprachiasmatic nucleus (Akiyama et al., 1999), although extensive testing of effects of antisense has not yet been reported, for example, testing more phases during the subjective day and combinations of gene targets. Might *per* gene suppression be linked with activity and not with phase shifts?

Dissociation between *per* genes suppression and phase shifting occurrence is apparent in data from our lab, where a NPY Y1/Y5 receptor agonist, which does not phase shift the clock, still suppresses subjective day *per1* expression (Fukuhara et al., 2001). If activity is the crucial factor involved in the suppression of clock genes expression, then other phase shifting behavioral stimuli not linked to locomotor activation, like sleep deprivation (Antle and Mistlberger, 2000) and chlorodiazepoxide (Biello and Mrosovsky, 1993) should not affect *Per* expression. The effect of such stimuli on clock-genes expression remains to be investigated.

Light following a non-photic pulse consisting of 3 h of running in a novel wheel is able to block the phase advancing effect of the activity pulse; this appears to be a direct modulation of the phase shifting response and not a consequence of the effect of light on the phase delaying portion of the phase–response curve (Mrosovsky, 1991). Could dissection itself block the wheel-induced shift in some comparable manner? We do not posit that the SCN has a time window of sensitivity to this dissection effect, because animals with wheels for 3 h compared to animals with wheels for 5 h, both dissected at ZT9, show different phase shifting responses. It remains possible, however, that some effect of dissection might be responsible for a blocking effect of the non-photic stimulation 3 h after, but not 5 h after, the beginning of the novel wheel stimulation.

The paradigm employed in this study (i.e., exposure to a novel wheel using animals previously housed without wheels) induces phase advances when a rhythm other than the wheel running activity is recorded, like general activity or temperature. As shown by Wickland and Turek (1991), the amount of activity over baseline correlates with the magnitude of the phase shift at CT9 but only during the first hour of the stimulation. An average of 2 h phase advance was obtained with lengths of wheel pulses that range 0.5–3 h, and with varied amounts of total wheel revolutions. In agreement with these previous results, in our study, after exposure to the wheel for the first time the animals did not run more than 2–3 h, so it

appears that the length of the pulse is relevant mostly for the extra time the system as a whole is allowed to express or complete the phase shift. This suggests that under the 'first wheel' paradigm, the arousal induced by novelty might be an important signal for the circadian clock, as much as the level of activity is for phase shifts induced by dark, triazolam or novel wheel-induced activity. It is possible to achieve phase advances using a novel wheel pulse that can be observed when tracking other rhythms, like activity, temperature or, in our case, the *in vitro* neural firing rhythm of the SCN. Our '*in vivo/in vitro*' paradigm, although non-conventional, represents the possibility of dissecting a behaviorally induced phase shift to elucidate more precisely the time course and the anatomical requirements of this response.

Acknowledgements—This work was supported by NIH Grant NS26496 and AV Davis Foundation.

REFERENCES

Akiyama, M., Kouzu, Y., Takahashi, S., Wakamatsu, H., Moriya, T., Maetani, M., Watanabe, S., Tei, H., Sakaki, Y., Shibata, S., 1999. Inhibition of light- or glutamate-induced *mper1* expression represses the phase shifts into the mouse circadian locomotor and suprachiasmatic firing rhythms. J. Neurosci. 19, 1115–1121.

Antle, M.C., Marchant, E.G., Niel, L., Mistlberger, R.E., 1998. Serotonin antagonists do not attenuate activity-induced phase shifts of circadian rhythms in the Syrian hamster. Brain Res. 813, 139–149.

Antle, M.C., Mistlberger, R.E., 2000. Circadian clock resetting by sleep deprivation without exercise in the Syrian hamster. J. Neurosci. 20, 9326–9332.

Best, J.D., Maywood, E.S., Smith, K.L., Hastings, M.H., 1999. Rapid resetting of the mammalian circadian clock. J. Neurosci. 19, 828–835.

Biello, S.M., 1995. Enhanced photic phase shifting after treatment with antiserum to neuropeptide Y. Brain Res. 673, 25–29.

Biello, S.M., Janik, D., Mrosovsky, N., 1994. Neuropeptide Y and behaviorally induced phase shifts. Neuroscience 62, 273–279.

Biello, S.M., Mrosovsky, N., 1993. Circadian phase-shifts induced by chlordiazepoxide without increased locomotor activity. Brain Res. 622, 58–62.

Bobrzynska, K.J., Mrosovsky, N., 1998. Phase shifting by novelty-induced running: activity dose-response curves at different circadian times. J. Comp. Physiol. A 182, 251–258.

Challet, E., Scarbrough, K., Penev, P.D., Turek, F.W., 1998. Roles of suprachiasmatic nuclei and intergeniculate leaflets in mediating the phase-shifting effects of a serotonergic agonist and their photic modulation during subjective day. J. Biol. Rhythms 13, 410–421.

Ebling, F.J., 1996. The role of glutamate in the photic regulation of the suprachiasmatic nucleus. Prog. Neurobiol. 50, 109–132.

Fukuhara, C., McKinley Brewer, J., Dirden, J.C., Bittman, E., Tosini, G., Harrington, M.E., 2001. Neuropeptide Y rapidly reduces Period1 and Period2 mRNA levels in the hamster suprachiasmatic nucleus. Neurosci. Lett. 314, 119–122.

Gannon, R.L., Rea, M.A., 1995. Twelve hour phase shift of hamster circadian rhythms elicited by voluntary wheel running. J. Biol. Rhythms 10, 196–210.

Gillette, M.U., 1991. SCN electrophysiology *in vitro*. In: Klein, D.C., Moore, R.Y. Reppert, S.M. (Eds.), Suprachiasmatic Nucleus: The Minds Clock. Oxford University Press, New York, pp. 125–143.

Hall, A.C., Earle-Cruickshanks, G., Harrington, M.E., 1999. Role of membrane conductances and protein synthesis in subjective day phase advances of the hamster circadian clock by neuropeptide Y. Eur. J. Neurosci. 11, 1–9.

Harrington, M.E., Mistlberger, R., 2000. Anatomy and Physiology of the Mammalian Circadian System. In: Kryger, M.H., Roth, T., Dement, W.C. (Eds.), Principles and Practice of Sleep Medicine, 3rd edn. WB Saunders, PA, pp. 334–345.

Hastings, M.H., Duffield, G.E., Smith, E.J., Maywood, E.S., Ebling, F.J., 1998. Entrainment of the circadian system of mammals by nonphotic cues. Chronobiol. Int. 15, 425–445.

Horikawa, K., Yokota, S., Fuji, K., Akiyama, M., Moriya, T., Okamura, H., Shibata, S., 2000. Nonphotic entrainment by 5-HT1A/7 receptor agonists accompanied by reduced *per1* and *per2* mRNA levels in the suprachiasmatic nuclei. J. Neurosci. 20, 5867–5873.

Janik, D., Mrosovsky, N., 1994. Intergeniculate leaflet lesions and behaviorally-induced shifts of circadian rhythms. Brain Res. 651, 174–182.

Joy, J.E., Turek, F.W., 1992. Combined effects on the circadian clock of agents with different phase response curves: phase-shifting effects of triazolam and light. J. Biol. Rhythms 7, 51–63.

Maywood, E.S., Okamura, H., Hastings, M.H., 2002. Opposing actions of neuropeptide Y and light on the expression of circadian clock genes in the mouse suprachiasmatic nuclei. Eur. J. Neurosci. 15, 216–220.

Maywood, E.S., Mrosovsky, N., 2001. A molecular explanation of interactions between photic and non photic circadian clock resetting stimuli. Gene Expr. Patterns 1, 27–31.

Maywood, E.S., Mrosovsky, N., Field, M.D., Hastings, M.H., 1999. Rapid down-regulation of mammalian period genes during behavioral reseting of the circadian clock. PNAS 96, 15211–15216.

McKinley Brewer, J., Yannielli, P.C., Harrington, M.E., 2002. Neuropeptide Y differencially suppresses *per1* and *per2* mRNA induced by light in the suprachiasmatic nuclei of the golden hamster. J. Biol. Rhythms, 17, 28–39.

Mead, S., Ebling, F.J., Maywood, E.S., Humby, T., Herbert, J., Hastings, M.H., 1992. A nonphotic stimulus causes instantaneous phase advances of the light-entrainable circadian oscillator of the Syrian hamster but does not induce the expression of c-fos in the suprachiasmatic nuclei. J. Neurosci. 12, 2516–2522.

Meyer-Bernstein, E.L., Morin, L.P., 1998. Destruction of serotonergic neurons in the median raphe nucleus blocks circadian rhythm phase shifts to triazolam but not to novel wheel access. J. Biol. Rhythms 13, 494–505.

Mintz, E.M., Gillespie, C.F., Marvel, C.L., Huhman, K.L., Albers, H.E., 1997. Serotonergic regulation of circadian rhythms in Syrian hamsters. Neuroscience 79, 563–569.

Morin, L.P., 1999. Serotonin and the regulation of mammalian circadian rhythmicity. Ann. Med. 31, 12–33.

Mrosovsky, N., 1991. Double-pulse experiments with nonphotic and photic phase-shifting stimuli. J. Biol. Rhythms 6, 167–179.

Petersen, S.L., McCrone, S., 1994. Characterization of the receptor complement of individual neurons using dual-label *in situ* hybridization histochemistry. In: Eberwine, J.H., Valentino, K.L., Barchas, J.D. (Eds.), *In situ* Hybridization in Neurobiology. Advances in Methodology. Oxford Universuty Press, New York, p. 82.

Reppert, S., Weaver, D., 2001. Molecular analysis of mammalian circadian rhythms. Ann. Rev. Physiol. 63, 647–676.

Sumova, A., Illnerova, H., 1996. Melatonin instantaneously resets intrinsic circadian rhythmicity in the rat suprachiasmatic nucleus. Neurosci. Lett. 218, 181–184.

Wickland, C., Turek, F.W., 1994. Lesions of the thalamic intergeniculate leaflet block activity-induced phase shifts in the circadian activity rhythm of the golden hamster. Brain Res. 660, 293–300.

Wickland, C.R., Turek, F.W., 1991. Phase-shifting effects of acute increases in activity on circadian locomotor rhythms in hamsters. Am. J. Physiol. 261, R1109–R1117.

Yannielli, P.C., Harrington, M.E., 2000. Neuropeptide Y applied *in vitro* can block the phase shifts induced by light *in vivo*. NeuroReport 11, 1587–1591.

Yokota, S.I., Horikawa, K., Akiyama, M., Moriya, T., Ebihara, S., Komuro, G., Ohta, T., Shibata, S., 2000. Inhibitory action of brotizolam on circadian and light-induced per1 and per2 expression in the hamster suprachiasmatic nucleus. Br. J. Pharmacol. 131, 1739–1747.

Zylka, M.J., Shearman, L.P., Weaver, D.R., Reppert, S.M., 1998. Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. Neuron 20, 1103–1110.

(*Accepted* 20 *February* 2002)