Circadian Organization Is Governed by Extra-SCN Pacemakers

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Abstract In mammals, a pacemaker in the suprachiasmatic nucleus (SCN) is thought to be required for behavioral, physiological, and molecular circadian rhythms. However, there is considerable evidence that temporal food restriction (restricted feedisng [RF]) and chronic methamphetamine (MA) can drive circadian rhythms of locomotor activity, body temperature, and endocrine function in the absence of SCN. This indicates the existence of extra-SCN pacemakers: the Food Entrainable Oscillator (FEO) and Methamphetamine Sensitive Circadian Oscillator (MASCO). Here, we show that these extra-SCN pacemakers control the phases of peripheral oscillators in intact as well as in SCN-ablated PER2::LUC mice. MA administration shifted the phases of SCN, cornea, pineal, pituitary, kidney, and salivary glands in intact animals. When the SCN was ablated, disrupted phase relationships among peripheral oscillators were reinstated by MA treatment. When intact animals were subjected to restricted feeding, the phases of cornea, pineal, kidney, salivary gland, lung, and liver were shifted. In SCN-lesioned restricted-fed mice, phases of all of the tissues shifted such that they aligned with the time of the meal. Taken together, these data show that FEO and MASCO are strong circadian pacemakers able to regulate the phases of peripheral oscillators.

Key words peripheral oscillators, methamphetamine, MASCO, restricted feeding, FEO, period 2, luciferase reporter, mammalian, mouse

Since the 1970s, it has been believed that mammalian circadian rhythms are under the strict control of the suprachiasmatic nucleus of the hypothalamus (SCN) (Moore and Eichler, 1972; Stephan and Zucker, 1972). Ablation of the SCN results in total behavioral arrhythmicity and loss of other circadian rhythms. Recently, however, new evidence has emerged to challenge the view of the SCN as the sole circadian pacemaker.

Scheduled daily meals are able to entrain the activity rhythms of rodents in the absence of the SCN (Krieger et al., 1977; Marchant and Mistlberger, 1997;

Stephan, 1984). Animals anticipate the timing of a single daily meal and display continued food-anticipatory activity (FAA) when released into fasting conditions (Stephan, 1992). The ability of restricted meals to drive daily rhythms of locomotor activity in animals with complete SCN ablation demonstrates the existence of a food entrainable oscillator (FEO).

Circadian rhythms of locomotor activity in SCN-lesioned animals have also been observed when animals are administered methamphetamine, either chronically in their drinking water or via subcutaneously implanted

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minipumps (Honma et al., 1987; Tataroglu et al., 2006). These data imply the existence of yet another circadian oscillator, which we have called the Methamphetamine Sensitive Circadian Oscillator (MASCO). MASCO represents an endogenous circadian oscillator and not the output of an hourglass mechanism, as persistent rhythmic behavior can be observed for several cycles even after the stimulus (drug) is withdrawn (Mohawk and Menaker, 2009; Tataroglu et al., 2006), and circadian rhythms of locomotor activity recur when methamphetamine is delivered continuously (Honma et al., 1987). Both FEO and MASCO are capable of producing circadian rhythms of locomotor activity, body temperature, and hormone secretion (Angeles-Castellanos et al., 2010; Honma et al., 1988; Honma et al., 1992).

This study tests the hypothesis that both MASCO and FEO can regulate the phases of peripheral oscillators in intact and SCN-lesioned mice. We show that FEO and MASCO can shift the phases of peak PER2 expression in individual tissues more robustly in SCN-lesioned than in intact mice, demonstrating that, under some conditions, the SCN competes with FEO and MASCO for control of peripheral circadian rhythmicity. Our results support the conclusion that both FEO and MASCO are true circadian pacemakers, capable of organizing circadian rhythmicity throughout the body.

MATERIALS AND METHODS

Animals

Adult *mPeriod2^Luciferase* mice (*mPer2^Luc*; originally derived from animals kindly given to us by Dr. Joseph Takahashi) were obtained from our breeding colony. These mice carry a PER2::LUC fusion protein, which has been engineered to produce light when expressed in the presence of luciferin (Yoo et al., 2004). Reports indicate that PER2::LUC expression from cultured explants is a reliable measure of in vivo expression and circadian organization (Yamazaki and Takahashi, 2005; Yoo et al., 2004; Yoshikawa et al., 2005). We used both male and female mice and observed no robust differences between the sexes. Procedures were approved by the University of Virginia Animal Care and Use Committee.

Experimental Design

Effects of methamphetamine. mPer2^Luc mice were assigned to 1 of 3 groups: intact control, sham-lesioned control (sham), or SCN lesion (SCNX). The SCN was ablated electrolytically as previously described (Mohawk et al., 2009), and only animals exhibiting total behavioral arrhythmicity (as determined by χ^2 periodogram analysis) were included in the experiments. The sham-lesioned animals received the same procedure as the SCN-lesioned ones except that the current was not passed through the electrode lowered into the animal's brain. Following 1 week of postoperative recovery in an entraining 12:12 LD cycle, the animals were released to constant darkness (DD). The intact group was treated identically but received no surgery or anesthesia. After 2 weeks in DD, the animals either received methamphetamine (MA) ad libitum in their drinking water (0.005% MA) for 3 more weeks or were left on pure water. A separate group of SCN-lesioned animals was treated with MA for 4.5 additional weeks (total MA treatment = 7.5 weeks) to investigate the long-term effects of the drug. The animals were sacrificed and tissues harvested at CT11.5 (approximately 30 min prior to activity onset) as determined by each individual's wheel-running activity record.

Effects of restricted feeding. mPer2^Luc mice were assigned to the same 3 groups as above. One week after the surgeries, animals were released to DD. After 2 weeks in DD, half of the sham-operated group as well as half of the intact group were subjected to a temporally restricted feeding (RF) schedule with food only available for 6 h from CT3 to CT9. SCN-lesioned animals were fed between local time 0900 to 1500 h. The rest of the animals were fed ad libitum. Following 9 to 11 days of restricted feeding, animals were sacrificed, and tissues were cultured 1 h before the end of restricted feeding. The groups of intact pure water–treated animals and SCNX pure water–treated animals from the MA experiment were used as the intact ad libitum and SCNX ad libitum controls.

Locomotor Activity Analysis

Wheel-running activity was recorded and analyzed using ClockLab (Actimetrics, Evanston, IL). Activity onset was calculated independently for each animal to determine circadian time. Free-running period (τ) was calculated using χ^2 periodogram analysis on at least 1 week of continuous data over a range of 16 to 42 h, with α set at 0.01. Food-anticipatory activity (FAA) was also measured by wheel-running activity. Data were compressed into 5-min bins and daily activity profiles generated in ClockLab (Actimetrics) for the 3 days prior to the onset of RF (ad libitum or AL days) and the 3 days of RF prior to the day of

euthanasia (RF days). Mean activity profiles were generated for AL days and RF days and plotted as a function of time ($n = 5$ sham; $n = 4$ SCNX).

Tissue Culture

Tissue culture and preparation were performed as described elsewhere (Yamazaki and Takahashi, 2005). Following treatment, mice were anesthetized with carbon dioxide, and tissues were harvested and placed in chilled Hanks' balanced salt solution. We collected SCN, pineal gland, salivary gland, pituitary gland, liver, lung, kidney, and cornea. SCN was taken from a 300-μm coronal brain section. Pineal glands were flattened and cultured whole. The anterior pituitary gland was also cultured whole. The salivary gland, liver, lung, and kidney were hand sliced into thin sections. The whole cornea was removed from the eye. Explanted tissues were placed on Millicell culture inserts (Millipore, Billerica, MA) in 35-mm culture dishes in medium containing luciferin. Cultures were incubated for 6 days under constant conditions (constant darkness, constant temperature of 35 °C, and no medium change). Light emitted from each culture was recorded with photomultiplier detectors (Hamamatsu, Bridgewater, NJ).

Analysis of PER2::LUC Bioluminescence Data

Bioluminescence data were detrended by subtracting the 24-h running average from the raw data. The detrended data sets were smoothed by taking 3-h running averages. In this smoothed data set, the time corresponding to the highest level of bioluminescence that occurred between 24 and 48 h in culture was considered the peak phase. Peak phases were converted into phase angles relative to the previous locomotor activity onset or, in the case of SCNX ad libitum and SCNX RF mice, relative to local clock time. Mean vectors of circular distributions based on phase of individual tissues were calculated. The Rayleigh uniformity test was applied to determine if there was significant clustering of the peak phases for each tissue. The Watson-Williams F test was applied to evaluate the differences among treatments. Circular statistics were performed using mean vectors, and average phases of PER2::LUC expression are reported as arithmetic means in circadian time (CT). In a few cases, tissues were lost during tissue harvesting; only healthy, rhythmic cultures were used for analyses.

RESULTS

In order to avoid confusion, we first present operational definitions for the parameters we have used in analyzing our data. *Phase* refers to the time of peak PER2::LUC expression in a particular tissue relative to the activity onset of the animal from which it came (or relative to clock time in SCN-lesioned animals since they are arrhythmic). *Phase synchrony* is defined as a nonrandom distribution of phases of a particular tissue among individual animals in a group. The loss of clustering around the mean phase is characterized as *phase desynchrony*. *Phase relationship* is defined as the relationship among the phases of different tissues within an individual animal.

Methamphetamine

Effects of MA on circadian locomotor activity. As expected, MA treatment resulted in significantly lengthened circadian periods of locomotor activity (Suppl. Fig. S1A). Sham-lesioned mice that received MA in their drinking water for 3 weeks had an average free-running period of 24.41 ± 0.16 h, approximately 40 min longer than their prior free-running period. No change in period length was observed in sham-lesioned mice on pure water.

SCN lesion resulted in arrhythmicity, and MA administration reinstated rhythmicity in SCNX mice. The average free-running period of SCNX animals following 3 weeks of MA availability was 24.88 ± 0.29 h (Suppl. Fig. S1B). After 7.5 weeks of MA availability, it was 26.22 ± 0.61 h (Suppl. Fig. S1C). None of the SCN-lesioned mice on pure water recovered rhythmicity.

Effects of MA on the SCN and peripheral oscillators of intact and sham-lesioned mice. All tissues from intact control animals drinking pure water displayed tight phase synchrony ($p < 0.05$ for all tissues) (Fig. 1A) with most tissues having peak PER2::LUC expression in subjective night. In addition, the within-animal phase relationships between individual tissues were consistent among animals.

MA treatment resulted in phase desynchrony in intact animals. We did not observe significant phase clustering of SCNs, corneas, or salivary glands from MA-treated intact animals ($p > 0.10$) (Fig. 1C). The mean phases of kidney and salivary gland were delayed in MA-treated intact animals, and the mean phase of SCN was significantly advanced. We also observed altered phase relationships among tissues from individual MA-treated animals (Fig. 1C).
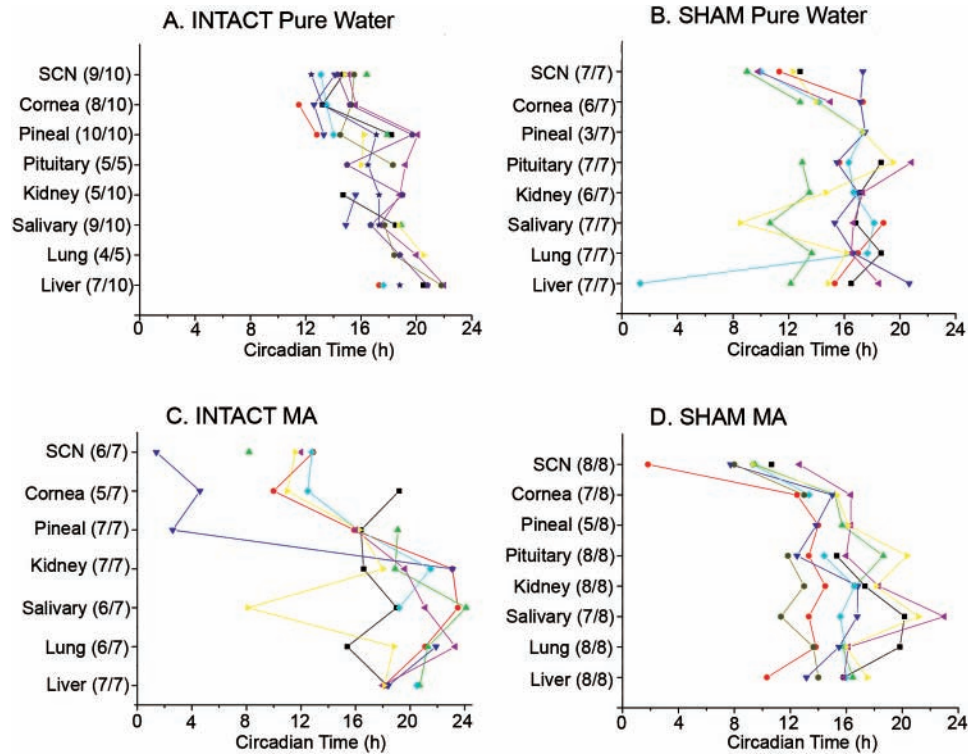
Figure 1. Phase maps of peak PER2::LUC expression in intact and sham-lesioned mice exposed to MA or pure water. Each color represents an individual animal (see online version of this article for colors). Circadian time (CT) is plotted along the x-axis, where CT12 is the onset of locomotor activity. Tissues are listed on the y-axis along with the *n* for each tissue (number of rhythmic cultures over total number of animals; see Materials and Methods). Intact mice display consistent phases relative to activity onset and consistent phase relationships (tissue to tissue) within animals, with PER2::LUC in most tissues peaking in the subjective night (A). Sham surgery resulted in increased phase spread (B). Methamphetamine administration (0.005% available in the drinking water) resulted in a delayed phase of kidney and salivary gland and an advance of the SCN, relative to activity onset (*p* < 0.05) (C). Methamphetamine administration in sham-lesioned mice had a less robust effect (D), with only the pineal gland demonstrating a significant phase shift compared to sham controls on pure water (*p* < 0.05) (B). The small sample size of pineal in the sham-lesioned pure water–treated group (*n* = 3), however, makes the interpretation of this effect difficult.

analyses of phase synchrony of a single tissue across animals.

In SCNX mice, methamphetamine treatment resulted in phase synchrony after 3 weeks and more robust phase synchrony after 7.5 weeks (Fig. 2B and 2C). After 3 weeks, we observed clustered phases of peak PER2::LUC expression in cornea, kidney, salivary gland, and lung (*p* < 0.05), whereas after 7.5 weeks, the phases of pineal glands and livers were also clustered (*p* < 0.01). Furthermore, in the 7.5-week methamphetamine-treated group, the peak phases of all tissues occurred in the subjective night, with phase relationships similar to those of intact animals drinking pure water (Suppl. Fig. S2), and there were consistent, normal phase relationships among tissues from single animals.

Surprisingly, sham surgery resulted in decreased phase synchrony among salivary glands (*p* = 0.062) and livers (*p* = 0.104) from mice drinking pure water (Fig. 1B). In contrast to the widespread phase desynchrony following MA treatment of intact animals, the only phase desynchrony observed in MA-treated sham-lesioned mice was among the salivary glands (*p* = 0.134) (Fig. 1D). MA treatment also had a less robust effect on the mean phase of peripheral tissues in sham-lesioned mice.

Effects of MA on peripheral oscillators of SCN-lesioned mice. As expected, tissues from SCNX mice drinking pure water displayed disrupted phase relationships (Fig. 2A). Since these mice did not exhibit free-running locomotor rhythms, peak phase is plotted relative to clock time. Thus, there is no reference point to use in

Restricted Feeding

Effects of RF on circadian locomotor activity. In agreement with recent reports, we observed robust FAA in response to 6 h of daily food availability (Mendoza et al., 2010; Moriya et al., 2009). SCNX and sham-lesioned mice both exhibited appreciable FAA; SCNX mice had larger amounts (Suppl. Fig. S3).

Effects of RF on the SCN and peripheral oscillators of intact and sham-lesioned mice. In intact mice, RF affected phase synchrony, mean phase, and within-animal phase relationships of PER2::LUC expression (Fig. 3). Phase synchrony of pituitary glands was significantly affected, with no significant clustering observed among the pituitaries from RF animals (*p* > 0.05). Restricting feeding to midsubjective day shifted the peak phases in almost all tissues examined.
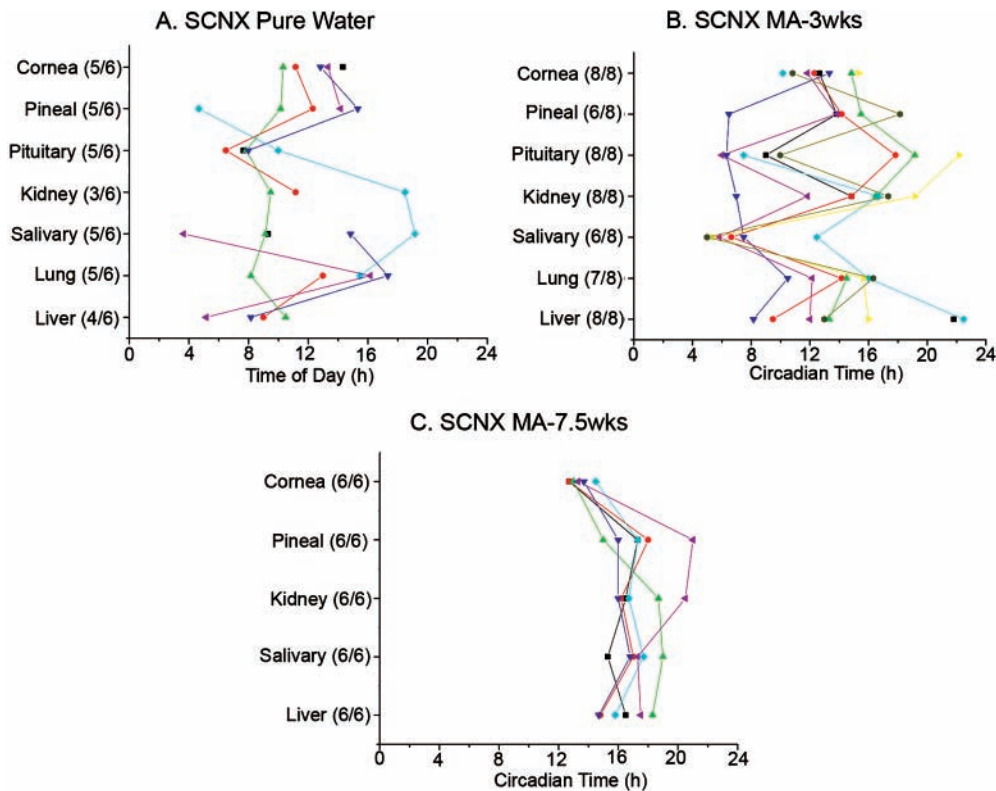
Figure 2.   Phase maps of peak PER2::LUC expression in SCNX mice treated with MA or pure water. Each color represents an individual animal (see online version for colors). SCNX mice on pure water had arrhythmic locomotor activity, and therefore, peak PER2::LUC expression is plotted arbitrarily with regard to the animal's locomotor behavior. For the purposes of examining phase relationships among different tissues within animals, the data for peak PER2::LUC expression in SCNX mice on pure water are plotted in local time (A). For SCNX mice on methamphetamine, circadian time (CT) is plotted along the x-axis, where CT12 is the onset of activity (B, C). In all cases, tissues are listed along the y-axis along with the *n* for each tissue (number of rhythmic cultures over total number of animals; see Materials and Methods). Following 3 weeks on methamphetamine, there was significant clustering of the phases of cornea, kidney, salivary gland, and lung relative to activity onset (Rayleigh test, *p* < 0.005) (B). Following 7.5 weeks on methamphetamine, pineal gland and liver also demonstrated stable phase relationships relative to activity onset (Rayleigh test, *p* < 0.005), and peak PER2::LUC expression for all tissues occurred in the subjective night (C), as in SCN-intact mice on pure water (see Fig. 1A).

mean phases of salivary glands, lungs, and livers of RF sham-operated mice differed significantly from those of the sham-operated ad libitum–fed controls (*p* < 0.05 for all tissues and *p* = 0.051 for cornea). There was not, however, a significant shift in the phase of pineal gland or kidney in restricted-fed sham-operated mice.

Effects of RF on peripheral oscillators of SCN-lesioned mice. As expected, SCN lesion resulted in disorganization of the normal phase relationships among tissues (Fig. 5A). In restricted-fed SCNX mice, all tissues exhibited stable phase angles of PER2::LUC expression peaking around the time of the meal (Fig. 5B).

DISCUSSION

We have shown that peripheral tissues respond to the extra-SCN oscillators MASCO and FEO. MASCO and FEO shifted the peak phase of PER2::LUC in peripheral tissues and influenced the phase relationships among tissues. Moreover, the effects of MASCO and FEO on peripheral oscillators were more robust when the SCN was ablated, indicating interaction between these extra-SCN oscillators and the pacemaker in the SCN.

Peripheral oscillators normally require SCN-driven signals to maintain synchrony (Balsalobre et al., 1998, 2000; Oishi et al., 1998; Sakamoto et al., 1998). In the absence of the SCN, peripheral tissues still produce daily oscillations; however, the normal phase relationships among them are disrupted (Yoo et al., 2004). The ability of MASCO and FEO to impart phase synchrony and reinstate consistent phase relationships
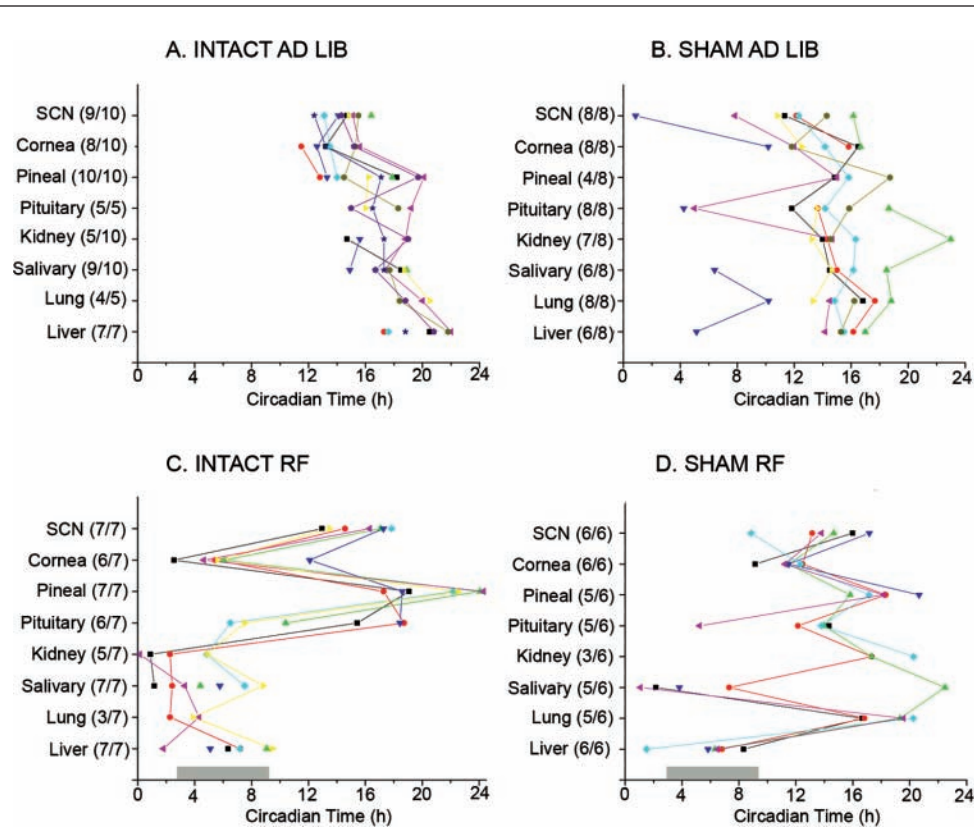
Cornea, pineal, kidney, salivary gland, lung, and liver all had significantly different phases in RF mice compared to intact ad libitum controls (*p* < 0.05) (Fig. 3C), with peak expression in most tissues shifted towards the time of the meal, whereas peak phase of the SCN remained unaffected. The effect of RF was robust in liver and salivary gland; there was an almost 180° phase difference between the livers and salivary glands of ad libitum and RF-treated intact animals (Fig. 4).

In ad libitum–fed sham-operated animals, phase desynchrony was observed in pituitary, salivary gland, and liver (*p* > 0.05) (Fig. 3B). Sham surgery alone also decreased SCN phase synchrony (*p* = 0.058). RF had a less robust effect on sham-operated mice than on intact controls (Suppl. Table S2). The

Figure 3.   Phase maps of peak PER2::LUC expression in intact and sham-lesioned restricted- or ad libitum–fed mice. Each color represents an individual animal (see online version for colors). Circadian time (CT) is plotted along the x-axis, where CT12 is the onset of activity. Tissues are listed on the y-axis along with the *n* for each tissue (number of rhythmic cultures over total number of animals; see Materials and Methods). Restricted feeding time (between CT3-9) is indicated with gray blocks on the x-axis. The data in Figure 3A are the same as those in Figure 1A. Sham surgery disrupted the normal phase relationships (tissue to tissue) within animals (B). Restricted feeding resulted in a phase shift of cornea, kidney, liver, lung, pineal, and salivary gland ($p < 0.05$), with peak PER2::LUC expression in most of the tissues shifting towards the time of the meal (C). The livers, lungs, and salivary glands of restricted-fed sham-lesioned mice also displayed phase shifts ($p < 0.05$) (D) relative to sham-lesioned mice drinking pure water.
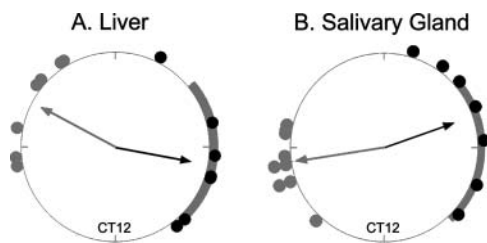

Figure 4.   Rayleigh plots of the peak phases of PER2::LUC in liver (A) and salivary gland (B) from restricted (black circles)– and ad libitum (gray circles)–fed SCN-intact animals. The onset of locomotor activity (CT12) is indicated. The gray arc around a portion of each circle represents the timing of the meal. Restricted feeding results in a shift in peak phase of PER2::LUC expression in both tissues from the subjective night to the subjective day (i.e., towards the time of the scheduled meal). The arrows represent the mean vectors for the circular distributions. The length of the vector represents the strength of the phase clustering, while the angle of the vector represents the mean phase.

among peripheral oscillators demonstrates that MASCO and FEO can act as strong circadian pacemakers.

Effects of Methamphetamine on Peripheral Oscillators

As previously reported for both rats and mice, methamphetamine significantly lengthened circadian periods of locomotor activity in intact and sham-operated animals and restored rhythmicity in SCN-lesioned mice (Honma et al., 1987; Mohawk et al., 2009; Tataroglu et al., 2006). Our results further demonstrate that the phases of peripheral oscillators respond to methamphetamine. In intact animals, all tissues except liver and lung were affected by MA treatment (Fig. 1). The lack of effect on liver we report here is consistent with previous reports that, in SCN-intact animals, *Per2* expression in liver is nonresponsive to methamphetamine treatment (Iijima et al., 2002). *mPer1* and *mPer2* gene expression in the SCN has also been reported to be refractory to MA treatment in some studies (Nikaido et al., 2001; Iijima et al., 2002), which is in contrast to the phase advance we observed in the SCN of MA-treated animals. However, previous experiments utilized either a single injection or daily injections of MA for 7 days, while our animals were exposed to chronic MA. Perhaps only prolonged exposure to MA affects the phase of the SCN pacemaker (Fig. 1).

Surprisingly, we found that sham surgery itself impacted phase synchrony among salivary glands and livers. Sham surgery also attenuated the ability of MA to affect phase synchrony in peripheral oscillators.
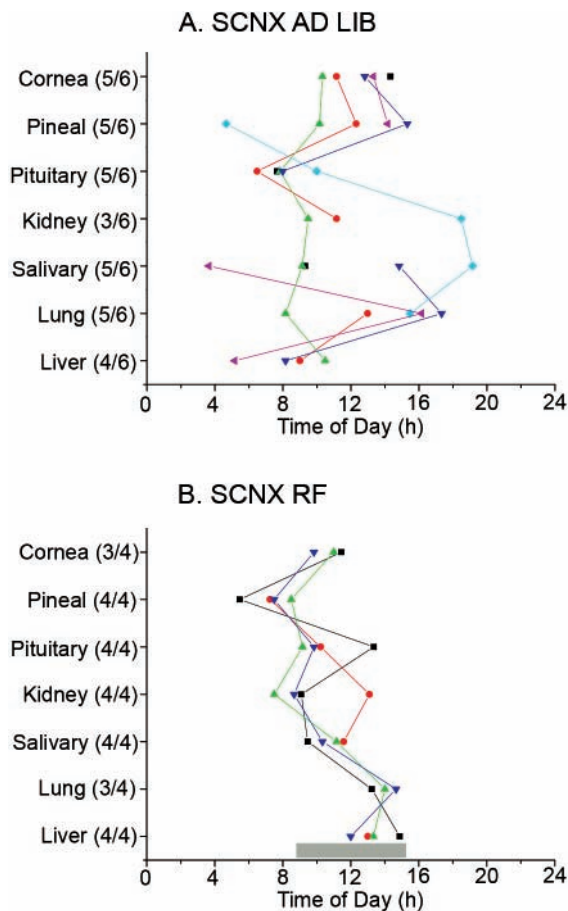
Figure 5. Phase maps of peak PER2::LUC expression in SCNX mice. Each color represents an individual animal (see online version for colors). Tissues are listed on the y-axis along with the *n* for each tissue (number of rhythmic cultures over total number of animals; see Materials and Methods). To demonstrate phase relationships among tissues in individual animals, the data for PER2::LUC expression in SCNX mice on pure water are plotted in local time (A). The phase map for SCNX mice on restricted feeding is also plotted in local time, and the timing of the meal (0900-1500 h) is indicated by the gray block (B). In SCN-lesioned animals, restricted feeding resulted in phase synchrony both within and between animals, with all tissues exhibiting stable phase angles of peak of PER2::LUC expression relative to time of the meal presentation (Rayleigh test, $p < 0.05$ for all tissues).

Together, these results suggest that the ability of both the SCN and MASCO to impart phase information to peripheral tissues is impaired following surgical manipulation. Indeed, the SCN tends toward phase desynchrony in sham-lesioned animals, suggesting that perhaps it is affected by the surgery. The mechanism behind the impaired response to pacemaking cues is unknown. It is possible that the sham procedure may damage the SCN or its nearby targets. However, we did not observe any visible damage to

SCN in our sham groups, and all the SCN cultured from these groups were still robustly rhythmic. Furthermore, we observe a similar effect of laparotomy alone (unpublished data), which suggests that the observed circadian disruption is a response to surgery itself, which may induce a systemic inflammatory response or introduce metabolic changes, resulting in disruption of peripheral rhythms.

In order to explore the contribution of SCN to the effects of MA on peripheral oscillators, we tested MA in SCN-lesioned animals. SCN lesion results in disrupted phase relationships among tissues, but following MA treatment, normal phase relationships and tight phase synchrony were recovered in SCNX mice. As is the case with behavior, the effects of MA on the periphery were more pronounced following an extended period of time on the drug. Three weeks of MA treatment synchronized the phases of kidney, salivary gland, and lung, while 7.5 weeks of treatment synchronized also pineals and livers (Fig. 2). Different peripheral oscillators are known to entrain to the same stimuli with different kinetics (Yamazaki et al., 2000), which may explain the differences among the tissues we studied.

The phase relationships among tissues from SCN-lesioned animals were further stabilized by extended MA treatment. While salivary gland was clearly synchronized by 3 weeks of MA exposure, the mean peak of PER2::LUC expression occurred in the subjective day. In contrast, salivary glands from intact animals on pure water normally exhibit peak PER2::LUC expression in the subjective night. When MA was continued for 7.5 weeks in SCN-lesioned animals, salivary glands, as well as the other peripheral tissues examined, exhibited peak PER2::LUC expression in the subjective night. Thus, extended MA treatment resulted in phase relationships relative to activity onset similar to those of intact animals on pure water (Suppl. Fig. S2), lending further support to the hypothesis that MASCO is capable of acting as a pacemaker, not only for locomotor activity but also for peripheral organs.

Effects of Restricted Feeding

We replicated previous observations reporting FAA in response to restricted feeding in both intact and SCN-lesioned animals (Davidson and Stephan, 1999; Davidson et al., 2003; Krieger et al., 1977; Stephan et al., 1979). We also found significant effects of restricted feeding on peripheral oscillators, with most of the tissues shifting towards the time of the meal.

In intact and sham-lesioned mice, we found robust effect of food restriction on peripheral oscillators. The peak phase of PER2::LUC expression was shifted significantly in cornea, pineal, kidney, salivary gland, lung, and liver of RF-treated animals (Fig. 3). In intact mice, the phase of PER2::LUC was shifted towards the time of food presentation in cornea, kidney, salivary gland, lung, and liver. This result is consistent with previous reports that limiting food availability to the light portion of the LD cycle is able to reverse the phases of liver, lung, kidney, heart, and pancreas (Damiola et al., 2000; Stokkan et al., 2001).

The phase shift of cornea that we observed in response to RF is surprising, as this tissue seems to be resistant to most phase-shifting stimuli (Yoshikawa et al., 2005). The salivary gland of rats has been reported to be resistant to RF-induced phase shifts unless denervated (Vujovic et al., 2008). The rats used in that study were held in a 12:12 LD cycle, while the mice in the current study were housed in constant darkness. Disrupting the pathway necessary for light-driven entrainment via sympathetic denervation (as in the rat study) or depriving mice of light cues by maintaining them in constant darkness (as in the present study) may both render salivary glands more susceptible to RF-induced entrainment.

We did not find any effects of RF on the phase of SCN, which is consistent with previous reports (Akiyama et al., 2004; Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001; Wakamatsu et al., 2001). Restricted feeding does not appear to have a strong effect on the SCN pacemaker itself.

Sham surgery alone disrupted phase synchrony in peripheral tissues, suggesting that entrainment of peripheral oscillators is impaired by surgical manipulations. Much as with MA, the tissues of sham-lesioned animals were less impacted by restricted feeding than were tissues from intact animals. Both pineal gland and kidney were affected by RF treatment in intact animals but not in sham-surgery controls. Sham surgery may impair entrainment to RF in pineal gland and kidney, and thus, these tissues may need more than 10 days of restricted meals to entrain in sham-lesioned animals. Indeed, entrainment of peripheral tissues to all pacemaking cues (from FEO, MASCO, or SCN itself) may be impaired or delayed in sham-operated animals.

When SCN-lesioned animals were subjected to RF, the phases of peripheral tissues were synchronized. It has been previously reported that RF affects *Per1* and *Per2* expression in the livers of SCN-lesioned as well as in intact animals (Hara et al., 2001). Here, we show that timed feeding also synchronizes the phases of cornea, pineal, pituitary gland, kidney, salivary gland, and lung (Fig. 5). All tissues peaked around or during the time of the meal. Even tissues that did not synchronize to meal time in sham-lesioned or intact animals (e.g., pineal gland) readily shifted towards the time of feeding when the SCN was ablated. This suggests that in intact animals, restricting feeding to the middle of the subjective day results in competition between the SCN and FEO for control of peripheral oscillators, with meal time prevailing as the dominant zeitgeber for most but not all tissues. After SCN lesion, there is no longer a competitive signal from the "master circadian pacemaker," and RF acts on FEO alone (Fig. 5B). It has been reported recently by Angeles-Castellanos et al. (2010) that SCN lesions cause elevated behavioral, temperature, and hormonal anticipatory response to restricted feeding. In the intact ad libitum–fed condition, FEO and the SCN probably work in concert, reinforcing each other to provide harmonious time keeping throughout the circadian system. It should be noted that tissues involved in digestion and metabolism (liver, kidney, salivary gland) were particularly robustly affected by RF, suggesting that metabolic signals may be a key mechanism in phase control of peripheral oscillators by FEO.

Conclusion

Here, we demonstrate that MASCO and FEO are capable of regulating the phase of peripheral oscillators. In intact animals, MASCO and FEO appear to compete with the SCN for the control of peripheral tissues; thus, MA treatment or restricted feeding results in phase desynchrony and abnormal phase relationships within the circadian system. However, in the absence of the SCN, chronic MA or RF leads to increased phase synchrony and stable phase relationships among peripheral tissues. It remains to be seen if, as is the case with behavior, FEO and MASCO can continue to drive peripheral rhythms after the stimuli (RF or MA, respectively) are withdrawn. We propose that MASCO and FEO are normally coupled to the master pacemaker in the SCN and that these circadian oscillators function together to provide adaptive phase synchrony throughout the organism. The molecular mechanism and anatomical location of both MASCO and FEO are unknown (reviewed in Honma and Honma, 2009). Neither MASCO (Honma et al., 2008; Mohawk et al., 2009) nor FEO (Pendergast et al., 2009; Storch and Weitz, 2009) appear to require

the SCN or the canonical molecular clock mechanism to drive circadian outputs.

Despite the fact that canonical clock genes are not required for FEO or MASCO, here, we show that the phase of PER2::LUC expression is affected by MA and RF in peripheral oscillators. Our data suggest that these extra-SCN circadian pacemakers interact with the canonical molecular clock, at least at the level of peripheral circadian organization. It is possible that there is a direct effect of MA or RF on *Per2* similar to the acute induction of *Per1* by light (Tischkau et al., 2003). Alternatively, and perhaps more likely, peripheral oscillators may be influenced indirectly by the altered activity and feeding rhythms observed in MA and RF-treated animals. Indeed, as is the case for some SCN-driven rhythms, MASCO and FEO may be affecting peripheral oscillators via body temperature or endocrine signals, such as glucocorticoids, both of which are known to be altered by MA and RF treatment (Angeles-Castellanos et al., 2010; Honma et al., 1988; Honma et al., 1992).

The integrity of the circadian system, expressed most clearly in the consistent phase relationships among its oscillators, is important for a wide variety of normal functions (Mahoney, 2010; Marcheva et al., 2010). Our results make it clear that such integrity can be affected—indeed, under some circumstances maintained—by inputs other than light, entering the system through routes only secondarily involving the SCN. This information should ultimately be useful in designing treatments to counteract circadian dysregulation caused by space travel, shift-work schedules, or aging.

NOTE

Supplementary material for this article is available on the *Journal of Biological Rhythms* Web site: http://jbr.sagepub.com/supplemental.

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