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Suprachiasmatic Regulation of Circadian Rhythms of Gene Expression in Hamster Peripheral Organs: Effects of Transplanting the Pacemaker

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Neurotransplantation of the suprachiasmatic nucleus (SCN) was used to assess communication between the central circadian pacemaker and peripheral oscillators in Syrian hamsters. Free-running rhythms of haPer1, haPer2, and Bmal1 expression were documented in liver, kidney, spleen, heart, skeletal muscle, and adrenal medulla after 3 or 11 weeks of exposure to constant darkness. Ablation of the SCN of heterozygote tau mutants eliminated not only rhythms of locomotor activity but also rhythmic expression of these genes in all peripheral organs studied. The Per:Bmal1 ratio suggests that this effect was attributable not to asynchronous rhythmicity between SCN-lesioned individuals but to arrhythmicity within individuals. Grafts of wild-type SCN to heterozygous, SCN-lesioned tau mutant hamsters not only restored locomotor rhythms with the period of the donor but also led to recovery of rhythmic expression of haPer1, haPer2, and haBmal1 in liver and kidney. The phase of these rhythms most closely resembled that of intact wild-type hamsters. Rhythmic gene expression was also restored in skeletal muscle, but the phase was altered. Behaviorally effective SCN transplants failed to reinstate rhythms of clock gene expression in heart, spleen, or adrenal medulla. These findings confirm that peripheral organs differ in their response to SCN-dependent cues. Furthermore, the results indicate that conventional models of internal entrainment may need to be revised to explain control of the periphery by the pacemaker.

Key words: suprachiasmatic; circadian; period gene; Bmal1; entrainment; neurotransplantation

Introduction

The circadian system of mammals contains multiple oscillators that are governed by a central pacemaker localized to the suprachiasmatic nucleus of the hypothalamus (SCN) (Hastings et al., 2003). Ablation of the SCN eliminates circadian rhythms of locomotor activity, and transplantation of fetal SCN leads to recovery of behavioral oscillations whose periods match that of the donor (Ralph et al., 1990). Encapsulated grafts that make no axonal connections with the host appear competent, indicating that humoral products of the pacemaker sustain behavioral rhythms in the host (Silver et al., 1996). SCN grafts appear incompetent to sustain endocrine rhythms, suggesting that different pathways may be responsible for pacemaker control of physiological rhythms (Meyer-Bernstein et al., 1999).

On a molecular level, the operation of a transcriptional-translational feedback loop within the SCN appears critical to rhythmicity (Albrecht and Eichele, 2003). The protein products of the Bmal1 and Clock dimerize and stimulate transcription of Period and Cryptochrome genes. The protein products of Per and Cry inhibit transcriptional activation by the BMAL1:CLOCK dimer. Rhythms of Per1/2, Cry1/2, and Bmal1 expression occur not only in the SCN but also in peripheral organs including heart, lung, liver, skeletal muscle, and spleen (Oishi et al., 1998; Zylka et al., 1998; Bittman et al., 2003; Carr et al., 2003; Tong et al., 2004). Not only do the peaks of transcription lag that of the SCN by 6–9 h, but ablation of the central pacemaker leads to loss of these peripheral rhythms in rats (Sakamoto et al., 1998) and mice (Akhtar et al., 2002; Iijima et al., 2002; Terazono et al., 2003). Experiments using reporter constructs indicate that peripheral tissues can sustain many circadian cycles after isolation from the pacemaker (Yamazaki et al., 2000; Yoo et al., 2004).

Although these observations support the multioscillatory model of the circadian system, they raise new questions about the role of the SCN as a central pacemaker and the way in which it may entrain the periphery. Although SCN transplants can reinstate behavioral rhythms in knock-out mice incapable of sustaining peripheral circadian rhythms (Sujino et al., 2003), central regulation of the peripheral oscillators is poorly understood. The ability of SCN transplants to reinstate rhythms of clock gene expression in peripheral organs has not been established. Parabiotic linkage of SCN-lesioned mice to intact partners can reinstate circadian rhythms of mPer and mBmal1 gene expression in some, but not other peripheral organs (Guo et al., 2005). This suggests...
that different peripheral oscillators may be controlled by different pathways, with blood-borne signals being of predominant importance for some but not others. In the present studies, we used SCN transplants to investigate the role of this hypothalamic structure in regulation of circadian oscillations in peripheral organs.

Materials and Methods
Wild-type Syrian hamsters (LAK:LVG strain) were purchased from Lakeview hamstery or bred in our laboratory. All procedures were approved by the University of Massachusetts Institutional Animal Care and Use Committee. Tau mutant hamsters used in these studies were heterozygotes that were bred locally by crossing homozygote male males with wild-type females. Phenotype was determined by maintaining animals in constant darkness (DD) for several weeks and recording locomotor activity using running wheels and Clocklab software (Actimetrics, Evanston, IL). Tau mutant hamsters exhibited circadian periods between 21.5 and 22.5 h (mean 22.20 ± 0.14 h SEM). A schematic illustration of the experimental protocol is provided in Figure 1.

Heterozygous tau mutant hamsters were subjected to SCN lesions as previously described (Matsushita et al., 1996). Briefly, a tungsten rod electrode (A-M Systems, Carlsborg, WA), insulated except at the tip with Epoxytite resin, was lowered to a position 0.75 rostral to bregma, 7.85 mm below the dural surface, and 0.2 mm on each side of the midline. Lesions were made by passing 1.5 mA of direct current for 15 s. Arrhythmicity was confirmed by assessment of wheel-running behavior during at least 3 weeks of DD following surgery. Two wild-type hamster pups were killed on the 15th day of embryonic life for use as donors for each behaviorally arrhythmic hamster as previously described (Meyer-Bernstein et al., 1999).

Of the tau mutant SCN-lesioned animals, one group of controls (n = 22) received cortex transplants to allow assessment of peripheral rhythms of circadian gene expression in the absence of the SCN. These animals were maintained in 14:10 h light/dark (14L:10D) for up to 1 week after graft surgery and then were transferred to DD for up to 7 weeks to confirm that arrhythmic locomotor patterns persisted. To determine a nonrandom killing time, these animals were next placed in 14L:10D for 2 weeks. They were then returned to DD and killed 3 d later at zeitgeber time (ZT) 3, 9, 15, or 21 to permit comparison with intact and behaviorally recovered, SCN-transplanted lesioned animals killed at the corresponding circadian times (CTs).

Another group of experimental animals (n = 24) received SCN transplants. These animals were placed in DD several days after surgery. Animals that recovered free-running locomotor rhythms within 8 weeks were considered to have received successful transplants. A minimum of five such hamsters were killed at each of four circadian times (CT3, 9, 15, or 21).

Wild-type intact hamsters served as additional controls. These animals were transferred from 14L:10D to DD for 3 d (acute DD) or 11 weeks (chronic DD) before being killed. These intervals were chosen to match the duration of DD exposure of cortex-grafted (acute) and SCN-grafted (chronic) animals, respectively, during the final phase of the experiment. Intact hamsters were killed at CT3, 9, 15, or 21.

Animals were killed by rapid decapitation under very dim red light. Brains, livers, kidneys, testes, heart, adrenal (cortex plus medulla), spleen, and skeletal (vastus) muscle were rapidly dissected and immediately frozen on dry ice. Tissues were stored at −80°C until mRNA assessment. Brain sections were cut on a cryostat at a thickness of 20 μm, thaw-mounted onto Superfrost-Plus slides (Fisher, Pittsburgh, PA) and processed for in situ hybridization to evaluate the completeness of the lesion, the borders of the graft, and gene expression within the transplant. Adjacent series of sections were processed to detect expression of AVP and haPer1. Adrenal glands were sectioned in a similar manner and processed for in situ hybridization for haPer1, mPer2, and haBmal1 as described previously (Bittman et al., 2003). Briefly, sections were thawed for 10 min, fixed 15 min with 4% formalin in PBS, deaminated in 0.25% acetic anhydride/triethanolamine (0.1 M, 0.9% NaCl), dehydrated through graded alcohols, delipidated in CHCl3, briefly rehydrated, and air dried. Hybridization was performed using [35S]-UTP-labeled antisense cRNA probes that were prepared by in vitro transcription from the following templates: a ~758 bp transcript from an 1121 bp Syrian hamster per1 cDNA fragment (GenBank accession number AF249882, nt215-1336, homologous to AF02292 nt392-1150) cloned into pBluescript IIKS(-) (Stratagene, La Jolla, CA), created by Drs. P. L. Lowrey and J. S. Takahashi (Northwestern University, Evanston, IL); a 480 bp mouse per2 fragment (GenBank accession number AF035830 nt 9–489) in the pCRII vector (Invitrogen, Carlsbad, CA), a gift from Drs. L. Shearman and S. M. Reppert (University of Massachusetts Medical School, Worcester, MA), and Syrian hamster Bmal1 (GenBank accession number AF090177 nt1-1336) in pDNA3, a gift of Dr. N. Gekakis (Harvard University, Boston, MA). Plasmids were linearized for antisense and sense transcription, respectively, with the following indicated RNA polymerases: haPer1 with ApaI (T7) and BstXI(T3), Per2 with NotI(Sp6) and BamHI(T7), and Bmal1 with HindIII(Sp6) and XbaI(T7). Slides were incubated overnight in a humidified chamber at 37°C with 4 × 104 cpm/ml probe in hybridization buffer [0.01% sodium pyrophosphate, 10% dextran sulfate, 2× saline sodium citrate (SSC), 50% formamide, 0.1% yeast tRNA, 0.5% heparin, 50 mM DTT, and 1× Denhardt’s solution]. Posthybridization washes consisted of rinses in 1× SSC at room temperature followed by 2× SSC–50% formamide at 52°C, incubation in RNaseA (50 mg/ml; USB, Cleveland, OH) at 37°C, 2× SSC at room temperature, 2× SSC–50% formamide at 52°C, 2× SSC, an ethanol dehydration series, and air-drying. Slides were apposed to Kodak BioMax MR film to assess hybridization signal, and then dipped in NTB3 emulsion and exposed for 2 weeks to allow assessment of mRNA over adrenal cortex or medulla or the neural transplant. Sections were counterstained with Toluidine blue (brain) or hematoxylin/eosin (adrenals), coverslipped using Permoun, and examined under bright-field and dark-field microscopy. Images were captured using a digital camera (Magnafire; Optronics, Goleta, CA) attached to a Leica (Deerfield, IL) microscope and imported into Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA). Images were not altered in any way except for minor adjustments of brightness and contrast.
Total RNA was extracted from peripheral organs using the Ultraspec RNA isolation reagent (Biotex Laboratories, Friendswood, TX) according to the directions of the supplier. RNA (10 μg total) was separated by electrophoresis through a 1% agarose formaldehyde gel, blotted onto Zeta-Probe GT membrane (Bio-Rad, Hercules, CA), and hybridized with random primed 32P-labeled probe (specific activity 2 × 10^6 cpm/ml) as described previously (Tong et al., 2004). Blots were hybridized with ULTRAhyb buffer (Ambion, Austin, TX) and washed following the protocol of the manufacturer. Probes were haPer1 (homologous to nt 950-1586, GenBank accession number AF022992, a gift of Dr. T. Hamada, Columbia University, New York, NY), haPer2 (homologous to nt 841-1620, GenBank accession number AF035830), and haBmal1 (nt 300-1770, GenBank accession number AF070917). Blots were exposed at –80°C to Kodak (Rochester, NY) BioMax MS film with two intensifying screens. Individual blots were repeatedly probed and stripped with haPer1, haPer2, haBmal1, and haGAPDH (homologous to nt 692-1160, accession number AF106860), respectively. Autoradiograms were quantified by densitometry. Ratios between each transcript and GAPDH were calculated. Statistical evaluation was performed using the Kruskal–Wallis test.

Results
As expected, significant temporal fluctuations occurred in relative haPer1, haPer2, and haBmal1 expression in liver, kidney, heart, spleen, and skeletal muscle of intact wild-type hamsters on the third day of constant darkness (Fig. 2). In each of these organs, haBmal1 mRNA peaked in antiphase to haPer expression. The effect of duration of exposure to DD (3 d vs 11 weeks) on the phase and amplitude of circadian gene expression differed considerably between tissues. Differences between organs in the amplitude of clock gene expression and reduced amplitudes after prolonged exposure to DD may have made it more difficult to assess fully the restoration of rhythmicity by SCN transplants. In liver, haPer1 expression was similar in acute and chronic DD, although the duration of the peak lengthened with prolonged exposure to constant darkness. The amplitude of the liver haPer2 peak was reduced in chronic DD, but the pattern of haBmal1 expression was unchanged. In kidney, the haPer1 peak tended to be broader in chronic than in acute DD, but expression of haPer2 and haBmal1 was similar after 3 d and 11 weeks of DD. In spleen, the amplitude of the haPer1 rhythm tended to decrease after long-term exposure to DD, whereas the haPer2 mRNA rhythm was little affected and relative levels of haBmal1 at CT3 and CT9 were increased. The rise of haPer1 expression in the heart was more sustained in chronic than in acute DD, but duration of haPer2 and haBmal1 expression was similar in both conditions. In skeletal muscle, chronic DD reduced the amplitude of the haPer2 rhythm and extended the trough of haBmal1 expression but had little effect on haPer1 mRNA patterns. As reported previously (Tong et al., 2004), two transcripts of haPer1 were detected in Northern blots of hamster testes. Both transcripts showed a statistically significant low-amplitude rhythm in acute DD. Expression of both haPer1 transcripts was profoundly suppressed at all CTS after 11 weeks of DD. No rhythm of haPer2 expression was evident in testes of either acute or chronic DD hamsters. In contrast, haBmal1 mRNA levels showed a low amplitude oscillation in testes of hamsters maintained in DD for 11 weeks, but not in animals killed on the third day of constant darkness. In situ hybridization revealed that rhythms of haPer1, haPer2, and haBmal1 expression in adrenal medulla were of higher amplitude in acute than in chronic DD (Fig. 3). No significant rhythms of clock gene expression were evident in adrenal cortex in either acute or chronic DD.

SCN ablation eliminated circadian rhythms of locomotor activity (Fig. 4). As expected, these oscillations were reinstated by grafts of fetal SCN but not by transplantation of fetal cortex. The period of behavioral rhythms reinstated by SCN grafts corresponded to the wild-type donors (τ = 24.06 ± 0.04 h; n = 21) and differed significantly from the prelesion period in the same animals (τ < 0.001, paired t test). Histological inspection confirmed the existence of a complete lesion and a healthy, viable graft in the third ventricle in each case in which the rhythms disappeared after brain lesion and were restored after SCN transplantation. In situ hybridization revealed discrete overlapping clusters of AVP- and haPer1-expressing cells in CT3 and CT9 grafts (Fig. 5).

Figure 2. Normalized values (mean ± SEM) of haPer1 (diamonds and purple lines), haPer2 (squares and red lines), and haBmal1 (circles and blue lines) mRNA (relative to GAPDH) in peripheral organs of intact hamsters exposed to DD for 3 d (acute DD, left) or 11 weeks (chronic DD, right) before being killed at circadian time (as indicated by locomotor activity onset) 3, 9, 15, or 21. The asterisks indicate statistical significance (p < 0.05) compared with nadir values of the same transcript by Kruskal–Wallis test.
Discussion

Our results establish that ablation of the SCN eliminates circadian rhythms of clock gene expression in several organs and that its transplantation can restore these rhythms in some, but not other organs. The pacemaker regulates the periphery through diverse pathways, and revisions of the internal entrainment model may be necessary.

Syrian hamsters are highly seasonal. Daylength regulates reproduction, metabolism, and immunity (Banerji and Quay, 1986; Esquifino et al., 1994; Demas and Bartness, 2001; Goldman, 2001). Although our primary purpose was to provide appropriate intact controls for evaluation of the effects of cortex or SCN grafts in arrhythmic hamsters, the comparison of clock gene expression patterns in acute vs chronic DD-exposed hamsters sheds light on photoperiodic responses of peripheral organs. Our findings confirm that clock gene expression in testis is suppressed by protracted darkness (Tong et al., 2004). Short photoperiods were also reported to suppress the amplitude and duration of haPer1 rhythms in hamster heart and lung (Carr et al., 2003). Our data agree in that long-term DD altered clock gene expression, but we found that chronic DD elicited an increase in haPer1 mRNA in the heart during late subjective night. Suppression of haPer2 expression in liver and adrenal medulla in chronic DD may also reflect photoperiodic regulation. Clock gene expression was also suppressed in spleen, but muscle and kidney were little affected by duration of DD exposure. The physiological significance of these changes is unknown. Although there was good agreement in the phase of clock gene expression after 3 d versus 11 weeks in most peripheral organs, we observed interesting changes in the duration of gene expression that may correspond to the expansion of locomotor activity (a) that is commonly observed in protracted darkness.

Destruction of the SCN is known to eliminate circadian rhythms of clock gene expression in peripheral organs of rats (Sakamoto et al., 1998) and mice (Akhtar et al., 2002; Iijima et al., 2002; Terazono et al., 2003). Nevertheless, it is controversial whether SCN lesions cause arrhythmicity within individuals or asynchronous rhythmicity between individuals. Yoo et al. (2004) observed that peripheral tissues collected from SCN-lesioned mice, like those of intact animals, exhibit circadian rhythms of PER2:Luc in static explant culture. SCN lesions increased the variability of the phase of this oscillation in vitro. The authors concluded that peripheral oscillations persist in vivo after SCN ablation. Although these observations, like others on a rat Per1:Luc construct (Yamazaki et al., 2000), support the capacity of peripheral tissues to sustain circadian rhythms, they are also consistent with the interpretation that SCN lesions induce peripheral arrhythmicity in vivo. The capacity of tissues to oscillate in vitro does not require that they are also rhythmic in vivo. Damped rhythms of clock gene reporter expression are restarted by medium change (Yamazaki et al., 2000). Therefore, it is likely that setting up cultures will initiate oscillations of tissues taken from SCN-lesioned mice even if they are arrhythmic before they are killed. SCN ablation may have diverse and nonspecific effects that increase variability of the latency between the establishment of the culture and the onset of the oscillation (Cailotto et al., 2005). Ultimately it may be desirable to adopt an in vivo bioluminescence approach (Yamaguchi et al., 2001; Ishida et al., 2005), perhaps in combination with SCN transplantation, to more fully understand pacemaker control of peripheral phase.

Our data from intact hamsters can be used to test the idea that peripheral rhythms persist after SCN ablation with phases that
differ between individuals. If this is true, haPer and haBmal1 mRNA levels should remain in antiphase in both intact and lesioned individuals. A simulation of the effects of averaging together data from animals that are free running at widely distributed phases is provided by plotting the haPer:haBmal1 ratios of intact hamsters killed at CT3, 9, 15, and 21. These data (Fig. 7) show that combining data from hamsters that are free running asynchronously would produce intermediate haPer:haBmal1 ratios at all times at which the animals are killed, with a large SEM at every point. The actual ratios of SCN-lesioned, cortex-grafted animals killed at the corresponding Zeitgeber times do not correspond to this simulation. Instead, there is a very small SEM at every CT. The coefficients of variation at each CT and in each organ were invariably smaller in the cortex-grafted animals (0.33 for Per1:Bmal1 and 0.27 for Per2:Bmal1) than in the simulation performed by combining values from intact animals (0.92 for both Per1:Bmal1 and Per2:Bmal1). This outcome suggests the arrest of free-running peripheral tissue rhythms by SCN lesions. It is less consistent with the idea that peripheral tissues of SCN-lesioned hamsters free run asynchronously. Our results thus contradict the assertion (Yoo et al., 2004) that the SCN functions as a phase coordinator and instead suggest that the SCN serves as a pacemaker of peripheral oscillations. Although rhythms at a tissue level may be arrested when removed from the influence of the SCN, individual cells continue to oscillate but lose phase coherence (Nagoshi et al., 2004; Welsh et al., 2004). In the absence of communication between oscillating cells within a peripheral organ, the role of SCN may be to impose phase coherence from above.

We observed three patterns of response to SCN grafts. The liver and kidney exhibited rhythms that resembled those of intact hamsters. At the other extreme, the spleen and heart were similar in the SCN- and the cortex-grafted groups, despite the efficacy of the former in restoring liver, kidney, and activity rhythms. Skeletal muscle showed a third pattern: recovery of rhythmic clock gene expression with an unusual phase. This diversity of response patterns shows striking similarity to their response to parabiotic linkage of arrhythmic to intact mice. In those experiments (Guo et al., 2005), rhythms of mPer1, mPer2, and mBmal1 gene expression recovered in liver and kidney, but not in spleen, heart, or skeletal muscle, of SCN-lesioned mice surgically linked to brain-intact partners. This result suggests that blood-borne and/or other non-neural (e.g., behavioral or temperature) SCN-dependent cues are sufficient to drive circadian rhythms in liver and kidney but not other organs. Grafts that establish activity rhythms also lead to periodic feeding and thus may restore blood-borne cues sufficient for recovery of
rhythms in liver and kidney, but not other organs. The liver clock responds to dietary cues (Damiola et al., 2000; Stokkan et al., 2001), but the relative sensitivity of other organs to such signals is unclear. Alternatively, some by-product of periodic locomotion, e.g., a rise in metabolites or body temperature, might sustain synchrony among oscillators in liver and kidney but not in spleen and heart. Although circadian rhythms in some tissues (including liver) may be reset by adrenal steroid hormones, this seems unlikely to account for the recovery of rhythms observed here because SCN transplants fail to reinstate rhythms of glucocorticoid secretion (Meyer-Bernstein et al., 1999).

Our results indicate that, at least for the liver and kidney, a 2 h period difference is compatible with entrainment of the periphery to the pacemaker. These findings are consistent with the report that mouse embryonic fibroblasts can be influenced by humoral signals after implantation into a host of a different genotype, although it was not possible to resolve their phase and period in those experiments (Pando et al., 2002). Entrainment theory predicts that the phase angle difference between a slave oscillator and its master depends on the periods of the two oscillations (Pittendrigh and Daan, 1976; Aschoff and Pohl, 1978; Pittendrigh, 1981). This model of entrainment of a multioscillatory system predicts that a temporal program is generated as slave oscillators advance or delay phase.

Figure 6. Normalized values (mean ± SEM) of *haPer1* (diamonds and purple lines), *haPer2* (squares and red lines), and *Bmal1* (circles and blue lines) mRNA (relative to GAPDH) in peripheral organs of *tau* heterozygote SCN-lesioned hamsters that received intraventricular grafts of fetal cortex (left) or SCN (right) from embryonic day 15 wild-type donors. Recipients were maintained in DD for ∼11 weeks after transplantation surgery. Cortex-grafted animals, whose locomotor rhythms did not recover after surgery, were transferred to 14L:10D for 2 weeks before being returned to DD and killed at indicated zeitgeber times 3 d later. SCN-grafted animals were decapitated at CT3, 9, 15, or 21 as defined by locomotor activity rhythms that were restored after transplant surgery. Symbols indicate statistical significance (p < 0.05) relative to the same transcript (*, Per1; †, Per2; +, Bmal1) at one or more other times of day as in Figure 2.

Figure 7. Top, Simulation of liver *Per1:Bmal1* (A) and *Per2:Bmal1* (B) mRNA ratios that would be obtained by averaging values of hamsters showing asynchronous free runs. For each time point, data obtained in the present study from five intact wild-type animals were used; data were taken from equal numbers of animals killed at the different phases (CT3, 9, 15, and 21). Note that *Per:Bmal* ratios remained at intermediate values with large SEMs at each time point. Bottom, Actual *Per1:Bmal1* (C) and *Per2:Bmal1* (D) ratios obtained from intact animals killed after 3 d (diamonds and black lines) or 11 weeks (squares and dashed lines) of DD at the indicated CTS and of SCN-lesioned, cortex-grafted hamsters (circles and gray lines) killed at the corresponding ZTS. Note that little variation is evident in *Per:Bmal* ratios in cortex-grafted animals killed at various zeitgeber times, as would be expected if asynchronous free runs were occurring. Similar patterns were obtained using data from kidney, spleen, muscle, and heart.
pheral oscillators have a strong resetting curve to dexamethasone (Nagoshi et al., 2004). We are working to achieve better resolution of the phase of circadian rhythms re-established by SCN transplants and to systematically vary the periods of donors and hosts. Nevertheless, the present results establish the utility of this model in examination of mechanisms of internal entrainment.

References


